STUDIES ON SKIN DISEASES OF CROCODILES

Thesis submitted
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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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ABSTRACT

This study was conducted to determine the occurrence of skin diseases in farmed crocodiles in Australia. Six farms, three in Queensland and three in the Northern Territory were visited during the period May to August 1996. Samples of skin with lesions were obtained and data on morbidity and mortality, possible aetiological agents, and other likely predisposing factors were collected. The approximate number of crocodiles on these farms varied from 800 to 3,000. Furthermore, pathology case records of histopathological slides of crocodile skin lesions on file from 1989 to 1995 were included. All data gathered on recent (1996) cases, especially the gross and microscopic findings were combined for analysis with the data on retrospectively examined cases of skin diseases on file in the Australian Institute of Tropical Veterinary and Animal Science, James Cook University of North Queensland.

Two hundred and three lesions (119 were from retrospectively examined cases and 84 were recent) from 180, mostly young crocodiles were examined. The skin lesions were obtained from crocodiles on nine farms, from a group of experimental animals and from one adult found dead in the wild. Necropsies were performed either on the crocodile farms, at James Cook University, or at the Department of Primary Industries and Fisheries – Berrimah Veterinary Laboratory, Northern Territory. The histopathological and bacteriological examination of the 84 recent cases was carried out at the Australian Institute of Tropical Veterinary and Animal Science. Samples of skin with poxvirus lesions were examined with an electron microscope.

Five specific skin diseases in uncomplicated form including dermatophilosis, mycotic dermatitis, poxvirus infection, probable mycobacterial dermatitis and capillanasis were identified. Dermatophilosis was the most prevalent skin disease and was also frequently diagnosed, being present in 62 of 66 (94%) in mixed or dual infections. The lesions were discrete focal, 1 to 4 mm diameter 'brown spots' of variable prominence, predominantly on the ventral abdomen but also elsewhere in the body. The histopathological examination confirmed the presence of filamentous organisms mostly in debris that had accumulated on the ulcerated or eroded epidermis but also elsewhere in the subcutis, and in severe cases in the muscular layer.

Duplicate samples of skin confirmed histologically as dermatophilosis were homogenised for bacterial culture. *Dermatophilus* sp was positively identified initially based on the cultural characteristics which included haemolysis, pitting into the medium and white to grey colonies. The organisms were filamentous and branching, Gram-positive, non-acid fast, catalase positive and oxidase negative. Other biochemical tests also supported the identity of the organism as closely resembling *Dermatophilus congolensis*.

Several transmission experiments were carried out. In a pilot study on transmission of dermatophilosis, two isolates of *Dermatophilus* sp (strains TVS 96-366-5A and TVS 96-490-7B), both from cases of dermatophilosis in farmed crocodiles were used. Within several days after inoculation, the hatchlings developed typical 'brown spot' lesions not only at inoculation sites but also other locations both in infected and in-contact control animals. Histopathological examination revealed changes characteristic of dermatophilosis. *Dermatophilus* sp confirmed as TVS 96-490-7B

but not strain TVS 96-367-5A on the morphological, cultural and biochemical characterisation was isolated from 'brown spot' skin lesions on several occasions after infection.

In the second transmission experiment, the protocol was changed to evaluate possible control and treatment procedures. Although occasional minute skin lesions developed in crocodiles, the lesions quickly regressed so effective transmission was not achieved. A third transmission experiment was conducted using facilities and procedures as in the pilot study but with control and inoculated hatchlings maintained in separate tanks in separate buildings 60 metres apart. Both inoculated and in-contact controls in the principal group developed 'brown spot' lesions. Again only strain TVS 96-490-7B was isolated from both the principal and control groups. A few hatchlings in the control group however became infected with either 'brown spot' disease or poxvirus after 20 days post inoculation.

The *Dermatophilus* sp isolated from spontaneous outbreaks and recovered isolates from several transmission experiments was compared with *Dermatophilus congolensis* type strain (ATCC 14637) and *Dermatophilus chelonae* (DCH 2) by morphological, cultural and biochemical characterisation. In addition, more advanced comparison at the molecular level using 16S rDNA sequence and ribotyping were also used. The results showed that the isolate used for inoculation and the recovered isolates were identical, thus confirming its role in causing 'brown spot' disease. Overall, both the phenotypic and genotypic differences were sufficient to suggest that the crocodile isolate is a distinct species of *Dermatophilus*, thus a new species of *Dermatophilus* – *Dermatophilus crocodyli* sp nov. is proposed.

Following successful transmission of 'brown spot' disease and identification of the aetiological agent – *Dermatophilus crocodyli*, treatment and control studies were carried out. An initial *in vitro* study on the antibacterial properties of copper sulphate, salt and formalin showed that copper sulphate was effective against *Dermatophilus* sp. This was followed by experimental transmission of hatchlings as in the pilot experiment but the inoculated animals were kept in separate pens, one group was placed in pen with flowing water and the other group was placed in pen with static water to compare the development of 'brown spot' lesions. This experiment showed the flowing water had no effect on the control of 'brown spot' disease. In the second experiment, all infected animals were treated with either formalin or copper sulphate. Hatchlings treated with formalin developed severe lesions compared with those treated with copper sulphate. All infected hatchlings were treated with copper sulphate using three protocols. Of the three protocols, it was found that immersion of infected hatchlings for 15 minutes in medicated water containing 1 ppm copper sulphate was most effective.

Studies on poxvirus were carried out to include the gross and histopathological characterisation of the disease, electron microscopy of poxvirus and transmission of poxvirus. The gross and microscopic appearance of the skin lesions infected with poxvirus was consistent in all 11 hatchlings. Marked circumscribed grey-white lesions up to 3 mm diameter were present on the lower limbs, foot pads, tail and back, whereas the lesions on the neck, chest and abdomen were diffuse, irregular and translucent. Electron microscopy revealed the presence of 'dumb bell' shaped virus typical of poxvirus. Attempts to grow the virus in cell culture, chicken chorioallantois and crocodile embryos were unsuccessful. A transmission experiment was attempted but failed to produce lesions.

This study concludes that 'brown spot' disease is the most prevalent and probably the most important skin disease in farmed crocodiles in Australia. A newly proposed species – *Dermatophilus crocodyli* sp nov. was identified as the aetiological agent of 'brown spot' disease. Copper sulphate at 1 ppm concentration was proven to be an effective treatment for 'brown spot' disease.

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AITVAS	Australian Institute of Tropical Veterinary and Animal Science
ATV	Antibiotic versene and trypsin
BHK	Baby hamster kidney cells
bp	Base pairs
CAM	Chorioallantoic membrane
CFU	Colony forming units
CITES	Convention on International Trade in Endangered Species of wild fauna and flora
CTAB	Hexadecyltrimethyl ammonium bromide
DIG	Digoxigenin
DIG-dUTP	dTTP analogue
DMEM	Dulbecco's Modified Eagle's Medium
DPIF-BVL	Department of Primary Industry and Fisheries – Berrimah Veterinary Laboratories
EEV	Eastern encephalitis virus
kb	Kilo base pairs
LS	Loeffler's serum
MEGA	Molecular genetic analysis
nm	Nanometre
PAS	Periodic acid-Schiff stain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Post-inoculation
PT	Post-treatment
SDS	Sodium dodecyl sulphate
SSC	Sodium citrate
WS	Water sample

PUBLICATIONS

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CHAPTER 1

GENERAL INTRODUCTION

Crocodile farming was conceptualised in response to the continued threat of extinction of crocodiles. Illegal hunting and lack of understanding about this animal widely regarded as ferocious were the most likely cause of this decline in crocodile numbers. Initially, farming was thought to solve the problem of extinction, however, the outcome of farming was far greater than was expected. Through the intervention of the Convention on International Trade in Endangered Species of wild fauna and flora (CITES) and the Australian Government's initiative of protecting the crocodile population, the number of crocodiles in Australia particularly in the Northern Territory has steadily increased during the past 25 five years (Britton, Ottley and Webb, 1988). Farming of crocodiles became a sustainable, economically feasible and more rewarding business venture. From 1994 to 1997 report, the number of *C porosus* processed for skin and meat had increased from 3,000 to almost 8,000 in the Northern Territory (Simlesa,,1998).

Today, the crocodile industry is well recognised, and over a thousand crocodile farms have been established around the world. Although crocodile farming began in the late 1960's in Australia, the industry showed only limited development. The industry grew significantly in 1986 when exports of crocodile skin was permitted and the demand of crocodile leather had increased substantially. Two species of crocodiles, the indigenous freshwater crocodile (*Crocodylus johnstoni*) and saltwater crocodile (*Crocodylus porosus*) were originally farmed, however, the industry has concentrated more on saltwater crocodiles because of the higher economic value of their skin.

Presently, there are 16 commercial crocodile farms in Australia mostly located in Queensland and the Northern Territory. These farms produce their own stocks through captive breeding or the hatchlings are supplied by other farms or from the Parks and Wildlife Department. In the Northern Territory, the farmers are permitted to collect crocodiles or eggs from the wild and hatch the eggs on the farm. Other crocodile farms that are engaged in the tourism business raise other species of crocodiles such as alligators as an added attraction for the park.

Because crocodile farming has just emerged as a new form of 'wildlife agriculture', the technology used varies between farms. As there is no universal standard method to emulate a sound crocodile farm management, farmers have relied mostly on overseas concepts of animal husbandry, which were also a product of empirical methods. In 1986, the crocodile farms in Australia were constructed in such a way that the design of pens mimicked the natural habitat of

crocodiles in the wild. In addition, the nutritional requirement of crocodiles is still poorly understood, and is currently being studied in an attempt to produce optimum growth in a short period (Davis, Jack and Peuker, 1999).

Intensive farming of crocodiles is not different from conventional farm animals, in that there are many important requirements needed to maximise production. In my previous studies (Buenviaje, Ladds, Melville and Manolis, 1994), it was found that temperature, food and pen design were the main factors that influenced the occurrence of many diseases in farmed crocodiles. For example when the temperature of pens was maintained at 32°C and fish was excluded from diet, there was a lower incidence of bacterial and fungal infections and lungworm infections, respectively. The outcome of such research has had a tremendous impact on improved husbandry management of farmed crocodiles in Australia and probably elsewhere in the world.

Although there are still more aspects of the husbandry of intensive crocodile farming which need to be examined scientifically, it is also equally important to define the aetiological agents and the control and treatment of diseases, especially in regard to diseases affecting the skin. Based on the literature review on diseases of crocodiles, there is a paucity of information on the prevention and control and even treatment of diseases in crocodiles. Any outbreak of infection affecting the skin will caused heavy economic losses, considering that even a single blemish downgrades the market value of skin. It is particularly important because when one animal is sick, the infection can spread readily to other animals through the water. When optimal conditions and sound management techniques of crocodiles are applied, many diseases can be minimised if not eradicated.

The main objective of the research work documented in this thesis is to investigate the diseases affecting the skin of farmed crocodiles in Australia. This includes a complete description of the diseases but particularly the identification by gross and microscopic pathological examinations of the most important of the skin diseases. The specific objectives of this thesis include the following:

- To identify the most important skin disease of farmed crocodiles in Australia.
- To identify and characterise the aetiological agent of the most important skin disease and to show by transmission studies its role in causing the infection.
- To develop strategies for the control and prevention of the most important skin disease in farmed crocodiles.

CHAPTER 2

DISEASES OF CROCODILES: A REVIEW

2.1 Introduction

Because the crocodile farming industry has only been established recently, much of the research effort has focussed on husbandry aspects rather than on diseases. As a result, published reports on diseases of crocodiles, particularly diseases affecting the skin, are relatively few. Presumably, this reflects a general lack of understanding on the clinical manifestations of the disease, or alternatively diagnosticians have not been competent to recognise and diagnose diseases of crocodiles. Most of the diseases reported have been field cases, and the details on the pathology have been inadequately described, especially in regard to the histopathological lesions. The lack of a veterinary pathologist with a special interest in diseases of crocodiles, perhaps is the main reason for insufficient and inaccurate information about crocodile diseases. Despite all the problems of obtaining information, an attempt has been made to document what is known about diseases of crocodiles.

2.2 Comparative Anatomy and Physiology of Reptilian Skin with Particular Reference to Crocodiles

Crocodile farming for skin is an expanding industry because of the high demands for quality leather. The price of skin in the market differs according to the species of crocodile, for example skin of the saltwater crocodiles is in high demand and is most sought after by consumers. This is because the anatomical pattern and composition of the skin of saltwater crocodiles has a particular appeal that makes it more valuable leather as compared to leather from other species. An understanding of skin structure and its physiological functions and identifying differences in species is a necessary prerequisite to understanding diseases of the skin.

2.2.1 Basic structure of skin of reptiles and higher animals

The skin belongs to the integumentary system and is considered as the largest organ of the body, which serves several functions. The skin as an organ is made up of different tissues each with a specific function. For example in mammals, the skin has adnexal structures, which includes sebaceous, sweat and mammary glands and hair follicles. Such adnexal structures however are absent in reptiles.

In a diverse environment, reptilian skin may undergo certain modifications in structure and perform a unique physiological function (Avery, 1979) to survive in an environment in which they live. Amphibian skin for example is moist, slimy and warty in structure (Marcus, 1981).

Generally, the skin of reptiles is covered by scales or scutes and differs slightly in terms of structures and functions. In turtles for example, the skin at the back and the abdomen has been modified to a bony structure called carapace and plastron, respectively, while in other reptiles such as in crocodiles, the dorsal skin has distinct ridges called carina (Harris, 1963).

2.2.2 Variation of the shapes and forms of scales in different locations of the body

The skin of the rainbow lizard (*Agama agama*) is covered by mostly rhomboid shape scales arranged in rows that run diagonally around the body (Harris, 1963). Scales overlap one another in most locations of the body, especially the dorsal part. In imbricate scaling the caudal tip of one scale in front lies over the cephalic base of the next scale in the row. There are two different scale structures; those that possess a distinct ridge (carina) are called carinate and mucronate refers to the scales bearing a small spine or projection that extends back beyond the corner of the scale. The small spine or projection forms the major part of the scale of a spinose type scale. The crocodilian skin is almost similar to the skin of the rainbow lizard except for the absence of overlapping scales and small spines that extend backward beyond the corner of the scale.

Brazaitis (1987) described the skin structures in different locations of the body in various species of crocodiles. Individual scales are joined at their margins by thinner more pliable skin otherwise called 'hinge joint'. On some parts of the body, the scales are pitted, and have a smooth surface particularly in the belly as opposed to the skin at the back.

Understanding the differences of skin structure in crocodilians is important not only for identification of species but more importantly as a basis for the identification of normal structures of skin in different parts of the body as a guide for pathological interpretation of any skin lesions.

2.2.3 Distinguishing features of skin in some species of crocodiles

There are four subfamilies under the family of Crocodylidae namely: Alligatorinae, Crocodylinae, Tomistominae and Gavialinae. Alligatorinae have four genera namely: Alligator, Caiman,

Melanosuchus and Paleosuchus (Grenard, 1991). A total of 22 species of crocodilians is generally recognised throughout the world and all have differences in skin patterns specific to their own identity (Brazaitis, 1987).

King and Brazaitis (1971) described some characteristic features unique to various species of crocodiles. These unique structures of the skin which includes the osteoderms, integumentary sense organs or follicular glands and surface pitting of scales are useful anatomical features for the identification of species of crocodiles. In addition, these skin structures have special physiological functions in crocodiles. Osteoderm is a bony structure, which may be either single or double, present in the dorsal and belly skins in most members of the Alligatoridae (except alligators), *Osteolaemus* and *Crocodylus cataphractus*. The integumentary sense organs are small pit-like structures near the posterior margin of the scales of all crocodiles and gavials. Surface pitting is due to the wrinkled texture of the underlying osteoderms mostly seen in the dorsal scales of the hornback hides and the ventral scales. In addition, in some species and particularly in adults surface pitting is more obvious than juveniles and easily recognised in tanned skin. *Alligator mississippiensis* on the other hand has a very distinctive characteristic umbilical scar in the belly arranged in a 'spider web' pattern.

2.2.4 Histological structure of the skin

The epidermis

The histogenesis of the skin as presented by Sengel (1976) was based on the embryonic development of *Lacerta muralis*. During early development at 35 days post-laying, the epidermis consists of a single-layered cuboidal stratum basale and the outermost periderm. Gradually the epidermis forms into three strata, the stratum germinativum, the stratum intermedium and the periderm. Later, the nucleus of the flattened cells of the periderm is not visible and the cell becomes keratinised. Keratinisation terminates at the hinge region between scales. Below the periderm there is a layer of large squamous cells with round nuclei called the 'oberhautchen', followed by three to four layers of parakeratotic layers of the stratum corneum. The stratum intermedium is composed of undifferentiated cuboidal cells while the basal layer has cylindrical cells.

Normally during ecdysis, the whole outer generation of the epidermis consisting of periderm, 'oberhautchen' and stratum corneum is sloughed off. According to Maderson (1965) eosinophilic granulocytes migrate from the dermis through the stratum germinativum, up to the clear layer. Accordingly, the eosinophilic granulocytes secrete proteolytic enzymes responsible for breaking

up the outer generation layer from the inner generation before it sloughs off and the cycle of ecdysis is complete.

In the rainbow lizard (*A agama*), the epidermis is thickened and keratinised whereas, between two adjoining scales or 'hinge joint', keratinisation is absent which gives flexibility to the skin hence it is called flexible lamina. The protective outer layer of the skin that consists of dead structures produced by the epidermis during the process of cell division and keratinisation is called scales or periderm (Sengel, 1976).

In crocodiles, according to Heaphy (1989) the epidermis is composed of three layers namely: the basal stratum germinativum (innermost layer), the intermediate stratum granulosum and the stratum corneum. The basal stratum germinativum is composed of a single layer of columnar cells similar to mammals. In addition, the basal stratum germinativum varies from cuboidal to columnar cells, and is the site for cell division to form new cells (Tortora and Grabowski, 1993). The middle layer is the stratum granulosum and is composed of six layers of poorly differentiated compressed cells.

The keratin layer of the epidermis varies enormously in thickness in different locations of the body. The centre of the scales has the thickest keratin while between the scales, the 'hinge joint' has the thinnest keratin (Heaphy, 1989). According to Maderson (1965), the outermost region of compact keratinised material, presumably the 'periderm' is called the beta keratin, and beneath is a region of loose keratinised material, the alpha keratin.

The stratum corneum as the outer horny layer, consists of dead material of low plasticity; it cannot grow and is shed at intervals. Crocodilians however do not shed skin like other reptiles but instead merely lose isolated horny scutes (Wannawattanapong and Youngprapakorn 1994). Shedding of skin according to Marcus (1981) differs among reptilians; lizard skin is usually shed in several large pieces whereas snakes' skin is normally shed intact, including the spectacle over the eyes.

The dermis

The dermis in reptiles is thinner compared with amphibians, and is made up of collagen and elastic fibres and fat cells (Wolff, 1991). The melanocytes are present in the upper stratum of the dermis and are aligned along the adjoining basement membrane of the epidermis (Sengel, 1976).

The colour of the skin of reptiles and amphibians is determined by the distribution of three kinds of cells in the skin namely the iridocytes or guanophores that contain a white material called guanine; lipophores, which contain red and yellow pigments; and melanophores that contain melanin (Marcus, 1981). The change in colour of skin of amphibians, some lizards and chameleons is also influenced by pigments contained in the chromatophores situated beneath the epidermis (Fauca, 1973). In man and other animals the pigment melanin comprises about 8% of the epidermal cells and is contained in the melanoblast scattered particularly in the stratum basale of the epidermis (Tortora and Anagnostakos, 1987). Melanin is sometimes present in the dermis because chromatophores or melano-macrophages take up melanin (Ham and Leeson, 1961).

Crocodilians and some lizards have bony plates called osteoderms located in the dermis, which are readily visualised in radiographs (Marcus, 1981). According to Brazaitis (1987) the osteoderms were absent in *C porosus* and poorly developed in *C niloticus*, *C cataphractus*, *C siamensis* and *A mississippiensis*. The osteoderms are also called buttons, which may be either single or double buttons per scale depending on the species of crocodile. Other microscopic structures however, are similar to mammals.

The dermis in crocodiles consists of two layers, the superficial stratum proprium and the stratum corium that correspond to mammalian papillary layer, and reticular layer, respectively (Heaphy, 1989). The collagen fibres are loosely packed in the stratum proprium while in the stratum corium the fibres are tightly organised and running parallel to the skin surface. In some locations of the body where the skin is thin, the stratum proprium is absent. Mast cells in crocodiles are concentrated around the dermal component of the cutaneous papillae in contrast to mammals in which mast cells are mostly associated with the peripheral nerve endings and blood vessels.

Dorsal organs

The dorsal organs in *Crocodylus porosus* and *Crocodylus johnstoni* hatchlings are found beneath the antero-lateral edge of the first scale on either side of the dorsal midline and extend from a mid cervical region to the cloacal region. In *C johnstoni*, the dorsal organs have irregular lobules 2 mm in length and 1 mm in diameter (Heaphy, 1989). The dorsal organs vary in shape and size in other species of crocodiles (Chen, Jiang and Wang, 1991).

Histologically, each dorsal organ in *Alligator mississippiensis* (Cannon, Davis and Weldon, 1996) is covered with a capsule made up of dense collagenous and short elastic fibres, and is surrounded by skeletal muscles. The epithelium is composed of two to six layers of intact and

compacted organ cells found immediately next to the basal cells in *C porosus* (Chen *et al.*, 1991).

Cloacal glands

Marcus (1981) referred to cloacal glands that are all located in the cloaca of crocodilians of both sexes as scent glands. These paired glands have a holocrine type of secretion emptying their excretory products through minute openings visible in the skin. Histologically, these glands are divided into lobules by connective tissue; the lumen contains an oily substance, which functions as pheromones (Chen *et al.*, 1991).

Mandibular glands

The mandibular glands are cylindrical in shape, situated in the skin of the throat on the lateral sides of the jaw, in the Chinese alligator (*Alligator sinensis*). These glands are covered with a connective tissue capsule, and at least three regions of cells forming the inner part of the gland with its lumen at the centre (Chen *et al.*, 1991). The mandibular gland is a holocrine gland that secrets an oily substance with a pungent odour, especially during the breeding season.

2.2.5 Function of the skin

Crocodilian skin has dermal armour that is tough and leathery, presumably for protection against predators as well as enabling survival in fluctuating environmental conditions. The presence of osteoderms in the skin helps anchor the skin to the tendinous attachment of the epaxial musculature, thus promoting the lateral undulations generated in the trunk and tail of the crocodile (Seidal, 1979). The osteoderms also provide an efficient mechanism of heat distribution because of its association with the vasculature.

Davis, Spotila and Schefler (1980) have reported that the skin increases the evaporative water loss when the outside temperature increases, presumably as a means to regulate the temperature of the body.

The skin of crocodylids and gavialids contain darkly pigmented pits known as integumentary sense organs (ISO), on the post cranial scales. Although the function of the ISO is still not known, the presence of widely disperse fibrocytes, nerve terminals and chromatophores throughout the ISO region suggest that they are sensory (Jackson, Butler and Youson, 1996).

2.3 Diseases in Crocodiles

2.3.1 Bacterial Diseases

In crocodilians, most microorganisms encountered in any of the described bacterial infections whether systemic or localised infection have been identified as opportunistic pathogens rather than a primary agent causing the disease. Most of these organisms have been considered normal commensals in water, decaying material or probably in soil.

A survey on bacterial contamination in water from rearing ponds of crocodile hatchlings revealed the presence of different serotypes of *Salmonella* organisms. Samples from cloaca and faecal material were also cultured and found to have *Salmonella cerro*, *Salmonella singapore*, *Salmonella enteritidis* and *Salmonella arizona* (Manolis, Webb, Pinch, Melville and Hollis, 1991). *Aeromonas hydrophila*, *Edwardsiella* sp and *Pseudomonas* sp were recorded from a similar laboratory analysis on water samples in Queensland crocodile farms (Buenviaje *et al.*, 1994). The presence of salmonella in water and soil were from faecal contaminants and from leftover foods (Scanlan, 1988), and therefore crocodiles most likely became infected as soon as this microorganism gained its entrance to the body.

Bacterial septicaemia

Bacteria causing disease in crocodiles and in other reptiles are mostly Gram-negative microorganisms. Bacterial cultures of *Citrobacter freundii*, *Enterobacter agglomerans*, *Proteus* sp, *Morganella morganii*, *Serratia marcescens* and *Klebsiella oxytoca*, were obtained from organs with lesions in an American alligator (*A mississippiensis*) affected with Gram-negative septicaemia (Novak and Seigel, 1986). Affected animals had marked pink colouration of the skin suggesting septicaemia, dermatitis and stomatitis and traumatic injuries of the skin. Other clinical manifestations of the disease included intensive basking, anorexia, lethargy and flaccid paralysis.

Chakraborty, Basak and Majumber (1988) isolated a pleomorphic non-capsulated bacilli presumed to be *Proteus* sp from tissue samples of visceral organs of a two-year-old crocodile at the Zoological garden in Calcutta. Affected lungs were consolidated and had serofibrinous exudation. The tubular epithelium of the kidneys was desquamating, haemorrhagic and infiltrated with mononuclear cells. Other histopathological changes observed were coagulative necrosis in the liver and sloughing and ulceration of the intestinal villi.

Bacterial septicaemia/hepatitis has also been diagnosed in seven crocodile farms in Queensland and the Northern Territory (Buenviaje *et al.*, 1994). Subcutaneous oedema and ulcerations of the belly and tail particularly between scutes were common skin lesions. The liver, spleen and kidney were mottled and histological examination revealed the presence of Gram-negative bacteria in either or both intra- and extracellular locations in affected organs. Bacterial examination on the skin lesions revealed *A hydrophila* as the most frequently encountered pathogen, however other bacteria such as *Salmonella* sp, *Edwardsiella* sp, *Proteus rettgeri*, *Klebsiella* sp were also present. *A hydrophila* had been implicated in a number of disease outbreaks particularly septicaemia (Shotts, 1981). In contrast however Novak and Siegel (1986) were never successful in identifying *A hydrophila* from septicaemias in alligators.

Hatchlings that died of acute cases of septicaemia, a major cause of mortality in Nile crocodiles, were noticed to be in good body condition, with lethargy as the only notable sign (Foggin, 1987; 1992). Postmortem findings include fibrinopurulent peritonitis, pleuritis and pericarditis. The liver was sometimes swollen with petechial haemorrhages on the serosal surface, and the lungs had serous to purulent exudates particularly in the airways. *A hydrophila* and pure cultures of *Salmonella derby* were identified. Other Gram-negative bacteria including *S arizona*, *Chromobacterium* sp, *A hydrophila* and *Salmonella waycross* were identified (Ladds and Sims, 1990). The presence of an acute bacterial septicaemia in crocodiles in good condition implies that these bacteria were primary pathogens rather than secondary, in contrast to the report of Shotts (1981). It is impossible however to determine which bacteria are the primary pathogens in any of the bacterial septicaemia cases.

Localised bacterial infections

Similar opportunistic bacteria to those causing generalised infections (septicaemia) were also frequently isolated from localised lesions in various organs. It is likely that local infections could predispose to the spread of infections to the different parts of the body by way of the general systemic circulation or via lymphatic system causing septicaemia.

Several cases of chronic hepatitis and splenitis associated with fatty change in the liver were diagnosed in crocodile farms in Queensland and the Northern Territory (Buenviaje *et al.*, 1994). In some animals the liver and spleen showed variable degrees of mottling particularly on the surfaces. Histopathological examination revealed the presence of mild inflammatory response, only in the parenchyma.

A number of bacteria have also been identified from healthy crocodiles, particularly from the gular and cloacal glands in alligators (Weldon and Sampson, 1987; Williams, Mitchell, Wilson and Weldon, 1990). *Edwardsiella tarda* was considered the most common species, although the presence of these bacteria in the skin glands did not cause any trouble, although under favourable conditions to the microorganisms this would ultimately cause inflammation. *E tarda* was isolated in pure culture from the visceral organs of rainbow trout (*Oncorhynchus mykiss*) that died of septicaemia (Reddacliff, Hornitzky and Whittington, 1996).

Ophthalmitis

The pathology of ophthalmia encompasses any alterations involving the eye, and the aetiological agent involved could be parasitic, bacterial, fungal or viral. A vector such as flies (*Colesiota conjunctivae*) was shown to transmit *Rickettsia conjunctiva*, which causes ophthalmitis in cattle, sheep, goats, swine and chicken (Gillespie and Timoney, 1981a). Mild purulent conjunctivitis may recover within a week, however severe cases could progress to corneal ulceration, keratitis and vascularisation. The presence of ophthalmitis in malignant catarrhal fever is a consistent clinical finding in affected farms as well as zoo animals, mainly deer and bison (Wilcock, 1993a).

An epizootic form of ophthalmia in both hatchling and yearling crocodiles has been reported by Foggin (1987). The disease spreads extensively with initial serous discharge from the eyes and subsequently progressed to closure of the eyes as a result of caseous exudates accumulating in the conjunctival sac. The animals became blind and unable to eat properly and preferred to stay on land; this progressed to anorexia and the animal became severely emaciated. The lesions spread out and became more generalised affecting the skin of the head. Finally, the animals died of dehydration, malnutrition or from other secondary bacterial infections. Rickettsia-like organisms found intracellularly as demonstrated under the electron microscopy, were present.

Pneumonia caused by *Pasteurella multocida*

In farm animals, *Pasteurella multocida* is considered a normal commensal in the oropharynx, and causes disease only if the animals are exposed to predisposing factors such as poor sanitation, overcrowding, exposure to winter cold, intercurrent infections and stress (Gillespie and Timoney, 1981b). *P multocida* causes haemorrhagic septicaemia in cattle, goats and sheep, shipping fever in cattle, atrophic rhinitis in pigs and fowl cholera in birds. It has also been suggested that *P multocida* gain entrance through the eyes and skin abrasions in chickens.

Regarding possible infection with *P multocida* in reptiles, nothing has been reported yet (Cowan, 1968; Keymer, 1974; 1976; Goodwin, 1978; Jones 1978; Troiano and Roman, 1996). The first case of *P multocida* infection was reported in several American alligator (*A mississippiensis*) hatchlings in a zoological garden (Mainster, Lynd, Cragg and Karger, 1972). Post mortem examination revealed excessive accumulation of oedema in the lungs and the entire respiratory tract. These animals had some wounds on the skin and concussion on the head as a result of stoning by some people who broke into the garden. A few hatchlings died while others became progressively weaker and died later.

Salmonellosis

Salmonellosis is most frequently encountered as an enteric disease in young animals, and is a very important zoonotic disease. Transmission of the disease to humans can be carried out through ingestion of contaminated food and meat (Clarke and Gyles, 1986). Salmonellosis has been considered as one of the public health risks linked to the production of crocodile flesh of farmed crocodiles (Millan, Purdie and Melville, 1997). Salmonella species were commonly isolated from cloacal samples (Masden, Hangartner, West and Kelly, 1998) and the most likely cause of contamination in crocodile meat. Salmonella organisms are often associated with subclinical infections in reptiles particularly in snakes and tortoises. There have been 17 different serotypes of salmonella organisms isolated from the faeces of reptiles. The common manifestations of the disease are enteritis or septicaemia with necrotic foci in the liver and viscera (Marcus, 1971).

Huchzermeyer (1991) reported an outbreak of salmonellosis from a batch of almost 2,000 crocodiles in Eastern Transvaal. *Salmonella* sp and *Salmonella typhimurium* isolated from the affected animals were considered primary pathogens (Foggin, 1992). Post mortem examination revealed severe peritonitis, necrotic enteritis and focal liver necrosis as the outstanding lesions. In contrast, it was not certain if *Salmonella* sp was the primary cause of several reported cases of septicaemia where *Salmonella* sp was one of the bacteria identified from tissue samples (Shotts, 1981; Ladds and Donovan, 1989; Ladds and Sims, 1990; Buenviaje *et al.*, 1994).

Mycobacteriosis

Mycobacterial infection has been widely reported in wild species of animals, marine mammals and fish (Iudin, Lobuntsov and Grishchenko, 1972; Thoen, Richards and Jarnagin, 1977; Hardie and Watson, 1992). The occurrence of mycobacterial infections in a mixed group of animals

under confinement such as in zoos allows the spread of infection (Thorel, Karoui, Varnerot, Fleury and Vincent, 1998). Cross infection between man and animals and among species of animals is not uncommon (Pavlas and Mezensky, 1982; Cousins, Williams, Reuter, Forshaw, Chadwick, Coughran, Collins and Gales, 1993).

Among the reptilians, snakes appear to be commonly affected with mycobacterial infections (Cowan, 1968). The clinical signs of tuberculosis in reptiles are not specific and sometimes animals were only described as lethargic and exhibiting weight lost (Cooper, 1981). Postmortem findings revealed the presence of caseous nodules in internals organs, and on histopathological examination these were found to contain acid-fast organisms. Granulomatous lesions of the skin has been reported in side-nicked turtle (*Phrynops hilari*) (Rhodin and Anver, 1977). Involvement of the skin is crucial since *Mycobacteria* could find their way through the haematogenous route, and eventually throughout the body. The cutaneous lesions were described histologically as granulomatous inflammation in experimental infection of anole lizards (*Anolis carolinensis*) with *Mycobacterium ulcerans* (Marcus, Stottmeier and Morrow, 1975).

Reports of mycobacterial infection in crocodiles are limited, and the pathological description of the disease is inadequate. The reported case of mycobacteriosis in crocodiles by Zwart (1964) was presumably an incidental finding. Recently, 12 *C johnstoni* hatchlings with no external sign of infection except anorexia had caseous nodules in several visceral organs. A central zone of intensely eosinophilic amorphous debris surrounded with multinucleate giant cells, histiocytes and some lymphocytes contained acid-fast bacilli. A mycobacteria organism was also identified from infected tissues using the polymerase chain reaction (PCR) method (Ariel, Ladds and Roberts, 1997b).

Escherichia coli enteritis

The most common disease is septicaemic collibacillosis in which the portal of entry of the bacteria may not be necessarily from the alimentary tract. Collibacillosis has been a problem in captive wild animals that includes the American alligator (*A mississippiensis*), red neck wallaby (*Wallabia parma*), black-neck spitting cobra (*Naja nigricollis*), vampire bat (*Desmodus rotundus*), and a leopard cub (*Pantera nebulosa*) (Russell and Hernan, 1970). All these animals upon bacteriological examination showed almost pure culture of both non-hemolytic and hemolytic strains of *E coli*. Affected alligators had a swollen and haemorrhagic spleen, inflamed intestinal tract, purulent exudate throughout the lungs and the presence of straw-coloured fluid in the pericardial sac and abdomen.

E coli enteritis has been reported in crocodiles at the Crocodile Breeding Centre in Muta Ranchi, India (Sinha, Soman, Jha, Prasad, Chauhan and Prasad, 1987a). Nine of 40 crocodiles weighing 10 to 15 kg and about three to four years old died after exhibiting signs of anorexia, dullness, vomition and emaciation. On postmortem examination, the only significant lesion seen were in the small intestine in which there was noticeable catarrhal enteritis, and haemorrhagic enteritis was evident in the large intestines. Desquamation of the lining epithelial cells and severe infiltration of laminae associated with macrophages and neutrophils of affected intestines were seen microscopically. Bacteriological culture yielded *E coli* from organs with lesions.

Hepatitis due to Chlamydial infection

Chlamydia trachomatis of humans and *Chlamydia psittaci* are known to cause variety of diseases in mammals, birds and frogs (Newcomer, Anver, Simmons, Wilcke and Nace, 1982). This organism is a non-motile coccoid with a diameter ranging from 0.2 to 1.5 μm diameter, present within membrane-bound vacuoles in the cytoplasm of macrophages (Krieg and Holt, 1984).

Two separate cases of infections in *Chameleo fischeri* and captive juvenile puff adders (*Bitis arietans*) with chlamydia contained chlamydial organisms in the blood film, specifically from monocytes. Basophilic intracytoplasmic inclusion bodies within the macrophages from the liver and spleen were the characteristic appearance of developmental stages typical of a chlamydia organism (Jacobson and Telford, 1988).

Sudden deaths occurred in a group of 3,000 crocodile hatchlings in the Eastern Transvaal, Lowveld, South Africa. No other clinical signs were noticed except that the hatchlings found dead were in good body condition. Postmortem examination of all animals revealed a pale, mottled, and enlarged liver and spleen (Huchzermeyer, Gerdes, Foggin, Huchzermeyer and Limper, 1994a). Other findings were mild ascites, hydropericarditis and mild bilateral conjunctivitis. On histopathological examination the livers showed severe portal to diffused lymphoplasmacytic hepatitis with congestion, mild bile duct proliferation, vacuolar degeneration of hepatocytes and multifocal to coalescing necrosis. Typical chlamydial colonies at different stages of development were present in the cytoplasm of the hepatocytes on electron microscopical examination.

***Aeromonas hydrophila* infection**

There is much uncertainty whether or not *A hydrophila* is a primary pathogen but one thing that is very important is the frequent occurrence in most infections in reptiles (Shotts, 1981).

A hydrophila was implicated in various diseases like bacterial septicaemia in crocodiles (Buenviaje *et al.*, 1994), ulcerative stomatitis and septicaemia in snakes (Wallach and Boever, 1983) and high mortality rates among fish and reptiles in Florida (Shotts, Gaines, Martin and Prestwood, 1972). Affected alligators had several lesions affecting the respiratory system such as necrotic plugs in the bronchi and mucopurulent exudates in the lungs. Nodules 3 to 4 mm in diameter were scattered throughout the parenchyma of the lungs. *A hydrophila* was believed to be the offending agent because of its constant occurrence not only from defective tissues but also its presence in cultures from sewage treatment, citrus-processing plants and from the blood samples.

A hydrophila isolated from the internal organs of nine adult *A mississippiensis* was believed to have produced lytic toxins responsible for sudden and unexpected deaths in alligators without premonitory signs (Gorden, Hazen, Esch and Fliermans, 1979). The findings of bacteriological examination on normal animals revealed 80% had *A hydrophila*. Certainly, *A hydrophila* is a normal commensal of the body but under certain circumstances such as stress could favour rapid multiplication of the bacteria thereby causing a disease.

A disease called 'September disease' affecting farmed alligator hatchlings has been reported to be due to *A hydrophila* (Cardeilhac, 1990). In mild cases there were neurological signs such as nervousness, abnormal posture and 'stargazing'. In more severe cases accumulations of bloody fluid were found in the intestines and in the lungs associated with pulmonary congestion as well as bloody exudates from body openings.

Dermatophilosis

Dermatophilosis is worldwide in distribution involving countries in Africa, Europe, Middle East, America and Asia. *Dermatophilus* is a soil-borne bacterium, an important consideration regarding the possible source of infection in animals. It is a Gram-positive and filamentous bacterium classified under the Actinomycete group (Cottial, 1978), and divides both transversely and longitudinally to form pockets of coccoid cells (Gordon, 1976). A thorough discussion of *Dermatophilus* infection in man and animals emphasised above all the importance of skin disease particularly in cattle (Lloyd and Sellers, 1976). Other animals including goats and sheep (Munz, 1976), primates (Kaplan, 1976), marine and wild species of animals were also affected. Dermatophilosis is of great economic importance in domestic livestock, because once the skin is affected, the value of leather is downgraded (Lloyd, 1976). Dermatophilosis has also been reported in man. The lesions on the skin were characterised as pitted keratolysis, nodular or

pustular lesions (Albrecht, Horowitz, Gilbert, Hong, Richard and Connor, 1974; Kaminski and Suter, 1976; Gillum, Qadri, Al-Ahdal, Connor and Strano, 1988; Towersey, Martin, Londero, Hay, Soares Filho, Takiya, Martin and Gompertz, 1993).

Initial reports of Dermatophilosis in reptiles occurred in an Australian bearded lizard (*Amphibolurus barbatus*) (Simmons, Sullivan and Green, 1972). The lesions presented as a subcutaneous nodule 0.75 cm diameter on the ventral surface of the abdomen and the forelimbs. Microscopical examination confirmed the nodule as a subcutaneous abscess, which contained a caseous material extending to the abdominal musculature. In addition, the abscess had a central necrotic core encapsulated by granulation tissue, and some lesions had developed to a fibrous nodule with central calcifications. *Dermatophilus congolensis* was recovered from the central core of the abscess. The lesions in another case of dermatophilosis infection of an Australian bearded lizard showed multiple, raised, golden-brown cutaneous nodules on the head, body and extremities (Montali, Smith, Davenport and Bush, 1975). These nodules had numerous branching filaments of *D congolensis* actively infiltrating towards the outer epidermal layer accompanied by heterophilic granulocytes.

A proposed new species of *Dermatophilus chelonae* was identified from nodular lesions in turtles (Masters, Ellis, Carson, Sutherland and Gregory, 1995; Trott, Masters, Carson, Ellis and Hampson, 1995). The clinical findings and the pathology of the disease were not further described.

No reports on the occurrence of dermatophilosis have ever been documented in crocodiles. In the initial report of the incidence of 'brown spot' disease affecting the skin of farmed alligators (Newton, 1992), the lesions contained filamentous organisms resembling *Dermatophilus*. Bounds and Normand (1991) isolated *Dermatophilus* organisms from similar 'brown spot' lesions however, it was not mentioned in their report if the identified organism was the aetiological agent. An outbreak of 'winter sores' dermatitis in Nile crocodiles characterised as 'brown spots' or crust between scales of the belly skin, were presumably similar to 'brown spot' disease (Huchzermeyer, 1996). Frequent identification of a Gram-positive filamentous bacteria in skin lesions had been reported, however the identity of the organism was not confirmed (Ladds and Donovan, 1989; Ladds and Sims, 1990; Buenviaje *et al.*, 1994).

Other bacterial diseases

Two crocodiles (*Caiman crocodylus* and *Crocodylus acutus*) infected with *Erysipelothrix insidiosa* had an enlarged spleen, cirrhosis of the liver and irregularly diagonally brownish to blackened plaques ranging from 2 to 4 cm were present on the skin (Jasmin and Baucom, 1967). Histopathological examination of skin only revealed hyperplasia of the epidermis associated with necrosis while the spleen and liver showed no significant lesions. Bacteriological examination however, revealed the presence of *E insidiosa* in liver, spleen and skin. Other bacteria such as *Proteus* sp and coliforms, and fungi identified as *Aspergillus*, *Mucor* and *Rhizopus* sp were regarded as secondary pathogens.

Mohan, Foggin, Muvavarirwa, Honeywill and Pawandiwa (1995) reported outbreaks of polyarthritis in farmed crocodiles (*Crocodylus niloticus*) on five farms in Zimbabwe. Animals affected were between one and three years old and consistently displayed swollen limbs and joints, which eventually progressed to lameness and paresis. *Mycoplasma* sp was isolated in pure culture from animals with affected joints. *Mycoplasma crocodyli*, characterised through microbiological and DNA tests, was isolated from the lesions of the joints and lungs of infected crocodiles (Kirchhoff, Mohan, Schmidt, Runge, Brown, Brown, Foggin, Muvavarirwa, Lehmann and Flossdorf, 1997).

Clostridium limosum has been isolated from the kidney and liver of alligators with symptoms of paralysis (Cato, Cummins and Smith, 1970). *Clostridium* sp was also isolated in pure culture from the oedematous fluid of ailing *Gavialis gangeticus* (Misra, Kumar, Patnaik, Raman and Sinha, 1993). This organism was the cause of heavy mortality of fresh water Indian crocodiles at the Gaharial Research and Conservation Unit, Orissa, India. Initial signs and symptoms showed oedematous swelling of the abdomen and all limbs, and necrotic ulceration with oozing of fluid from the lower eyelids.

2.3.2 Fungal Diseases

Fungi are known as ubiquitous microorganisms commonly present in soil especially in association with decaying materials. These organisms can readily adapt to cause infection in man and other animals. Under favourable conditions such as during cold weather (Huchzermeyer, 1992), fungi can rapidly multiply in their host into which they usually gain entrance by way of skin abrasions or bite wounds. Surprisingly, some fungi identified as plant

and insect pathogens were reported as pathogenic to reptiles including crocodiles (Hibberd and Harrower, 1993).

Fungal infections have been considered a serious disease problem of captive reptiles when there was mismanagement especially regarding temperature and inadequate aseptic procedures. In one crocodile farm in Rockhampton, Queensland, fungal disease was the main cause of high mortality and economic losses due to devaluation of leather and high cost of treatment (J Lever, 1990, personal communication).

Infection of the skin initiated by fungi eventually develops as a severe dermatitis complicated by secondary infections. In such situations arriving at a definitive diagnosis of fungal infection can be very difficult unless the appropriate lesion associated with fungi is sampled. Detection of fungi in histological sections plays a very significant role in the diagnosis of deep fungal infection (Campbell, Davis and Mackenzie, 1985). Reported cases of fungal diseases in crocodiles were mostly diagnosed through histopathological examination of affected tissues, and less often by isolation and identification of fungi from tissues with lesions.

Trichoderma sp infection

Trichoderma, a member of the *Fungi imperfecti* group that normally lives on soil are a common cause of diseases in plants and animals (Landecker, 1972). Although many cases of fungal infection were reported, nothing appears to have been described about the occurrence of *Trichoderma* infection in reptiles (Hoff, Frye and Jacobson, 1984).

A single case of a female American alligator covered with a fungus-like material throughout the surface of the skin has been reported to be infected with *Trichoderma* sp (Foreyt, Leathers and Smith, 1985). In stained sections of the skin the fungal hyphae appeared as pale, eosinophilic filaments on the superficial layer of the skin as well as adjacent to the cutaneous layer of keratin. Fungal culture performed by swabbing the lesions on the skin inoculated into Sabouraud's medium and incubated at 30°C confirmed the presence of *Trichoderma* sp.

Fusarium solani infection

F solani has been considered a non-systemic pathogen in reptiles, however it can readily colonise damaged skin surfaces and invade the underlying tissues (Austwick and Keymer, 1981). This fungus has demonstrated its ability to shift to a pathogenic type under favourable

conditions. *F solani* infection had also been reported in man (Austwick, 1986) and a diverse range of species of animals including elm bark beetles (*Scolytus scolytus*) (Barson, 1976) and California sea lions (*Zalophus californianus*) (Montali, Bush, Strandberg, Janssen, Bonèss and Whitla, 1981). The high prevalence of *F solani* infections in crocodiles was also noted by Buenviaje *et al.* (1994) in crocodile farms in Rockhampton and Innisfail, Queensland.

A high mortality rate of *C porosus* hatchlings at a crocodile farm in Rockhampton was caused by mycotic infection (Hibberd and Harrower, 1993). In 1989 and 1990, a 50% mortality was recorded each year. *F solani* was the most common fungus identified in lesions (Hibberd, Pierce, Hill and Kelly, 1996). Another species of *Fusarium* was also identified particularly in the hypertrophic chorioallantoic membranes of *A mississippiensis* eggs (Schumacher and Cardeilhac, 1990). A strong proteolytic and lipolytic substance produced by *F solani* allows fungal hyphae to penetrate the eggshell of the snake, *Elaphe guttata* (Kunert, Chmelik and Bic, 1993).

Juvenile crocodiles affected with *F solani* showed signs of dehydration and loss of condition and appetite (Hibberd and Harrower, 1993). There were abundant hyphae observed on the jaw, around the eyes and within the buccal cavity. Multifocal necrotic lesions of varying size were noted on the feet and abdomen of some animals. In advanced cases, severely affected animals had lost their teeth and claws. Lesions of the liver, lung and intestines and skin had fungal hyphae. Samples from tissues with gross lesions were examined further by isolating the fungi using Potato Dextrose agar and Sabouraud's Dextrose agar. The mycelia on the plates were subsequently identified as *F solani*. Samples of air from the farm environment, both externally and inside the farm buildings, and from the soil, well water, scrapings from paths, floors and walls (external and internal), food preparation benches and indoor growing pens were found to contain *F solani*.

Cephalosporiosis

Cephalosporium, classified with the *Deuteromycetes*, is a fungus considered ordinarily as saprophytic and an opportunistic pathogen for certain trees, insects and plants (Pisano, 1963). Cephalosporiosis has been reported to cause disorders such as allergies (Blumstein, 1945) and arthritis (Ward, Martin, Ilvins and Weed, 1961) in humans. In reptiles, the first reported *Cephalosporium* sp infection was from a snake (Rodhain and Mattlet, 1950). Hibberd (1994) also identified *Cephalosporium* sp as one of the most common species of fungi in crocodiles.

Several caimans affected with cephalosporiosis had miliary nodules measuring approximately 2 to 10 mm in the lungs (Trevino, 1972). The lesions were discrete greyish-white nodules protruding on the surface of the lungs. Similar circumscribed greyish-white areas measuring 1 to 8 mm in diameter were also observed in the liver in addition to greyish thick-walled cysts present in both the liver and kidney. In some animals the muscle layers of the small intestine had minute white nodules while other organs had no other visible lesions. A granuloma filled with caseation necrosis surrounded by a mixture of heterophils, lymphocytes and large epithelioid cells associated with masses of tangled fungal hyphae was seen microscopically. Another significant lesion observed in this study was the presence of thrombosed vessels infiltrated with mats of fungal hyphae, indicating intramural invasions of fungi. Identification of the isolated fungi based on morphological characteristics confirmed *Cephalosporium* sp as the aetiological agent.

Pulmonary Aspergillosis

Aspergillus organisms are abundant in the environment and live in the soil as a saprophyte, deriving nutrients from dead plants and animal matter. All species of *Aspergillus* are cosmopolitan in distribution enhanced by the dissemination of spores into the air by wind currents (Chandler, Kaplan and Ajello, 1980) and therefore the organ most commonly affected is the lung. According to Gillespie and Timoney (1981c) pulmonary aspergillosis has been described mostly in birds, horses, lambs, calves and man; a few cases have been reported in amphibians and reptiles (Reichenback-Klinke and Elkan, 1965).

In reptiles, aspergillosis was reported in chelonia with nodular lesions containing hyphae in the lamina propria and cartilage of the lungs (Andersen and Ericksen, 1968), and in severe cases the lungs became consolidated and gangrenous (Hamerton, 1934; 1937; 1938; 1939). *Aspergillus* sp has also been recovered from skin lesions in crocodile hatchlings (Hibberd and Harrower, 1993; Buenviaje *et al.*, 1994; Hibberd, 1994). In most instances however it is not certain whether the lesions were caused primarily by *Aspergillus* sp because other possible aetiological agents were also present.

Jasmin, Carroll and Baucom (1968) described in detail the pathology of aspergillosis of two to six-week-old American alligators from the zoological gardens in Florida. The mortality rate was high and affected hatchlings were lethargic with no other remarkable gross lesions noted. The entire skin, particularly the scales of the abdomen and around the jaws and nostrils had slight desquamation. There were small foci of necrosis on the skin between the tail and the dorsal spines. Postmortem findings included pneumonia associated with greyish nodular areas of

necrosis and retention of the yolk material in the remnants of the urachus. A mixture of various fungi that were not considered significant was isolated from the skin whereas two distinct colonial types of fungi that conformed to the descriptions of the *Aspergillus fumigatus* and *Aspergillus ustus* groups respectively were isolated from necrotic lung nodules.

Fatal *Beauveria bassiana* Infection

Beauveria bassiana, a ubiquitous soil saprophyte is a well-recognised entomopathogen responsible for causing a 'muscardine disease' in silkworms where its thick mycelial filaments cover the entire body of the affected silkworm (Landecker, 1972). This fungus was also reported to cause skin infection in a young woman (Freour, Lahourcade and Chomy, 1962). The first reported case of Beauveriosis in reptiles was in the giant tortoise (*Testudo elephantina gigantica*) (Georg, Williamson and Telden, 1962).

In crocodiles, a distinctive pulmonary 'fungus ball' formation in the lungs caused by *B bassiana* has been reported by (Utz, Austwick and Loeffler, 1977). Another case of fatal disseminated Beauveriosis in captive American alligators on necropsy examination revealed large masses of white fluffy materials contained in the thoracic cavity (Fromptling, Jensen, Robinson and Bulmer, 1979). An estimated 60% of the pulmonary tissue was damaged by fungal growth transforming the normal pulmonary parenchyma into a dark consolidated tissue. Further microscopical studies revealed an unusual sporulation of colonies, which were prominent in the pleura and air spaces as well. Fungal isolation on Sabouraud's medium showed small creamy-coloured colonies with a fluffy texture and some colonies contained diffusible red-brown pigments. On microscopical examination the fungi were described as hyaline, septate, branching hyphae (3 to 5 mm in diameter) and confirmed the appearance as typical of *B bassiana*. Histological examination of lung tissue also contained various multifocal granulomas associated with fungi. Inflammatory cells present were a combination of mononuclear cells, heterophils, and occasional multinucleate giant cells in the granulomatous lesions.

Paecilomyces infection

Several infections with *P lilacinus* have been reported in man causing chronic facial lesion and mycotic keratitis (Chandler *et al.*, 1980). *Paecilomyces* is the most common fungus associated with the systemic mycoses of reptiles, as it accounted for nine of the 59 isolates reported (Austwick and Keymer, 1981). There were 63 cases of fungal infection confirmed from 111 cases of suspected mycoses from the Zoological Society's garden in London (Goodwin, 1974).

P lilacinus was recovered in five of six crocodiles and alligators and one of four turtles with chronic respiratory and systemic mycosis. Likewise, *P lilacinus* was also isolated from lungs of spectacled caiman and alligator in the same zoological garden (Keymer, 1976).

Affected American alligators had a multifocal miliary or nodular caseating pneumonia with mucoid exudate, and associated within the granuloma were a mixture of other inflammatory cells, caseation necrosis and hyphal mats of *P lilacinus* (Keymer, 1974). Similar gross and microscopical lesions in the lungs of affected *C crocodylus* and *C niloticus* were also described by Keymer (1974; 1976). Other species of *Paecilomyces* such as *Paecilomyces farinosus* was isolated in *A mississippiensis* that died of chronic mycotic pneumonitis (Goodwin, 1978; Jones, 1978).

In Australia, *Paecilomyces* sp infections in crocodiles were recorded from a farm at Rockhampton (Hibberd and Harrower, 1993) and on several farms in the Northern Territory (Buenviaje *et al.,* 1994). Skin lesions observed in those cases however, were not specific for *Paecilomyces* infections because other microorganisms were isolated concurrently from them. A relatively precise account on the pathology of *Paecilomyces* infection in *C porosus* was described by Maslen, Whitehead, Forsyth, McCracken and Hocking (1988). Affected hatchlings were weak associated with mild hind limb and tail paralysis, sometimes found dead without premonitory signs. One crocodile had fibrous adhesions present in the viscera and multiple granulomas in the liver, lungs and spleen. Sections from the liver and spleen showed granulomatous inflammatory response and the granuloma consisted of a very distinct inner core made up of necrotic cellular debris and faint basophilic filaments of fungal hyphae. On Grocott's stain, the hyphae appeared clearly as branching septate fungi approximately 2 µm wide infiltrating the outer core of the granuloma. The result of the fungal isolation from lesions in the liver was typical of *P lilacinus*.

Other fungal infections

There have been a number of reported cases of fungal skin infection in crocodiles, however, the pathology was not clearly described. Moreover, microscopical examination of mostly superficial skin lesions in crocodiles reveals invasions by a mixture of various bacteria, fungi and protozoans, and to implicate any one organism as the primary pathogen is very difficult. In this section however fungi whether isolated from a lesion or identified from skin scrapings are hereby presented purposely to record their presence in crocodile diseases.

Trichosporon cutaneum was identified in smears taken from brownish discolouration on the oral mucosa of caimans of the species *Paleosuchus palpebrosus* and *Paleosuchus trigonatus* at Kosice zoo (Ladzianska, Pauerova, Lasanda and Svec, 1989).

In North Queensland, Thomas (1995) reported two separate skin infections associated with *Chrysosporium tropicum*. In the first infected crocodile *C tropicum* was the only organism isolated and thus considered the aetiological agent. The creamy cheese-like lesions under the scales of the head, back and feet were present in affected animals. *C tropicum* was identified concurrently with *A hydrophila* in the second infected crocodile.

Metarhizium anisoplae was also reported by Goodwin (1974) and Keymer (1974) as responsible for the formation of miliary nodules in the liver and lungs accompanied by plaque-type lesions on the mucosal lining of the bronchioles.

Goodwin (1978) and Jones (1978) reported the presence of *Mucor circinelloides* causing greenish-raised ulcers of the stomach measuring up to 20 mm in diameter. Some areas of the mucosal lining were necrotic, associated with inflammatory cells and numerous hyphae. *Mucor* sp was identified from lesions in the lungs and intestines of infected crocodiles (Silberman, Blue and Mahaffey, 1977).

Buenviaje *et al.* (1994) reported two types of fungi from skin lesions examined at Berrimah Veterinary Laboratory, Darwin, Northern Territory. These two fungi were identified as *Curvularia lunata varaeria* and *Penicillium oxalicum*, but because of their superficial nature incriminating any particular fungus as the primary pathogen was not possible.

Scott and Simpson (1996) reported the presence of white moulds with a slight brownish tint in colour, present in the lungs of two American alligators. Affected lungs contained fungal masses growing within the air spaces, and *Scopulariopsis* sp was identified based on the morphology and growth characteristics. This fungus had septate and non-pigmented hyphae and the mature conidia were non-pigmented, approximately 7 to 8 mm in diameter. The conidia were thick-walled and lemon shape with a short 'neck' or flattened area at their base.

2.3.3 Parasitic Diseases

The study of parasitic diseases in traditional farm and companion animals is well understood compared with such diseases in reptiles particularly crocodiles. Although a greater number of

parasites had been identified in reptiles there is still very limited information on the pathological changes they cause. Moreover, reptilian parasites especially protozoans were incorrectly identified and await further description (Keymer, 1981). Such taxonomic confusion had compromised definitive diagnosis of the disease. Several reports on the presence of parasites in crocodiles have resulted from surveys either in the field or in zoos usually on samples collected from apparently healthy animals rather than from animals with overt disease. In addition, the majority of published reports describe the morphological structure of the parasites and very few described the lesions produced.

Parasites associated with pathological changes in tissues

Coccidiosis

Where large numbers of animals are raised under intensive husbandry management, coccidiosis may be among the major disease problems encountered. Young animals generally are most susceptible, as are crocodile hatchlings. Parallel to coccidiosis in traditional farmed animals, genera of *Eimeria* and *Isospora* are common in reptiles (Keymer, 1981). They have been reported to cause cholecystitis and intensive catarrhal and diphtheroid inflammation involving the small and proximal part of the large intestine.

In Southern Texas 67% of the 30 *A mississippiensis* were infected with coccidiosis identified as *Eimeria alligatori* (McAllister and Upton, 1990). Aquino-Shuster and Duszynski (1989) identified three species of coccidia in caimans namely, *E paraguayensis* and *E caimani* from *Caiman yacare*, and *Isospora jacarei* from *Caiman latirostris* in Paraguay.

Outbreaks of coccidiosis in Zimbabwe caused severe swelling, congestion and haemorrhages on the serosal surfaces of the intestine (Foggin, 1987). Histopathological changes included atrophy of the villus and inflammatory cell response in the lamina propria associated with various stages of coccidia. Several parasites present in the liver, spleen and lungs were presumed to have predisposed to secondary bacterial enteritis and granulomatous hepatitis.

The main cause of serious illness and death in seven crocodiles from Papua New Guinea was coccidiosis (Ladds and Sims, 1990). The sporulated coccidian oocysts that resembled *Goussia*-like organisms (Gardiner, Imes, Jacobson and Foggin, 1986) were found in the red pulp of the spleen, the interstitium of the lungs and within sinusoids of the liver. The affected wall of the intestines had fusion of the villi, epithelial hyperplasia and mononuclear infiltration of the lamina

propria. In addition, foci of suppurative pneumonia associated with the parasites were present, but other organs with obvious coccidiosis had no inflammatory response.

Filariasis

Fifteen genera and fifty species of filarial nematodes have been found in snakes and lizard (Hoff *et al.*, 1984). The microfilariae were present in the subcutaneous tissue, in hepatic sinuses, the heart chambers, major vessels, intestinal mesentery and the coelomic cavity. Diagnosis of filariasis can be made by the detection of microfilaria in blood smears of affected animals several days after infected mosquitoes have bitten them.

Several species of filaria were identified in crocodiles, however the report did not indicate any pathological changes associated with the parasites. A number of filaria was identified including *Micropleura vazi* in *Caimans sclerops* in Paraguay (Goldberg, Bursey and Aquino-Shuster, 1991), *Oswaldofilaria kanbaya* in *C porosus* from Australia (Barrow, 1988) and *Micropleura vivipera* in *C niloticus* in Zimbabwe (Foggin, 1987).

Telford (1984) reported the presence of adult worms in major blood vessels including the mesenteric and carotid arteries, renal portal veins, and posterior vena cavae in pythons (*Python reticulatus*). Such occlusion to the blood vessels resulted in thrombarteritis verminosa that led to gangrene on the tail tip and dermal ulceration (Frank, 1981). Other lesions seen in affected blood vessels included arterial granulomas containing filarial worms accompanied by calcification. A granulomatous inflammation of the sub-pleura of the lungs in young captive crocodiles in Papua New Guinea was associated with *Micropleura* sp (Ladds and Sims, 1990). *Micropleura* sp was implicated as the cause of severe parasitic infections of farmed young crocodiles in Irian Jaya (Ladds, Mangunwirjo, Sebayang and Daniels, 1995).

Capillariasis

Capillariasis has been reported in a wide range of hosts affecting different parts of the body (Cheng, 1986). Several deaths in humans in the Philippines were due to intestinal capillariasis. Migrating larvae of *Capillaria* sp produced a granulomatous reaction in the liver and pneumonitis in the lungs. Subcutaneous nodules, oedema and blisters were diagnosed in monkeys affected with *Capillaria cutanea* (Soulsby, 1969).

In reptiles, *Capillaria* spp were diagnosed in livers of lizards and snakes, and in oviducts of the snake *Coluber constrictor priapus* (Frank, 1981). The eggs of *Capillaria* were seen microscopically in the laminar layers of the stratum corneum of the skin in an American

crocodile (*Crocodylus acutus*). There are two species identified as the cause of 'skin capillariasis', *Paratrichosoma recurvum* (Moravec and Vargas-Vasquez, 1998) and *Paratrichosoma crocodilus* (Ashford and Muller, 1978). 'Skin capillariasis' can be easily recognised because it presents clinically as a very distinctive appearance of zigzag trails or 'squiggles' on the skin. It was previously thought to be a normal characteristic of the Orinoco crocodile (*Crocodylus intermedius*) (Brazaitis, 1987).

The morphological characteristics of the nematode *Paratrichosoma crocodylus* described by Ashford and Muller (1978) was reviewed and later renamed as *Capillaria crocodylus* by Spratt (1985). The results of the recent review on the morphological characteristic of *Paratrichosoma recurvum*, believed to be a congeneric species of *Paratrichosoma crocodilus*, was different and thus suggested to be retained as another species of *Paratrichosoma* (Moravec and Vargas-Vasquez, 1998). Several cases of capillariasis in *C niloticus*, *C porosus*, *C johnstoni*, *Crocodylus moreletii* and *C intermedius* have been documented by Telford and Campbell (1981). The presence of similar serpentine tunnels on the skin of an Indian mugger (*Crocodylus palustris*) presumed to be *Paratrichsoma* infection was also observed by Jacobson (1984). Adult worms, fourth stage larvae and eggs of the parasite were recovered from serpentine tunnels on affected skin.

Ascarid infection

As in mammals, ascariasis is a major parasitic infection in reptiles and evidence of host specificity has also been noted in some species of reptiles (Sprent, 1984). The species of *Ophidascaris* and *Polydelphis* are only present in snakes; *Paraheterophylum* are only in sea snakes while *Sulcascaris* and *Angusticaecum* are found only in chelonians. Other ascaridoids, such as *Orneoascaris*, *Goezia* and *Terranova*, however, have a wide range of reptilian hosts.

Among the ascarids, the genera of *Dujardinascaris* are often implicated as the cause of parasitic diseases in crocodilians. According to Scott (1995) *Dujardinascaris waltoni* had the highest prevalence rate among nematodes in a disease surveillance study conducted on *A mississippiensis* in Texas and Louisiana. Infection was highest in wild immature alligators, less in farmed immature alligators kept outdoors, and absent in farmed immature alligators found indoors.

In the Northern Territory, Barrow (1988) reported the occurrence of *Dujardinascaris taylorae* in wild *C porosus* only, while *Gedoelstascaris australiensis* was found in both *C porosus* and *C johnstoni*. No gross lesion associated with the presence of these parasites in the stomach was

noted. In contrast, Ladds and Sims (1990) reported the presence of *Dujardinascaris mawsoni* attached to the ulcerated mucosa of the stomach. Gastric ulceration and granuloma were present in 41% of the crocodiles affected with *Dujardinascaris mawsoni*. A severe necrotising ulcerative gastritis was also observed in alligators from South Carolina affected with *Dujardinascaris* (Jacobson, 1984).

Pentastomiasis

Pentastomiasis is a parasitic disease affecting the respiratory tract in several species of reptiles. This lungworm can be highly pathogenic to its host, and sometimes fairly specific to its host (Telford, 1971). In snakes, *Armillifer* sp is found commonly in pythonids and viperids, *Kiricephalus* sp in colubrids and *Porocephalus* sp from boid and crotalids (Hendrix, 1988). The adult pentastomes in reptilian definitive hosts can perforate the lungs and migrate to different sites of the body. Erratic migration of the parasites leads to systemic eosinophilia with signs of toxaemia or septicaemia. Riley (1994) recognised *Sebekia* sp as pathogenic to chelonians and fish. Of the 23 dwarf crocodiles (*Osteolaemus tetrapis*) only two were not infected with lung worms. *Sebekia okavangoensis* and *Alofia parva* were present in the lungs and body cavity of the 22 dwarf crocodiles (Riley and Huchzermeyer, 1995). The pentastome, *Sebekia wedli* collected from naturally-infected fish, Mosambique bream (*Oreochromis mossambicus*) and red-breasted bream (*Tilapia rendalli swierstrai*) were recovered from the lungs of two young Nile crocodiles (*C niloticus*) after an experimental transmission (Junker, Boomker and Booyse, 1998).

Pentastomiasis has also been increasingly reported in various species of crocodilians. Pentastomes of the genera *Sebekia* and *Alofia* were found in the lungs of African dwarf crocodiles (*O tetrapis*) in the Congo Republic (Huchzermeyer and Agnagna, 1994). *Sebekia oxycephala* was identified in alligators from South Carolina (Hazen, Aho, Murphy, Esch and Schmidt, 1978) and *Alofia platycephala* from the lungs of *Caiman crocodylus yacare* (Troiano, Martinez and Bravo Ferrer, 1996). *Selfia porosus* that closely resembled *Alofia* sp in regard to its morphological appearance, was obtained from the bronchioles of *C porosus* in Australia (Riley, 1994). Pentastomes identified in crocodiles in the Northern Territory included *Sebekia* sp and *Leiperia* from *C porosus* and *C johnstoni* both wild and in captivity, and *Alofia* sp from saltwater crocodiles (Barrow, 1988). A new species called *Leiperia australiensis* collected from infected lungs of *C porosus* and *C johnstoni* has recently been identified (Riley and Huchzermeyer, 1996).

On gross examination of pentastomiasis in *C porosus*, there were multiple dark foci sometimes reddish in colour embedded in the lungs but slightly protruding on the surface of the pleura.

Eggs and adult pentastomes present histologically were surrounded by multinucleate giant cells and other mononuclear cells (Buenviaje *et al.*, 1994). *S oxycephala* was present in the lungs, and in the liver associated with severe hepatic lipidosis in captive alligator hatchlings (Boyce, Cardeilhac, Lane, Buergelt and King, 1984). The clinical manifestations of crocodiles were reduced weight and respiratory distress which eventually resulted in death within a few days due to necrosis and haemorrhage in liver and lungs (Hazen *et al.*, 1978).

When exposed to stressful conditions, especially if the supply of oxygen in a reptilian host is inadequate, the pentastomes tend to become active and are stimulated to wander around the body (Hendrix, 1988). In snakes in particular, pentastomes of the genera *Kiricephalus* can migrate to dermal or subcutaneous sites beneath the scales. The pentastome *Kiriciphalus* can infect other animals such as dogs, cats and even humans as incidental hosts.

Fluke infestation

The digenean flukes are well recognised as a cause of parasitism in domestic animals while the monogenean flukes are chiefly parasites of cold-blooded aquatic vertebrates such as fishes, amphibians and reptiles (Soulsby, 1969).

Although flukes are present in reptiles, a limited number of reports have been published regarding the pathology of the disease (Frank, 1981). In snakes (*Boa constrictor*), a renal fluke *Styphlodora horrida* occluded the ureter, which resulted in the accumulation of cellular debris, mucus and urates leading to nephritis and visceral gout. Hyperplasia of the epithelial lining of the renal ducts associated with fibrosis and encapsulation of the parasites was also present (Brooks, 1984). Other flukes such as *Gogatea serpentium* caused blockages of the bile ducts in water snakes (*Erpeton tentaculatum*), which resulted in severe jaundice that eventually killed the snake.

A total of 64 digenetic trematode parasites was identified from crocodilians (Catto and Amato, 1994), and a greater occurrence of trematode infection was observed among mature alligators in the wild than in their immature counterparts (Scott, 1995). Different species of flukes from crocodiles both in the wild and under captivity were identified in the Northern Territory (Barrow, 1988). Flukes identified in different quarters of the small intestine were *Polycotyle ornata*, *Pseudocrocodilicola americaniense*, *Acanthostomum coronarium* and *Archeodiplostomum acetabulum* (Hazen *et al.*, 1978). Two flukes, *Deurithitrema gingae* (Blair, 1985) and *Renivermis crocodyli* (Blair, Purdie and Melville, 1989) were recovered from the kidneys of *C porosus* and *C johnstoni*, respectively. Of these flukes, a blood fluke (*Griphobilharzia amoena*)

and an intestinal fluke (*Cyathocotyle crocodili*) were significant in regard to their pathogenicity (Ladds and Sims, 1990; Ladds *et al.*, 1995).

A granulomatous inflammatory response associated with blood flukes was found in various organs of the body (Buenviaje *et al.*, 1994). Disseminated focal granulomatous pneumonia in *C novaeguineae* was associated with massive infestation numbers with blood flukes (Ladds *et al.*, 1995). According to Barrow (1988) crocodiles as young as two weeks of age were infected leading to serious illness due to secondary uraemia. The intestinal fluke (*C crocodili*) caused ulcerative enteritis characterised microscopically by hyperplasia of the remaining epithelium and loss and irregularity of the mucosal layer of the intestine. The immediate vicinity of the parasites had fibro-cellular exudation and the lamina propria was infiltrated with mononuclear inflammatory cells (Ladds and Sims, 1990).

Parasites identified with low or doubtful pathogenicity

Blood protozoan infection

Several species of blood protozoans have been reported in reptiles (Ball, Chao and Telford, 1969; Telford, 1984). *Hepatozoon* sp was found in red blood cells of crocodiles (Huchzermeyer and Agnagna, 1994). Another blood parasite, *Progarnia archosauriae* was found to infect not only the red blood cells but also leucocytes and thrombocytes of *C crocodylus* (Lainson, 1995).

Haemogregarina sp parasitised various crocodilian species such as, *C porosus* and *C novaguinea* (Ladds and Sims, 1990), *C johnstoni* (Buenviaje *et al.*, 1994), and *Crocodylus mindorensis* (Villapa, Jamerlan and Tsubouchi, 1990). *Haemogregarina crocodilinorum* was identified in *A mississippiensis* (Cherry and Ager, 1982). In addition to haemogregarines, a different genus of Hepatozoon was found in blood smears of infected Nile crocodiles (Telford, 1984). Different stages of *Hepatozoon* sp were also demonstrated in histopathological sections of the mucosa of the duodenum, jejunum, stomach and lung of infected African dwarf crocodiles (*O tetrapis*) (Huchzermeyer and Agnagna, 1994).

Haemogregarines are found within the cytoplasm of red blood cells however, extracellular forms were observed occasionally (Ladds and Sims, 1990). The intracellular location of the parasite caused enlargement of red blood cells and subsequent peripheral displacement of the nucleus. No obvious pathological changes were associated with the parasites other than haemosiderosis, however, there is some uncertainty as to its relationship with the parasites (Ladds and Sims, 1990). Haemolysis of red blood cells was apparent in snakes infected with haemogregarine (Hull

and Camin, 1960). The parasite *H crocodilorum* was detected in a leech (*Placobdella multilineata*) in alligators in Florida (Forrester and Sawyer, 1974). The hemogregarine gametocytes and sporozoites were present in histological sections of leeches (Glassman, Holbrook and Bennett 1979).

Trypanosoma infections

Trypanosomiasis is a blood parasitic disease common in various species of animals including humans. The pathogenicity of these parasites varies according to the species of *Trypanosoma* and the number of parasites present in the blood circulation (Jubb, Kennedy and Palmer, 1985). At least 58 species of *Trypanosoma* have been reported in crocodilians, turtles, lizards and snakes (Telford, 1984). The majority of the species of trypanosoma are non-pathogenic to their natural host, however, parenteral inoculation always produced fatal infections.

Trypanosoma grayi reported in adult crocodiles (*C niloticus*) in Uganda was confirmed through laboratory examination of the internal organs of tsetse flies (*Glossina* sp) (Hoare, 1929). Transmission of *T grayi* can be achieved through bites on the soft skin between scales of crocodiles. Crocodiles infected experimentally and examined postmortem showed a pale and watery blood, and the liver was yellowish in colour. Histopathological changes in affected organs were not mentioned in this report. Recent identification of *Trypanosoma* sp has been reported in a Nile crocodile and *C crocodylus yacare* however no apparent harmful effects have been observed (Telford, 1984; Nunes and Oshiro, 1990; Minter-Goedbloed, Pudney, Kilgour and Evans, 1983).

Leeches and other ectoparasitic infestations

Ectoparasites that are commonly found in reptiles include mites, ticks, blow flies, leeches and chiggeras; all are potential vectors for blood parasites (Soifer, 1978). A leech called *P multilineata* had been reported in crocodilians (Frank, 1981). A significantly higher eosinophilic count in alligators infected with leeches was noted compared with captured animals, which were not leech infected (Glassman *et al.*, 1979).

The leech can cause cutaneous wounds during feeding and transmit *Haemogregarina*. A substance called hirudin secreted by the leech during feeding can cause continuous bleeding providing entry of opportunistic organisms (Frye, 1981a).

A nymphal tick believed to be either *Amblyomma limbatum* or *Amblyomma moreliae* was identified from an emaciated freshwater crocodile (*C johnstoni*) during a survey study in the Lynd

river of North Queensland (Tucker, McCallum, Limpus and McDonald, 1994). According to Tucker (1994) there was an abundant goanna population in the area and it would appear likely as the potential vector.

Parasites not associated with disease

Generally, the parasites reported here were identified from surveys or on studies of faecal samples collected either from crocodile farms, animal zoos or field cases. In addition, the parasites were also identified from organs that had no pathological alterations, suggesting that the parasites did not cause any discomfort.

Frank (1984) mentioned amoebiasis in a crocodile (*C porosus*) caused by *Entamoeba envadens*. Although a fatal disease is particularly observed in snakes and lizards, it seems that crocodiles are considered as symptomless carriers similar to chelonians. *Cryptosporidium* sp has also been reported in Nile crocodiles but no further mention on the disease it could cause was made (Siam, Salem, Ghoneim, Michael and El Refay, 1994). *Blastocystis* sp has also been reported in apparently healthy *C porosus* in Singapore (Teow, Ng, Chan, Chan, Yap, Zaman and Singh, 1992).

Other parasites identified are all digenean trematodes collected from intestinal scrapings namely *Timoniella absita* from *C porosus* (Blair, Brooks, Purdie and Melville, 1988), *Acanthostomum quaesitum* from *C johnstoni* (Brooks and Blair, 1978) and several species of *Proterodiplostome* parasites from caimans (Catto and Amato, 1994). Nematodes of the genera *Brevimulticaecum, Ortleppascaris* and *Contracaecum* (Goldberg *et al.,* 1991) and Pseudodiplostomes (Catto and Amato, 1994) were identified in *C yacare*. A new species of *Crocodyloscapillaria, Crocodylocapillaria longiovata* has been reported from the stomachs of *C johnstoni* and *C porosus* from Northern Australia and Papua New Guinea (Moravec and Spratt, 1998). *Trichinella spiralis nelsoni* was found in the meat of slaughtered Nile crocodiles (Foggin and Widdowson, 1996).

2.3.4 Viral Diseases

Reported cases of viral diseases in reptiles and amphibians are extremely limited (Hoff *et al.,* 1984), and even in the standard textbooks of virology, viruses of reptiles are not mentioned (Fenner, Gibbs, Murphy, Rott, Studdert and White, 1993; Levy, Contrat and Owens, 1994). Most cases of viral infections were considered as incidental findings in an attempt to find the

causative agents of the disease being studied. The scenario regarding reported cases of viral infections in other animals is similar in crocodiles. Isolation and characterisation of the virus are needed to study further the pathogenesis of viral diseases especially in crocodiles.

Poxvirus infection

The first reported case of poxvirus infection was in Florida (Jacobson, Popp, Shields and Gaskin, 1979) where three juvenile spectacled caimans (*C sclerops*) were affected. Skin lesions were described as grey-white papules (1 to 3 mm in diameter) which eventually coalesced into grey-white patches that were particularly prominent on the eyelids, phalanges, and integument overlying the mandible and maxilla. In one caiman, the lesion progressed to conjunctivitis with palpebral oedema and poor body condition. Histopathological findings revealed the presence of epithelial hyperplasia accompanied by acanthosis, hyperkeratosis and necrosis. The majority of infected cells had ballooning degeneration with very distinctive eosinophilic intracytoplasmic inclusion bodies resembling Borrel bodies for smaller inclusions and Bollinger bodies for larger inclusions. Electron microscopy revealed that virions within the inclusions had a round to oval shape, measuring approximately 100 by 200 nm. Each virion contained a 'dumbbell shape' body typical of poxvirus.

A second outbreak of poxvirus infection was in a commercial crocodile farm in South Africa where one thousand Nile crocodile (*C niloticus*) hatchlings were affected (Horner, 1988). Initially, 40% of crocodiles in the rearing unit were affected, then the disease spread to all 16 pens. Although the gross lesions of affected Nile crocodiles differed from infected caimans described as brownish wart-like pox lesions, the microscopic skin lesions and viral morphology were similar. Another difference was the absence of skin lesions on the tail in affected Nile crocodiles. Furthermore, a concurrent mixed infection was also observed.

In the same year in Lake Tanganyika, Zambia, 300 yearlings from a group of 400 crocodiles were infected with poxvirus, and 82 of those crocodiles died (Pandey, Inoue, Oshima, Okada, Chihaya and Fujimoto, 1990). Similar skin lesions as described in previous reports were present in various parts of the body. The presence of a 'dumbbell shape' body within the eosinophilic intracytoplasmic inclusion bodies of affected skin was again observed.

Further reports on cases of poxvirus infections in crocodiles were documented until the beginning of 1990. For example, there were reports on poxvirus outbreaks in Zimbabwe and elsewhere in South Africa particularly in Transvaal Lowveld (Huchzermeyer, Huchzermeyer and

Puterill, 1991). Poxvirus infection affecting eight caimans (*Caiman crocodylus fuscus*) from the National Zoological Garden in South Africa was reported by Penrith, Nesbit and Huchzermeyer (1991). The skin lesions in this case were similar to previous reports however the infection spread to the oral mucosa, gingiva, tongue and the palate. Seven Nile crocodiles (*C niloticus*) infected with poxvirus has also been reported in one of the commercial farms in Kenya (Buoro, 1992). Recently, an outbreak of poxvirus infections in house Nile crocodiles (*C niloticus*) was reported by Gitao and Mwendia (1999). Again, similar lesions were observed and the poxvirus was identified by electron microscopy.

In Australia the first report of poxvirus infection was by Buenviaje, Ladds and Melville (1992) affecting single *C porosus* and *C johnstoni* hatchlings. Both cases were 'complicated'; the first with fungal infiltration, which was presumed as secondary, while the second lesion involved the epithelium that directly overlaid a dermal granuloma of undetermined aetiology.

The morphological features of poxviruses identified from two crocodiles and one caiman were almost indentical. Both strains of poxviruses have regular criss-cross surface patterns, however, the virions differ slightly in (Gerdes, 1991). The presence of a criss-cross surface pattern and the characteristic 'dumb-bell' shaped virions observed in crocodile poxviruses were similar in avian viruses, except that the crocodile poxviruses were slightly smaller than the avian poxviruses (Tripathy and Cunningham, 1984). The occurrence of poxvirus infection in crocodiles (Jacobson *et al.*, 1979) could be due to the close phylogenetic relationship of crocodylia and aves, and in this way the crocodile poxvirus is probably closely related to avian poxvirus. The isolation and culture of crocodile poxvirus is very important in order to study in detail the morphological features of the virus, the pathogenesis of the disease, and comparison with other poxviruses particularly from birds. To date, this has not been successful.

Adenovirus-like infection

Adenoviral infection was first reported in four species of captive snakes including a four-lined rat snake, a boa constrictor, an aesculapian snake, and a gaboon viper (Jacobson, Gaskin and Gardiner, 1985). Microscopically, the most significant lesion in the liver was severe diffuse hepatic necrosis, with an infiltrate of heterophils and small mononuclear cells. Throughout the hepatic parenchyma there were numerous large basophilic intranuclear inclusion bodies resulting in the ballooning and margination of the chromatin material in the nuclei of affected cells.

Adenoviral infection has also been reported in Nile crocodiles (Jacobson, Gardiner and Foggin, 1984; Foggin, 1987; Huchzermeyer, Gerdes and Puterill, 1994b). Of two affected eight-month-old Nile crocodiles, one was found moribund and was euthanased. The second one was a runt and had conjunctivitis and blepharitis four months before death (Jacobson *et al.*, 1984). On postmortem examination there were small foci scattered throughout the liver parenchyma of the first crocodile while the second had no significant gross lesions. The histopathological findings of the liver revealed multifocal to diffuse areas of necrosis associated with mostly mononuclear infiltrates. The hepatocytes had basophilic intranuclear inclusions resulting in ballooning of the nucleus and margination of chromatin. Similar inclusion bodies were present in the crypt epithelial cells of the intestine but not in the liver of the first crocodile. Electron microscopy of hepatic and intestinal inclusions showed the presence of crystalline arrays of viral particles. In some cells the nucleus had ruptured releasing the viral particles into the cytoplasm. The viral particles measured 75 to 80 nm in diameter, had hexagonal outlines and electron-dense cores, and were not enveloped. All these features were compatible with mastadenovirus, the mammalian adenovirus described by Fenner *et al.* (1993).

Other viral infections

Some viruses in crocodiles were identified from samples such as faeces while suspected viral infections were also observed from tissues examined histologically. In many instances, viruses identified from samples taken from crocodiles were suspected to be the cause of the disease but until transmission studies are undertaken it cannot be sure that these viruses are the aetiological agents of the disease. Ladds and Sims (1990) for example observed lymphocytic perivascular cuffing from brain sections of two crocodiles. The presence of this type of lesion in the brain is usually pathognomonic of a viral infection. In this report however the aetiological agent was not identified.

Viruses from faeces of farmed Nile crocodiles (C niloticus)

A thorough investigation of the possible aetiological agents causing mortality in farmed crocodiles of South Africa was conducted by Huchzermeyer *et al.* (1994b). Faecal samples were collected from crocodiles in pens where a high mortality had occurred. The result of electron microscopical examination revealed the presence of an adenovirus, coronavirus-like particles, influenza-c-virus and paramyxovirus. The paramyxovirus was identified through the typical spherical and filamentous forms enclosed by an envelope bearing clearly visible spikes. In addition this viral particle was found over a period of three months in crocodiles ranging from 32 to 144 cm. The influenza-c-virus particle was identified through its filamentous forms with the

surface projections being in a regular hexagonal arrangement. The virus particle was still present in faeces for one month in eight crocodiles ranging from 31 to 81 cm from pens with high mortality. The coronavirus-like particles were described as small, pleomorphic particles bearing longer and well-spaced projections. The presence of viral particles in faeces was found over one month, in four out of five crocodiles from a farm with high mortality in two- to three-year-old animals.

Eastern encephalitis virus (EEV)

A survey on the occurrence of eastern encephalitis virus in reptiles using blood samples for the detection of the antibody of EEV showed positive results in seven of 99 reptiles (Karstad, 1961). Animals positive for EEV included three species of snake (*Elaphe guttata, Coluber constrictor, Ancistrodon piscivorus*) one turtle (*Kinosternon subrubrum*) and one alligator (*A mississippiensis*). Signs of illness attributable to infection with EEV were not observed, therefore it was speculated that reptiles could possibly be a reservoir host of EEV (Shortridge and Oya, 1984).

2.3.5 Neoplastic Diseases

Neoplastic diseases in reptilians are rare and poorly described compared with mammals. The majority of the neoplasms examined came from animals, which were privately owned or held in zoological parks, thus a relatively small number of the animals that died reached the pathologist. Also the number of reptiles kept in zoos is limited, which may also account for low incidences of neoplastic diseases in reptiles. A general misconception that reptiles are not susceptible to neoplastic diseases could be another reason for a limited report on neoplasms in reptiles (Machotka, 1984).

Among the reptilians, crocodiles have the least number of reported cases of neoplasia probably because crocodiles are large and more difficult to handle thus zoos keep them in small numbers. While neoplasia is common in adult animals, the great majority of adult crocodiles are still in the wild with only few kept in farms and zoos, thus also contributing to limited cases of neoplastic diseases.

Polycystic ovarian mesothelioma

Mesothelioma is a rare neoplasm that occurs with greatest frequency in cattle and dogs but occasionally in horses, cats, pigs and other species of animals (Barker, 1993). The occurrence of mesothelioma was frequently associated with asbestosis in man but not in animals. The first

reported case of mesothelioma was in snakes *Pituophis melanoleucus* and *Crotalus horridus* (Machotka, 1984). Mesothelioma was also diagnosed from the ovary of an American crocodile (*C acutus*) that died after showing signs of increased coelomic distension (Obaldia, Brenes, Alvarez and Gale, 1990).

Round cell sarcoma

A single case of a round cell sarcoma from young *C porosus* reported by Scott and Beattie (1927) was comparable to lymphosarcoma according to Schlumberger and Lucke (1948). The clinical signs observed initially was the inability of the animal to rise and it usually leaned over and fell on its right side. Slight twitching of the forelimbs was observed a few hours before death. The necropsy examination revealed slightly nodular and haemorrhagic tumours (1 to 1.5 cm in diameter) in the cerebellum, heart and liver. Microscopically, these tumours were composed mainly of round cells of varying sizes with several mitotic figures and occasional multinucleate giant cells.

Fibroma and fibrosarcoma

Fibroma occurs in a wide range of animal species and is characterised by benign nodular dermal masses made up of 'whorls and bundles' of relatively mature cells (Yager and Scott, 1993). This neoplastic disease has been reported in turtles (*Chelonia mydas*) and certain species of snakes such as *Phython molurus*, *C horridus* and *Elaephe obsoleta spilloides*. A single case of fibroma in an alligator snapping turtle (*Macrochelys temminki*) had spherical masses on the palmar and plantar surfaces of the feet (Frye, 1981b). On microscopy, the tumour was made up of interlacing spindle-shaped fibrocytes and fibroblast, which sometimes formed into 'whorls and bundles' interspersed with collagen fibres.

An adult crocodile on unknown species had a whitish oval mass firmly attached to the inner side of the footpad under the digit diagnosed as a fibroma (Youngprapakorn, Ousavaplangchai and Kanchanapanka, 1994). The cut surface revealed tough, whitish fibrous tissue covered with a thin smooth skin and the microscopical examination showed characteristic interlacing bundles of fibroblasts. The first reported case of a fibrosarcoma of periosteal origin was identified in the oral cavity of a 22-year-old male captive Siamese crocodile (*Crocodylus siamensis*) (Janert, 1998). There was invasive growth of neoplastic cells of mesenchymal origin, which produced collagen fibres.

Lipoma

Lipomas occur in a diverse range of species of animals including budgerigars, rats, mice, dogs, cattle, horses and primates (Yager and Scott, 1993). Machotka (1984) reported two cases of lipomas in reptiles, one was diagnosed from a snake *Constrictor constrictor* and the other one was from an American crocodile (*C acutus*). In the case of *C acutus*, the lipoma was present in the liver hence was called intra-hepatic lipoma or fat storage disease.

Another case of lipoma was on the dorsal skin surface of the thoracic area of a 25-year-old crocodile of unknown species (Youngprapakorn *et al.*, 1994). The tumour was oval, soft and a whitish yellow colour on a cut surface. The tumour was slow growing and took several years before attaining its size. A characteristic large unilocular fat vacuole within the neoplastic cells made up the entire tumour on microscopical examination.

Cutaneous papilloma

The typical cutaneous papilloma or 'warts' appear as multiple rough projections on the skin and in severe cases form into a cauliflower-like mass. Papilloma virus is the aetiological agent affecting a wide range of animal species. The lesions on the skin are benign and normally do not affect the animal, however traumatised papillomas may lead to secondary infections. Papillomas located on the genitals can cause reproductive problems (Ladds, 1993) and under certain circumstances may undergo malignant change to squamous cell carcinomas (Smith, Jones and Hunt, 1972).

Cutaneous papillomas are reported to be very common in reptiles, which includes turtles, lizards and snakes (Machotka, 1984). There have been only two cases of warts reported in crocodiles, one from *C porosus* (Schlumberger and Lucke, 1948) and another one was from *A mississippiensis* (Wadsworth and Hill, 1956). Neither gross nor histopathological finding was included in their reports.

Seminoma

Seminomas occur in older animals but most frequently diagnosed in animals with cryptorchidism especially in dogs (Ladds, 1993). Seminoma in man is often deadly compared with seminomas in animals (Smith *et al.*, 1972). In reptiles, a case of seminoma was reported from an adult *A mississippiensis* with concurrent cutaneous papilloma (Wadsworth and Hill, 1956). The tumour

was contained in the dorsal wall of the body cavity, which obscured the adrenal gland and the testes. Microscopically, the tumour was made up of well-defined groups of large polyhedral cells resembling spermatocytes.

2.3.6 Diseases Caused by Nutritional Deficiencies

Reptiles living in their natural habitat live longer compared with those artificially reared in captivity, because of the poor understanding on the physiology and nutritional requirements (Wallach, 1971). The increasing popularity of keeping reptiles in captivity may be the reason for the high incidence of nutritionally-induced vitamin and mineral disorders (Langham, Zydeck and Bennett, 1971). Runt crocodile hatchlings commonly observed in crocodile farms had been the result of inappropriate nutrition or poor quality feeds (McInerney and Cert, 1994). Not much is known about the nutritional requirements of reptilians in captivity particularly in crocodiles. Diseases resulting primarily from unbalanced diets become a concern because of associated secondary disorders. A number of diseases present in farms and zoos have been attributed to improper nutrition. Research on nutrition, feed requirements and different types of feed preparation have recently been undertaken to address these problems (Stallmann, 1996).

Vitamin E and selenium deficiency

Steatitis or fat necrosis is a syndrome associated with vitamin E and selenium deficiency among domesticated animals including omnivorous zoo animals. Improper diet and food containing an excess amount of polyunsaturated fatty acids, which oxidise vitamin E making it unavailable to animals were the common cause of deficiency. Nutritional myopathies mostly in herbivores and pigs (Hulland, 1993) and vitamin responsive dermatosis in goats (Yager, Scott and Wilcock, 1993) have been implicated in vitamin E and selenium deficiency.

Crocodiles reared in captivity including *C porosus* (Ladds *et al.*, 1995), *C siamensis* (Youngprapakorn, 1988) and *A mississippiensis* and spectacled caiman (*Caiman sclerops*) (Wallach and Hoessle, 1968) have been reported to be commonly affected with vitamin E and selenium deficiency. Affected animals show clinical signs of anorexia for several weeks before death and most often preferred to stay in the water until their bodies were covered with lichen (Frye and Schelling, 1973). The clinical signs of paralysis, incoordination and anorexia were more pronounced in an affected captive Marcy snake (*Thamnophis marcianus*) than in crocodiles (Langham *et al.*, 1971).

The gross pathological findings observed were characterised by discrete yellow and brown 'woody' masses throughout the body particularly in adipose tissues around major blood vessels, myocardium, thoracic and abdominal cavities, lingual area and around the neck and flank (Youngprapakorn, 1988). The lesions of the tail affected by vitamin E deficiency had caused severe damage to the muscle tissues which resulted in a significant loss of meat suitable for harvest (Larsen, Buergelt, Cardeilhac and Jacobson, 1983). Calcification involving the perirenal fat and adipose tissues of the abdominal wall and the tail was accompanied by the infiltration of histiocytic cells (Ladds *et al.*, 1995). Massive deposition of ceroid materials associated with ceroid laden multinucleate giant cells were present in the liver, spleen, pancreas, omentum and serosal surfaces of the large and small intestines (Frye and Schelling, 1973). A presumed vitamin E deficiency in lizards of unknown species showed characteristic steatitis around the eyes (Larsen *et al.*, 1983).

Metabolic bone diseases

A deficiency of calcium, phosphorus and vitamin D that can cause enormous abnormalities of the skeletal system in animals and man has been observed also in reptiles (Frye, 1981b). The disease is often aggravated in animals kept under confinement or minimal exposure to sunlight. In a crocodile farm in South Africa several young Nile crocodiles (*C niloticus*) affected with osteomalacia suffered kyphoscoliosis, 'glassy teeth', 'rubber jaws' and extreme weakness. Although mortality was nearly nil, one crocodile drowned because it had failed to swim properly due to severe spinal deformities (Huchzermeyer, 1986). A spectacled caiman (*C sclerops*) was observed to be severely rachitic and died because the soft skull was crushed during a friendly dispute with another animal. Because of the severity of the disease, the jaws of affected false gavial (*Tomistoma schlegeli*) could be bent nearly double without breaking it (Kuehn, 1974). Although most commonly reported were cases of vitamin D deficiency, a suspected case of vitamin D poisoning characterised by extensive exostoses of the femur of a farmed Nile crocodile has also been reported (Huchzermeyer, 1999)

Gout

The pathology of gout has well been described in humans, birds, dogs and cats (Smith *et al.*, 1972). There are two distinct forms of gout, the arthritic form that is easily recognised clinically by painful enlargement of the joints and the visceral form usually discovered during necropsy and histopathological examination. Gout in reptiles was reported in monitor lizards (*Varanus exanthematicus*), an unknown species of alligator and several species of tortoise (*Testudo*

sulcata, *Kinixys belliana*, *Testudo radiata*, *Testudo hermani* and *Testudo graeca*) (Appleby and Siller, 1980). Wallach and Hoessle (1967) also reported visceral gout in a captive lyre snake (*Trimorphodon biscutatis*), the banded rattle snake (*Crotalus l. klauberi*) and a red tegu lizard (*Tupinambis rubescens*). This condition is seen sporadically in crocodilians (Jacobson, 1984). Gout was the most serious disease problem of a crocodile farm in Queensland (Buenviaje *et al.*, 1994). Both the articular and visceral forms were diagnosed in *C johnstoni* hatchlings, the visceral form causing severe damage to the kidneys. Renal malfunction is expected to exacerbate the condition because of failure to excrete uric acid or urates. According to Coulson and Hernandez (1964) alligators with gout had 70 mg/100 mL of serum that is far greater than the normal concentration of uric acid in blood plasma that is 1.0 to 4.1 mg/100 mL.

Ariel, Ladds and Buenviaje (1997a) described three progressive stages of 'tophi' development from affected kidneys of *C porosus* and *C johnstoni* hatchlings. The presence of eosinophilic hyaline masses associated with macrophages was presumed as the initial lesion. Buenviaje *et al.* (1994) also described similar amorphous deposits surrounded mainly by mononuclear cells in the myocardium of *C johnstoni*. The eosinophilic hyaline masses eventually dispersed in the intermediate stage and finally were replaced by tophi in the third stage of gout formation. Furthermore, a presumed vitamin A deficiency that had caused squamous metaplasia of the renal tubules was a key factor for the occurrence of gout.

Vitamin A deficiency

Vitamin A deficiency was very common in reptiles (Voprsalek and Simunek, 1996). Among the reptilians, the chelonians had the highest rate of avitaminosis A (Wallach, 1971). It is characterised by the presence of palpebral oedema as a result of the accumulation of keratinised debris in the ducts draining the Harderian glands. In severe cases, avitaminosis A can cause generalised oedema in turtles. Deficiency of vitamin A in reptiles inhibits tear glands from producing the saline wash that normally lubricates the eyes and nasal passages (Blake, 1974), which eventually leads to blindness (Wilcock, 1993a).

Both *C porosus* and *C johnstoni* hatchlings with suspected hypovitaminosis A accompanied with visceral gout, had multiple, pale brown nodules measuring up to 5 mm in diameter on the surface of the tongue (Ariel *et al.*, 1997a). Microscopically, the nodules on the dorsum of the tongue contained keratin debris trapped in the affected glands associated with prominent squamous metaplasia in the glands. Squamous metaplasia and hyperkeratosis were also seen in the large collecting ducts of the kidney. This epithelial transformation of the kidney had

compromised the excretion of urates, which accumulates in various organs of the body leading to gout formation.

Vitamin B1 or Thiamine deficiency

A deficiency of vitamin B1 or thiamine may occur as a result of low concentration in the diet, presence of thiaminase in some fish, heating the food to 212°F, and presence of sulfur dioxide used to preserve the fresh appearance (Jubb and Huxtable, 1993). Carnivores are more susceptible to thiamine deficiency compared with herbivores because the microbial flora in the rumen of herbivores can synthesise thiamine. The common cause of thiamine deficiency in horses was due to bracken fern and horsetail poisoning. Both plants contain thiaminase.

Water snakes, chelonians and crocodilians were commonly affected with vitamin B deficiency because their main diet consists of fish (Jackson and Cooper, 1981). The outstanding feature of the disease is sudden loss of righting reflex in a thiamine deficit in *C porosus* hatchlings (Jubb, 1992). These animals were often found floating, lying on their sides or back, listless with jaws open and unable to right themselves. A dramatic improvement was noted when treated with thiamine injections.

Other nutritional related diseases

Hypoglycaemic shock syndrome affecting captive alligators was associated with low blood glucose level during winter, hence is called physiologic winter hypoglycaemia (Wallach, Hoessle and Bennett, 1967). The clinical manifestation of the disease was progressive, characterised initially by 'star gazing' or circular swimming. As the disease becomes severe the animals showed signs of torticollis, catatonic seizures and death. The absence of lesions at necropsy and the quick recovery of animals after treatment with glucose strongly suggests a hypoglycaemia syndrome. The clinical signs in winter hypoglycaemia were similar in experimentally insulin-induced hypoglycaemia.

Frye (1984) mentioned vitamin K deficiency in crocodilians characterised by severe bleeding of the gums, and excessive alveolar bleeding as deciduous teeth are shed.

Huchzermeyer (1992) reported ulcerative gingivitis as a result of vitamin C deficiency in farmed crocodiles in South Africa. A deficiency of vitamin C in severe cases causes a spontaneous rupture of the skin, and a copious amount of caseous yellow to gray exudates accumulated

between the lips and the palate (Wallach, 1971). An epizootic incidence of infectious ulcerative stomatitis or 'mouth rot' was observed in captive snakes (Frye, 1984). Although vitamin C is necessary for collagen synthesis (Little, 1990) which would contribute better quality hides, the result of his study did not suggest that supplementation of exogenous vitamin C in the diet could be beneficial.

2.3.7 Developmental Anomalies

More developmental anomalies were reported in crocodiles compared with other reptiles presumably because of increased awareness on any crocodile diseases present due to the economic value of crocodiles. Likewise animals under captivity are closely monitored thus any abnormalities observed are recorded. Presumably the incidence of such anomalies is similar in wild animals, but monitoring such disorder would be very difficult and besides hatchlings with severe abnormalities are prone to predators and may not survive in a harsh environment.

The developmental anomalies observed in farmed crocodiles were mostly attributed to the failure in husbandry management (Hutton and Webb, 1993; Sumagaysay, 1993). Like other reptiles, developmental anomalies and embryonic death were linked to either low or high temperature during egg incubation (Kar and Bustard, 1982a; Hutton and Webb, 1993). During incubation in extremes of temperature, abnormalities in hydric or gaseous environment and variations in the orientation of eggs could predispose to malformations (Ferguson, 1985). Although the causes of many of the anomalies in farmed animals are essentially unknown, some viral infections, intra-uterine poisons ingested by the mother, vitamin deficiencies and genetic factors are known to cause embryonic anomalies (Smith *et al.*, 1972).

Other predisposing factors in the occurrence of congenital abnormalities in reptiles may be due to low oxygen, high humidity and the action of toxins and pollutants (Bellairs, 1981). It was observed that young and very old female crocodiles frequently produced abnormal embryos (Ferguson, 1985). Deeming and Ferguson (1991) experimentally demonstrated embryonic deaths after turning of crocodile eggs during incubation.

Malformations of the head

Several forms of malformations of the head have been described in all animal species including reptiles. Bellairs (1981) mentioned the common abnormalities in reptiles included shortening of jaws, cleft palate, and herniation of the brain and in more severe case absence of the head. A

1.2% incidence rate of jaw abnormalities has been reported in *C johnstoni* during a field survey in the McKinley river area at Northern Territory (Webb and Manolis, 1983). Similar jaw abnormalities were also reported in *C porosus* examined from the tidal rivers on the north coast of Arnhem land at Northern Territory (Webb and Messel, 1977). The types of abnormalities recorded were protrusions of the lower jaw beyond the anterior margin of the premaxillae, lateral displacement of the lower jaw, curved lower jaw dorsally around the premaxilla and underslung lower jaw relative to the upper jaw. The abnormalities were considered ontogenetic rather than a result of post hatching injuries.

Brachycephalic head is one of the most striking abnormalities of the head in most species of crocodile (Iordansky, 1973). Two adult males (*C novaeguineae*) with a brachycephalic head had a shortened snout and massively broadened lower mandibles (Hall, 1995). Other anomalies observed were the presence of calcified growths, which partly or completely filled several alveoli.

Youngprapakorn *et al.* (1994) reported a batch of newly hatched crocodiles, presumably *Crocodylus siamensis*, with a brachycephalic head characterised with short lower jaws. In addition affected hatchlings had docked tails and incomplete closure of the abdomen. Several other cranial abnormalities reported included developmental failure of the first branchial arch, face and abnormalities of part of the skull bones. Meningoencephalocele was diagnosed in five to six times per 5,000 newly hatched eggs. In some cases, the skin of the head above the brain failed to develop or was absent. Cleft lip (cheiloschisis), cleft palate (uranoschisis) and cleft chin was also reported in *C siamensis, C porosus* and hybrids. The occurrence of bulging of the midbrain on the cranial platform in crocodiles was attributed to high temperature during incubation (Webb and Manolis, 1983; Ferguson, 1985). Craniofacial malformations can also be induced experimentally through surgical excision, injection of teratogens and alteration of diet in captive breeding (Ferguson, 1985).

Eye abnormalities

Several eye defects in domestic animals are hereditary however some anomalies, particularly in ruminants, are associated with plant poisoning (Smith *et al.*, 1972; Wilcock, 1993b). Very few eye anomalies such as cyclopia, microphthalmia and exophthalmia had been mentioned in reptiles (Bellairs, 1981).

Singh and Bustard (1982) recorded various eye anomalies in hatchlings *Gharial gavialis gangeticus*, which were hatched from the eggs collected in the wild. An estimated 5 to 9% of

hatchlings with eye defects was the most prevalent abnormality recorded in three years. Various eye defects included complete absence of the eyes, corneal defect, unsmooth pupil and squint eyes. Approximately 95% of farmed *C siamensis* with anophthalmia had bilateral anomaly. Microscopically, there was derangement and malformation of the fibrous, vascular and neuro-epithelial coats of the eye (Youngprapakorn *et al.*, 1994). Those animals with unilateral anophthalmia also had asymmetry of face bone development. The nictating membranes of a crocodile hatchling were sealed resulting in accumulations of secretions inside the eye. Other eye abnormalities recorded were 'rusty eye', aniridia, exophthalmus and microphthalmia. Exophthalmus was thought to be possibly associated with a disorder of the thyroid gland.

Neck, body and tail deformities

One of the amazing congenital abnormalities reported in reptiles was the presence of two heads in snakes. It has also been reported in other reptiles such as turtles and lizards, and in an embryo of the American alligator (Bellairs, 1981). A radiographic examination in a snake (*Pituophis melanoleucus annectens*) showed a bifurcation of the thoracic vertebrae. Hunch back or scoliosis accompanied with multiple severe abnormalities has been described in *Gharial gavialis gangeticus* and *A mississippiensis* (Singh and Bustard, 1982; Elsey, Joanen and McNease, 1994). Embryonic tail deformities observed in crocodiles were reported to have been due to a high temperature during incubation of eggs (Kar and Bustard, 1982a). Various types of tail abnormalities including absence of tail (docked tail), coiled and crooked tails were observed. Docked tailed crocodiles can be raised normally on land but have a difficult time moving and swimming in deep water (Youngprapakorn *et al.*, 1994).

Limbs and digits abnormalities

Abnormalities of the limbs and digits are mentioned less often in reptiles. The cause is usually not known however some anomalies such as the absence of limbs in turtles and flippers in *Caretta* resulted from embryonic amputation, similar to the human foetus (Bellairs, 1981). Although the limbs were absent (amelia) or become mere stubs, the amelus crocodile can still move by twisting its body and pushing forward using its hindlimbs. Sometimes the limbs were very small or rudimentary (ectromelia) (Rainwater, McMurry and Platt, 1999).

Another limb abnormality described was the presence of an extra limb or 'fifth' limb attached to the dorsal surface of the pelvic region in a wild alligator (Bellairs, 1981). In a separate case the

extra limb was attached to the ventral side of the body near the umbilicus (Youngprapakorn *et al.*, 1994). The distal portion of the limb tapered with one finger and fingernail.

Several types of digit anomalies found were classified as follows; duplication of digits (dichirus), bony fusion or fleshy webbing of the digits (syndactyly) and extra digits (polydactyly) in *A mississippiensis* captured from the wild (Giles, 1948). Arthrogryposis was present in one animal with digit anomalies.

Skin colour abnormalities

Skin pigmentation included pigment-free (true albinism) to extreme dark and unpattemed (melanistic) has been found in most species of amphibians and reptiles (Dyrkacz, 1981; Frye, 1991a). A melanistic spectacled caiman (*C sclerops*) was completely black whereas an erythristic has reddish brown skin and albinistic alligator had pale non-pigmented skin (Allen, 1956). In a population survey of *C porosus* inhabiting the tidal mangrove creeks in Orissa, India, two adult females, one adult male and one juvenile of unknown sex were albinos (Kar and Bustard, 1982b). The colour of the skin of adult females was dull and not as white as the juvenile crocodiles presumably because of increasing age and size. Four albino *C siamensis* out of a clutch of 27 eggs have been reported from a farmed crocodile in Cambodia (Thouk, 1995). The four albinos were stronger and grew up faster than other animals.

Other abnormalities

According to Ferguson (1985) as the stage of embryonic development advances the visceral organs with the yolk sac are withdrawn into the body. Incomplete closure of the chest and abdominal wall will result in ectopia cordis and protrusion of yolk, umbilicus and other visceral organs (Elsey *et al.*, 1994; Youngprapakorn *et al.*, 1994).

Multiple abnormalities of the internal organs, mostly in farmed *C siamensis*, have also been described by Youngprapakorn *et al.* (1994). Initially, hatchlings were in good body condition but gradually became emaciated and died after two months. Several abnormalities detected were atresia of the duodenum and jejunum, esophageal stenosis, diaphragmatic hernia and cholescystectasia. In diaphragmatic hernia, the yolk projected into the pleural cavity and resulted in the compression of the heart and the lungs and the heart became twisted. Brockman and Kennedy (1962) described an interventricular septal defect in a male 18-month-old *A mississippiensis*.

2.3.8 Physical and Chemical Causes

Injuries as a result of either physical contact or exposure to chemicals inevitably could happen to all species of animals as well as human beings. Obviously, an animal's social behaviour that varies according to species is an important factor to consider for the presence of injuries. According to Margo (1990) only 1% of hatchlings in the wild make it to the adulthood. Moreover, young animals in the wild such as crocodiles have lesser chance of survival than those kept under captivity because of predators.

Injuries associated with the animal's social behaviour

An aggressive behaviour especially in those animals from the wild is obviously a response towards intruders. Crocodiles introduced to a new pen with other crocodiles or during the breeding season can acquire serious injuries due to fighting (Alcala, Ross and Alcala, 1987). Such vicious attacks can cause invalidity and even death. A survey on abnormalities and injuries in both *C porosus* and *C johnstoni* showed a greater percentage of wounds mostly rake and bite marks on the tail region (Webb and Messel, 1977; Webb and Manolis, 1983). Severe injuries such as broken jaws and mandible, and amputations of the tail tip and digits have also been recorded.

The result of the examination of the stomach contents of wild American alligator (*A mississippiensis*) during harvest revealed several web tags from alligators (Rootes and Chabreck, 1993). These tags were previously attached to web of smaller alligators for identification purposes. Web tags recovered mostly from bigger alligators indicate a cannibalistic behaviour or intense fighting in the wild, which presumably accounts for serious injuries and death of crocodiles.

Injuries associated with environmental factors

Several alligators along the coastal regions in Louisiana during winter were found bleeding from the nose and mouth and some were dead as a result of extremely freezing temperature (Joanen and McNease, 1988). Frostbite to severe tissue necrosis was a common sequela in reptiles exposed to freezing temperature (Frye, 1991b).

Injuries from bites and breaking of skin between segments in *C niloticus* were associated with noise from blasting during the construction of the national highway. The clinical signs included paralysis, dilated pupil, dehydration and septicaemia (Watson, 1990).

Webb and Manolis (1983) mentioned lacerations on the ventral surfaces in *C porosus* sliding on rocks. During the survey in the Northern Territory, one adult *C johnstoni* was found dead due to severe injury of the tail. The 25cm tree-root was broken off in the tail causing massive infection.

Management related injuries

Animals under captivity have a lesser chance of injuries if management is sound. A rough concrete floor can cause abrasion of the belly skin when animals slide in and out of the water (Coulson, Coulson and Hernandez, 1973; De vos, 1982). Mild injuries on the skin are often overlooked but it becomes important when the skin becomes infected with diseases such as dermatophilosis, fungal, viral and other bacterial diseases (Jacobson, 1989; Buenviaje *et al.*, 1994). Overcrowding induced pileups, suffocation, drowning and fighting may cause injuries and death (Joanen and McNease, 1976; 1977). A hysterical reaction to management procedures in crocodiles can cause traumatic injuries (Foggin, 1987). Other causes of injuries and mortalities are falling into empty ponds, faulty pen construction, and uncovered pens causing predation of hatchlings by birds and other animals (Crafter, 1986a).

The caretaker's sensitivity and awareness to the animals is very important to avoid or minimise death from injuries and diseases (Frye, 1981b). Severe injuries that almost amputate both limbs of a breeder *Crocodylus mindorensis* was due to mishandling by inept keepers during restraining (Ortega, Ortega and Tsubouchi, 1990). To reduce trauma during restraining, the eyes should be covered and the jaws taped but making sure not to obstruct the nostrils (Crafter, 1986b). The crocodile should not be tied with rope because of the danger of gangrene. Anaesthetics can be used for immobilisation especially in larger crocodiles (Bonath, Bonath, Haller and Amelang, 1990a; Bonath, Haller, Bonath and Amelang, 1990b).

Chemical and toxic effects

Poisoning in reptiles includes toxic plants common to herbivorous reptiles and chemical substances mostly coming from the households or automotive products. A fatal intoxication in a snake was due to accidental ingestion of antifreeze, propylene glycol, and a lead poisoning in a tortoise after eating a lead-based paint (Frye, 1991b). High concentration of heavy metals was

also detected in soils and vegetation, invertebrate and lizards from areas with previous mining activities (Avery, White, Martin and Hopkin, 1983).

In crocodiles, high mercury levels were detected in meat samples of alligators (Hord, Jennings and Brunell, 1990; Ruckel, 1993) and in other organs such as the kidney and liver (Jagoe, Arnold-Hill, Yanochko, Winger and Brisbin, 1998). A variety of organochlorine residues were found in eggs of American crocodile (*C acutus*) (Hall, Kaiser, Robertson and Patty, 1979). A high level of zinc concentration in serum was reported in crocodiles after ingesting coins. The clinical signs were anorexia and weight loss. The animal resumed feeding after the removal of coins (Lance, Cort, Matsuoka, Lawson and Saltman, 1995).

Mateo, Roberts and Enright (1984) experimentally inoculated turpentine subcutaneously in young *A mississippiensis* to observe the pathological changes. The blood vessels were congested and foci of heterophils and mononuclear cells were observed at the periphery of and near the inoculation sites four hours after inoculation. The underlying muscles had occasional foci of necrosis but mostly were degenerated. The number of heterophils and mononuclear cells increased in the inoculation sites, in necrotic muscle fibres and in congested vessels between eight hours and one day post-inoculation. A mixed population of heterophils and mononuclear cells aggregated around the blood vessels. At three days post-inoculation, the number of heterophils and mononuclear cells were approximately equal. Multiple foci were scattered throughout the dermis. From seven to fourteen days post-inoculation, the inflammatory cells present were predominantly macrophages, and occasionally there were multinucleate giant cells in palisade formation. The heterophils were still present but mostly were necrotic. At 30 days post-inoculation, the dermal foci were surrounded by macrophages, heterophils, fibroblast and collagenous fibres. The authors concluded that the general progression of events of inflammatory response in crocodiles was similar to that of acute and chronic response in mammals. The granuloma however was not a classical granuloma as in mammals because only a thin rim of macrophages and multinucleate giant cells were present around the foci.

2.3.9 Other Diseases

Cosgrove and Anderson (1984) described amyloidosis in several species of reptiles such as snakes, tortoises and in a ten-year-old American alligator (*A mississippiensis*). The amyloid deposits appeared as homogeneous eosinophilic material in a variety of tissue locations usually associated with blood vessels such as around cerebral blood vessels, in the spleen and glomeruli of the kidneys.

Sinha, Roy and Chaudhary (1987b) reported a 15-year-old female crocodile (*C palustris*) showing clinical signs of weakness, offensive smell from the mouth and small quantity of watery faeces after being fed beef and fish. The disease affecting the crocodile was assumed as gastro-enteritis. *Proteus rettgeri* was isolated from faeces.

Inflammation of the peritoneal cavity has been reported in a broad-fronted crocodile (*O tetrapis*) but the pathology was not described (Hamerton, 1938). An adult American alligator (*A mississippiensis*) was reported to have egg yolk serositis by McDonald and Taylor (1988). The affected alligator had diffuse, yellow, granular serosal thickening at necropsy. Tissue samples from the stomach, small intestine and spleen revealed a chronic proliferative serositis associated with 3 to 15 mm eosinophilic extracellular globules identified as egg yolk. The presence of egg yolk in the coelomic cavity could have been due to traumatic rupture of non-ovulated ovarian follicles and escape by reverse peristalsis of ovulated ovarian follicles.

According to Huchzermeyer and Penrith (1992), 52 crocodiles diagnosed with rhino-gastritis or so called 'white nose syndrome' were showing inappetence and the presence of a whitish area around the nostrils. On postmortem examination, the pyloric area of the stomach and the duodenal pouch had numerous ulcers of 2 to 4 mm in diameter. Sometimes glossitis and mild rhinitis were also observed. Multifocal lymphocytic infiltration in the stomach mucosa and in the dermis and mucosa of the nostrils and tongue were seen microscopically. Severe polyarteritis accompanied by thickening of the tunica intima and lymphocytic infiltration of the muscularis and adventitia of the arteries in various organs were the most striking features of the disease. No causal organism so far has been implicated.

Penrith and Huchzermeyer (1993) diagnosed thymic necrosis in a number of slaughtered Nile crocodiles (*C niloticus*) and 14 of 25 diseased crocodiles from four South African farms. Foci of necrosis associated with heterophilic granulomas in the medulla close to the centre of the lobules were present. Accordingly, the cause was presumed as stress related or immune system failure.

Pharyngitis was diagnosed in a number of one-year-old crocodiles that died after being transported by road over a very long distance (Huchzermeyer and Penrith, 1992). Severe swelling and reddening of the basihyal valves and the pharynx were the only lesions present. Bacteriological examination failed to identify any aetiological agents.

Giant cell enteritis has been diagnosed in saltwater crocodiles from several crocodile farms in Queensland and in one case from Papua New Guinea (Ladds, Donovan, Reynolds and Turton, 1994). No gross characteristic signs or symptoms were observed except for severe emaciation. On histopathological examination there was thickening of the proximal intestines associated with large numbers of multinucleate giant cells particularly in the lamina propria and often in deeper layers. Intracytoplasmic inclusion bodies of 1 to 2 mm in diameter present within the multinucleate giant cells, were identified as a protozoa resembling *Leishmania* sp in mammalian histiocytes. Metastatic giant cells were also present in the pancreas, liver and lungs.

Turton, Ladds and Melville (1990) reported inter-digital subcutaneous emphysema ('bubble foot') in *C porosus* hatchlings from crocodile farms in Queensland and the Northern Territory. The affected animals had gaseous inter-digital swelling involving limbs often extending to the base of the tail in severe cases. Microscopical examination of affected digits showed multiple, sharply defined spaced presumably filled with gases. No definite aetiological agents were associated with the disease.

Several cases of meningitis affecting hatchling saltwater crocodiles were reported on one farm during winter months in three consecutive years (Ladds, Bradley and Hirst, 1996). Nervous signs include swimming in circles and an elevated head swaying from side to side was observed. Apart from being lethargic, conjunctivitis involving one eye was also noted. On histopathological examination of the affected brain, acute meningitis by heterophilic infiltration, oedema and vascular congestion were present. *Providencia rettgeri* was isolated in pure culture from meningial vessels, liver, spleen and kidney.

2.3.10 Summary and Conclusion

In summary, the literature review has shown that a variety of diseases is present in crocodiles. The majority of the reports on diseases in crocodiles have been from animals raised in crocodile farms, zoos and fauna parks because their health condition can be easily monitored under intensive management. Although a relatively few and mostly incidental findings of the disease have been reported in wild animals, this does not mean that these animals were less susceptible to diseases.

Since intensive crocodile farming is still in its infancy period, the lack of understanding of husbandry of the animals has lead to higher incidences of diseases. Imbalances of the nutritional requirements and failure to maintain the required body temperature are the most

common cause of disease. Like other animals, young crocodiles are more susceptible to disease than adults, and secondary infections commonly occur as a result of stress or injuries related to their instinct territorial behaviour.

A problem encountered in this literature review is the paucity of information on the pathology of the crocodile diseases, especially the clinical signs and symptoms. There has been very limited research conducted on the epidemiology of crocodile diseases, nor has the role of suspected aetiological agents been verified through experimental transmission studies. As the crocodile farming industry is rapidly expanding, there is a need to focus research on diseases, and their prevention and control.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Source of Animals

Both saltwater (*Crocodylus porosus*) and freshwater (*Crocodylus johnstoni*) crocodiles (but mostly saltwater crocodiles) ranging from six months to three-year-old were used in various experiments. Queensland and the Northern Territory crocodile farms were the main source of crocodile specimens. Crocodile hatchlings were also supplied by the Northern Territory Department of Primary Industry and Fisheries, Darwin, Northern Territory and Queensland Department of Primary Industry, Oonoonba, Townsville. Other sources of crocodile specimens mostly in retrospective cases, came from parks or from a group of experimental animals, were also included. The permit to use these crocodiles for experimental purpose was approved by the Queensland Department of Environment and Heritage, Townsville Queensland (Permit number 001657/98SAA) and Northern Territory University Animal Experimentation Ethics Committee with project reference numbers 97-019 and A99006.

3.2 Collection of Skin Samples

Samples of crocodile skin with lesions were obtained either through biopsy or after the animals had been killed by barbiturate overdose injected directly into the heart. A post mortem examination was performed to ascertain the cause of illness on those crocodiles that had been killed or had died from disease. The origin of the animal including the species, age, sex and the length (cm) from snout to the tip of the tail, were recorded. In addition, the location, colour and size of the lesions were also included. A minimum of three samples of skin of at least $2cm^2$ with 'brown spot' lesions, and including a representative lesion from animals with two or more different types of lesion were collected. Skin samples were fixed in 10% neutral buffered formalin for histopathological examination or frozen in sterile plastic bags at –20°C for bacterial and viral culture.

3.3 Bacteriological Examination

3.3.1 Preparation of skin samples and preliminary screening of the aetiological agent of 'brown spot' disease

Frozen skin samples from suspected 'brown spot' lesions were thawed and carefully removed from the sterile plastic bags ('Whirl-Pak', sold in Australia by Lyppards Pty Ltd) to minimise contamination. The lesions were carefully excised from the skin not to include unaffected skin again to eliminate possible contamination. Using a sterile mortar and pestle, the skin samples were ground and mixed with 1 mL of a sterile pH 7.0 phosphate buffered saline (PBS). At least three drops of the suspension were spread onto five blood agar plates containing Polymyxin B (Sigma Chemicals Co. Steinheim, Germany) at 1000 units/mL of medium (Abu-Samra and Walton, 1977), and incubated at 37°C in an atmosphere of 5% CO_2 in air. After 48 hours of incubation, the plates were examined with a dissecting microscope for the presence of minute white to grey colonies that were pitting into the medium.

3.3.2 Further microbiological tests used for identification

Under a dissecting microscope, the white to grey pitting colonies amongst the contaminating bacterial colonies were picked out with a straight inoculating loop, subcultured onto blood agar plates and incubated at 37°C in an atmosphere of 5% CO_2 in air. After culture for 72 hours, the plates were examined for any white to pale yellow colonies surrounded with a zone of haemolysis. A Gram staining technique was used to describe the cellular morphology and to confirm if the bacteria were Gram-positive and filamentous. The acid fastness of the isolates was confirmed using a Kinyoun modified Ziehl Neelsen staining method. The presence of a capsule and the motility of the test isolates were verified using a nigrosin methylene blue stain and a hanging drop method (Baker and Silverton, 1976). Other bacteriological techniques used were conventional nitrate reduction, methyl red and Vogues-Proskauer, indole, spot oxidase, casein medium, Loeffler's serum medium, tyrosine and xantine agar for hydrolysis and carbohydrate media for fermentation tests and a rapid urease test.

3.4 Experimental Transmission

3.4.1 Preparation of inoculum

The bacterial isolates stored in beads (Pro-lab Diagnostics, Ontario, Canada) frozen at –80°C were thawed at room temperature. One or two beads were streaked onto blood agar plates and incubated at 37°C in an atmosphere of 5% CO_2 in air. After three or four days of incubation, a loopful of bacteria was subcultured onto another blood agar and incubated further. All colonies harvested after four days incubation were transferred into 10% bovine serum in tryptose phosphate broth and incubated for a further 24 hours. The broth was decanted but retaining at least 2 mL of broth and together with the bacterial sediments, were poured off into a sterile dounce homogeniser tube. After homogenizing the broth, the bacterial suspension was collected into 5 mL Bijoux bottles.

3.4.2 Animal inoculation

The animals used for the transmission experiments were young crocodiles ranging from six weeks to one year of age. All materials used including the pens, were disinfected with 1% chlorine solution, and allowed to air dry for at least 12 hours prior to commencement of the experiment. The animals were allowed at least one week of settling-in period before they were inoculated with *Dermatophilus* sp isolates or poxvirus. Prior to inoculation, the animals were randomly divided into treatment and control groups. Several locations of the body (the skin under the jaws, neck, chest, belly and tail) were gently scarified with a scalpel. A permanent felt marker pen was used to identify the areas marked and matching inoculation sites on the skin in non-scarified animals and control animals. For inoculation, a cotton swab dipped in the homogenised broth culture of *Dermatophilus* sp or homogenised pox virus in broth or pH 7.0 PBS was rubbed gently but repeatedly into the scarified and non-scarified sites marked as above. A sterile broth or pH 7.0 PBS was used in the sham-inoculated control animals. In order to avoid the inoculum being immediately washed off following inoculation, all animals were held out of water in plastic crates for two hours before they were returned to their respective pens. All crocodiles were individually restrained and examined clinically at post-inoculation (PI) days three, six, nine, 20 and 40, and finally at the termination of the study on the 47th day PI.

3.5 Strains Investigated

Seventeen isolates included 10 isolates from crocodiles (Nos. 1 to 10), three *D congolensis* from either sheep or deer, one *D congolensis* from cattle, two *D chelonae* from turtles and one type strain of *D congolensis* from cattle obtained from the American type culture collection (ATCC 14637) were used in the study.

Table 3.1 Bacterial isolates used in nucleic acid studies.

Nos.	Isolates	Identity	Source
1	TVS 96-490-78	*Dermatophilus* sp	'Brown spot' lesions from a saltwater crocodile in Queensland (see Chapter 4)
2	TVS 96-490-98	*Dermatophilus* sp	'Brown spot' lesions from a saltwater crocodile in Queensland (see Chapter 4)
3	TVS 96-367-5A	*Dermatophilus* sp	'Brown spot' from a saltwater crocodile in Northern Territory (see Chapter 4)
4	TVS 97-124	*Dermatophilus* sp	Recovered isolate from a pilot study (see Chapter 6)
5	TVS 97-124-WS	*Dermatophilus* sp	Isolated from pen water in a pilot study (see Chapter 6)
6	TVS 97-405	*Dermatophilus* sp	Recovered isolate from the treatment group (see Chapter 6)
7	TVS 97-412	*Dermatophilus* sp	Recovered isolate from the treatment group (see Chapter 6)
8	TVS 97-427-1	*Dermatophilus* sp	Recovered isolate from the treatment group (see Chapter 6)
9	TVS 97-472	*Dermatophilus* sp	Recovered isolate from the treatment group (see Chapter 6)
10	TVS 97-474	*Dermatophilus* sp	Recovered isolate from a control group (see Chapter 6)
11	ACM No. 530 (DCD1)	*Dermatophilus congolensis*	Hoof lesion from a deer (Department of Microbiology, University of Queensland, Brisbane, Queensland)
12	ACM No. 531 (DCS1)	*Dermatophilus congolensis*	Skin lesion from a sheep (Department of Microbiology, University of Queensland, Brisbane, Queensland)
13	ACM No. 532 (DCS2)	*Dermatophilus congolensis*	Strawberry footrot from a sheep (Department of Microbiology, University of Queensland, Brisbane, Queensland)
14	NO. 22204 (DCC1)	*Dermatophilus congolensis*	Skin lesion from cattle (Department of Primary Industries, Oonoonba, Townsville, Queensland)
15	WA 430 (DCH1)	*Dermatophilus chelonae*	Skin lesion from a turtle (Animal Health Laboratories, Department of Agriculture, South Perth, Western Australia)
16	WA 1305 (DCH2)	*Dermatophilus chelonae*	Skin lesion from a snapping turtle (Animal Health Laboratories, Department of Agriculture, South Perth, Western Australia)
17	ATCC 14637 (DCC2)	*Dermatophilus congolensis* (type strain)	Scab from infected cattle (American Type Culture Collection, Maryland)

3.6 DNA Extraction

3.6.1 DNA extraction method as described for *Aeromonas hydrophila* (Oakey, 1997)

Bacterial colonies harvested from pure cultures on blood agar plates were transferred to 500 mL aliquots of brain heart infusion broth (Oxoid Australia, Heidelberg, Victoria) and incubated at 37°C in an atmosphere of 5% CO_2 in air for three to five days. The bacterial cells were pelleted by centrifugation at 5,000× *g* for 15 minutes. The pelleted cells were resuspended in a lysis buffer (10 mM Tris pH 9.5, 100 mM EDTA, 1 mg/mL lysozyme), mixed gently and incubated on ice for five minutes. Sodium dodecyl sulphate (SDS) and proteinase K were added to a final concentration of 1% and 100 µg/mL respectively, and were incubated at 55°C until the mixture cleared. To precipitate proteins, 0.33 volume of 6 M NaCl was added. An equal volume of chloroform was added and tubes were mixed in an orbital shaker for 15 minutes, and centrifuged at 4,000× *g* for 10 minutes. The aqueous DNA was removed using a wide bore pipette and 300 µg/mL of DNase free RNase was added. The tubes were incubated at room temperature in order to denature the RNA. The DNA extraction was dialysed against TE buffer (100 mM Tns, 10 mM EDTA, pH 8) at 4°C for 36 hours to remove any residual EDTA that would interfere with the subsequent action of *Taq* polymerase. To precipitate the DNA, an equal volume of ethanol was added and incubated at –20°C overnight. The DNA was pelleted by centrifugation at 10,000× *g* for 15 minutes, and dried in the hot cabinet (80° to 85°C). Each pelleted DNA sample was resuspended in 500 µL TE buffer and stored at –20°C.

3.6.2 Quigen protocol for DNA extraction

A loopful of bacteria was placed into an Eppendorf tube and mixed with 180 µL of buffer ATL supplied in QIAamp tissue kit (Quigen Pty. Ltd., Clifton Hill, Victoria). Proteinase K stock solution (20 µL) was added, vortexed and incubated at 55°C until the tissue was completely lysed. Occasional vortexing of tubes was necessary to disperse the samples during incubation. The samples were added to 20 µL of RNase A (20 mg/mL), vortexed, and incubated for two minutes at room temperature. Buffer AL (200 µL) (supplied in QIAamp tissue kit) was added, vortexed, and was incubated at 70°C for 10 minutes. After adding 210 µL of absolute ethanol, the mixture was applied to a QIAamp spin column in the 2 mL collection tube, and centrifuged at 6,000× *g* for one minute. The QIAamp spin column was replaced in a clean 2 mL collection tube, 500 µL of buffer AW (supplied in QIAamp tissue kit) was added, and the tubes were centrifuged at 6,000× *g* for one minute. After the filtrate was discarded, another 500 µL of buffer AW was added, and centrifuged at 10,000× *g* for three minutes. The QIAamp spin

column was placed in a clean Eppendorf tube, and 200 µL of buffer AE (supplied in QIAamp tissue kit) preheated to 70°C, was added twice. After brief incubation at room temperature, the samples were centrifuged at 6,000× *g* for one minute. The DNA extracts were stored at –20°C.

3.6.3 Caesium chloride DNA extraction

Bacterial cells grown from the brain heart infusion broth were pelleted by centrifugation at 4,000× *g* for 10 minutes. The pelleted cells were resuspended in a lysis buffer containing 9.5 mL of TE (100 mM Tris pH 9.5, 10 mM EDTA), 0.5 mL of 10% SDS and 50 µL of proteinase K (20 mg/mL). After incubation at 37°C for one hour, the mixture was dialysed against 10 mM tris (pH 8) containing 100 mM EDTA. The gradients were generated by dissolving caesium chloride at 1.221 g/mL lysate, and subjected the mixture to ultracentrifugation at 400,000× *g* for 48 hours. The amount of DNA present at different gradients was determined by withdrawing 0.5 mL aliquots and dropping 50 µL portion of each onto 1% agarose gel containing ethidium bromide, and examined over UV light. All DNA samples were dialysed against TE buffer at 4°C overnight to remove the caesium chloride present in DNA extraction. An equal volume of absolute ethanol was added to the DNA extraction, and was incubated at –20°C overnight. The DNA was pelleted by centrifugation at 10,000× *g* for 15 minutes, and dried to remove any residual ethanol. The DNA pellets were added with 1 mL TE buffer and stored at –20°C.

3.6.4 Modified protocol 3.5.1 for DNA extraction

In the modified procedure, all the isolates were grown on blood agar containing 5% sheep blood, and incubated at 37°C in an atmosphere of 5% CO_2 in air for four days. Bacterial colonies were harvested into 200 mL of brain heart infusion broth (Oxoid Australia, Heidelberg, Victoria) and incubated for a further 24 hours at 37°C in a shaking incubator. The bacterial cultures were centrifuged at 5,000× *g* for 15 minutes to pellet the cells. The pelleted cells were subjected to thawing and freezing at least three times before adding 5 mL of lysis buffer (10 mM Tris pH 9.5; 100 mM EDTA; 5 mg/mL lysozyme). The bacterial suspension was gently mixed by vortex and incubated on ice for 15 minutes to digest the cell walls. SDS and proteinase K were added to the suspension to a final concentration of 1% and 10 mg/mL, respectively. The mixture was incubated at 57°C in a water bath at least two hours in 0.33 volume of 6M NaCl. To precipitate the proteins, 0.2 volume of hexadecyltrimethyl ammonium bromide (CTAB) was added, and the tubes were incubated ovemight. The nucleic acids were cleaned by adding equal volumes of chloroform, mixed in orbital shaker for 15 minutes and centrifuged at 5,000× *g* for 15 minutes.

The aqueous DNA was removed into a clean tube using a wide bore pipette, and mixed with an equal volume of phenol, before incubation at 68°C in water bath for 20 minutes. After centrifugation at 5,000× *g* for 15 minutes, the aqueous solution was finally cleaned following a similar procedure using phenol:chloroform (1:1). RNA was removed by the addition of 300 µg/mL of DNase free RNase, and incubated at room temperature for one hour. The remaining DNA extract was dialysed against 100 mM Tris-HCl overnight at 4°C. Final DNA extraction was obtained by ethanol precipitation where an equal volume of absolute ethanol was added, and then incubated at –20°C overnight. The extraction was centrifuged at 10,000× *g* for 15 minutes, and the pellets were dried in a hot room. Dried DNA pellets were resuspended in 1 mL TE buffer and stored at –20°C.

3.6.5 Estimation of DNA concentration through a gel electrophoresis

Approximately, 5 µL of DNA suspension mixed with 2 µL of loading dye and 5 µL of sterile distilled water was loaded into the wells of 0.8% agarose gel (DNA agarose, Progen Industries Ltd., Queensland). The agarose gel was placed in a HE 33 submarine agarose gel unit (Hoefer Scientific Instrument, San Franscisco, USA), and the electrophoresis unit (Bio-Rad Model 200/2.0 power supply, Bio-Rad, 3300 Regatta Boulevard, Richmond, CA 94804, USA) was set to 80 volts for 30 minutes to one hour. The concentration of DNA was determined by comparison of the staining intensity of ethidium bromide with the largest fragment of *Hind III* cut Lambda DNA marker (Promega Co., New South Wales, Australia). Aliquots of DNA were prepared by diluting to 5 ng/mL in TE, and stored at –20°C.

3.7 16S rDNA Sequencing Analysis

3.7.1 Primer design

The G+C % was calculated from the published *Dermatophilus congolensis* (ATCC 14637) type strain 16s rDNA sequence (Normand, Orso, Cournoyer, Jeannin, Chapelon, Dawson, Evtushenko and Misra, 1996). The melting temperature (°C) was determined as described by Sharrocks (1994) using the equation: $Tm = 59.9 + 0.41 (G{=}C\%) - 675/n$ where n = length of 16s rDNA sequence.

The 16S rDNA sequence was entered into the "Primer" program at the University of Minnesota (http://alces.med.umn.edu/rawprimer.html) with a suggested primer length of 21 nucleotides and a suggested primer melting temperature of 58°C (based upon the melting temperature

slightly lower than the melting temperature for the whole sequence). This program was chosen because it has few restrictions and final selection was achieved manually. Maximum 100 primer options were requested to achieve as many potential primer sequences as possible. However, in each event, the program was able to suggest more than 100 sequences, and so the sequence was fragmented prior to entering into the program to ensure all possible primers were included in the program's output.

The lists of possible primers were then analysed according to different criteria to eliminate primers that would obviously not work optimally in a PCR reaction (Oakey, 1997). Primers were eliminated if the primer sequence:

a) occurred more than once in the probe sequence;
b) contained any obvious folding sequences, taking a prudent value of four bases as significant for folding;
c) contained any repeat sequences, taking a value of four bases as a significant repeat sequence;
d) contained chains of the same base, taking four bases as a significant chain;
e) had homology with any previously reported sequence when entered into a Blast search;
f) was more than 150 bases from the end of the probe (forward primers more than 150 bases 3' end of the probe and reverse primers more than 150 bases from the 5' end of the probe);
g) 3' end did not consist of a G or C base.

The amended lists of forward and reverse primers for each probe were compared for incompatibility with respect to primer self-annealing, taking four bases as significant, and with respect to amplicon length which was kept to a maximum possible.

3.7.2 PCR amplification

***Modified PCR amplification protocol* (Marchesi, Sato, Weightman, Martin, Fry, Hiom and Wade, 1998)**

The forward primer (63 f) of 5'– CAG GCC TAA CAC ATG CAA GGC –3' and a reverse primer (387r) of 5' –GGG CGG WGT GTA CAA GGC –3' were reported to amplify approximately 1,300 base pairs of the consensus 16S rDNA gene of Gram-positive bacteria containing higher percentage of the G-C bases (Marchesi *et al.*, 1998). The PCR reaction was modified to consist of 10 ng template DNA, 1 µM of each primer, 200 µM dNTP, 2 mM $MgCl_2$, 1.1 unit *Taq*

polymerase (Bresatec Ltd., Therbaton, South Australia), 5 µL 10X buffer and sterile distilled water to a final volume of 50 µL. The PTC-100™ thermal cycler (Bresatec Ltd.) was preheated to 70°C before the tubes were added. After an initial three minutes at 94°C, the mixtures were subjected to 30 cycles consisting of one minute at 95°C, one minute at 50°C, 1.5 minutes at 72°C, and a final extension step of 72°C for five minutes.

***Modified PCR amplification protocol* (Oakey, 1997)**

The primers designed from the above protocol (primer design) were used to amplify 1,000 base pairs of the published *D congolensis* 16S rDNA gene. One forward primer (Fwd 1) of 5' –AGA GTT TGA TCC TGG CTC AG- 3') and two reverse primers, reverse 1 (Rvs1) of 5' –CGC TCG TTG GAC TTA ACC– 3' and reverse 2 (Rvs2) of 5' –ACG GCT ACC TTG TTA CGA CTT– 3') were tested for amplification abilities. All the parameters involved such as dNTP (0.75 mM, 1.0 mM, 1.25 mM, 1.5 mM and 1.75 mM final concentration), template (2.5 ng/µL, 5 ng/µL, 7.5 ng/µL and 10 ng/µL), magnesium chlonde (6.25 mM, 12.5 mM, 18.75 mM, 25 mM, 31.25 mM, 37.5 mM, 43.75 mM, 50 mM, 56.25 mM and 62.5 mM combined final concentration) and primer annealing temperature (42°, 44°, 46° and 48°C) were optimised. The second reverse primer (Rvs2) failed to produce a PCR product whereas a product of approximately 1,000 bp was obtained using primers Fwd 1 and Rvs 1. The final combination of PCR components obtained after optimisation was used to carry out the PCR reactions with template DNA from all *Dermatophilus* isolates. The final reactions consisted of 2.5 mM each dNTP (1.75 mM total final concentration); 50 pmols each of reverse and forward primers; 6.25 mM (final concentration) magnesium chloride; 2.5 ng template DNA; 1.5 units *Taq* polymerase; 4 µL 10X reaction buffer; sterile distilled water to 40 µL. A drop of mineral oil was added to each tube to prevent evaporation and the reaction mixtures were subjected to a total of 35 cycles of 95°C for two minutes, 46°C for 30 seconds and 72°C for 2.5 minutes. The amplicons were visualised in 1% agarose gel electrophoresis containing ethidium bromide staining. The size of amplicons was estimated by comparison with *Hind III* cut Lambda phage DNA markers.

3.7.3 Purification of PCR products

All PCR end products were purified using QIAquick PRC purification kit protocol (Qiagen Pty Ltd, Clifton Hill, Victoria). These kits have the ability to purify a single or double stranded PCR products ranging from 100 base pairs (bp) to 10 kilo base pairs (kb) from the primers, nucleotides, polymerases, and salts, using a QIAquick spin columns in a microcentrifuge. Five volumes of buffer PB (supplied in QIAquick purification kit) were added to one volume of the PCR post-reaction mixture, and mixed. To bind the DNA, the sample was applied into the QIAquick

column and centrifuged at maximum speed of 10,000× *g* for 30 to 60 seconds. The flow-through was discarded, and the QIAquick column was washed with 0.75 mL buffer PE (supplied in QIAquick purification kit) and centrifuged for 30 to 60 seconds. The flow-through was discarded and the QIAquick column was further centrifuged for one minute at a maximum speed. The DNA was eluted from the QIAquick column in a clean Eppendorf tube with 50 µL buffer EB (supplied in QIAquick purification kit) by centrifugation for one minute at 10,000× *g* for one minute.

3.7.4 Cycle sequencing of PCR products

The method used to perform cycle sequencing was obtained from ABI prism dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Scoresby, Victoria). Two reaction mixtures were separately prepared for each reverse and forward primers. The standard mixture was made up of 8 µL terminator ready reaction mix, 30 to 90 ng PCR product, 3.2 pmol primer and deionized water to 20 µL. The reaction mixture was overlayed with 40 µL of mineral oil before placing them into the PTC-100 thermal cycler which was programmed to perform 25 cycles consisted of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for four minutes.

3.7.5 Purifying extension products

An ethanol/sodium acetate precipitation procedure as described in ABI prism dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Scoresby, Victoria) was used to remove excess dye terminators. The extension product was transferred into an Eppendorf tube, and added with 2.0 µL of 3M sodium acetate (NaOAc) pH 4.6 and 50 µL of 95% ethanol (EtOH). To precipitate the extension products, the tube was vortexed and placed on ice for 10 minutes and then centrifuged for 15 to 30 minutes at 10,000× *g* for one minute. After the supernatant was discarded, the pellets were washed with 250 µL of 70% ethanol and were repelleted again. The pellets were air dried in the laminar flow cabinet for an hour. Dried pellets were sent to the Department of Biochemistry and Molecular Biology DNA sequencing unit (James Cook University) for sequencing. Direct sequencing of amplified 16S rDNA was performed through the ABI Prism sequencer machine (PE Applied Biosystems). All sequenced 16S rDNA isolates were aligned and matched with *Dermatophilus congolensis* type strain (ATCC 14637) using Sequencer™3.0 computer program.

3.8 Ribotyping

3.8.1 Restriction digestion

Thirteen DNA samples including *Dermatophilus congolensis* type strain (ATCC 14637) were digested with two different restriction endonucleases, *Eco R1* and *Sca 1.* Each reaction contained 4 µg of template genome, 10 units enzyme and 10% volume enzyme buffer. The mixture was incubated at 37°C overnight, and the reaction was terminated with 5 µL EDTA (200 mM). Digested DNA was concentrated by adding three volumes of ethanol and incubated at –20°C ovemight. The DNA was pelleted by centrifugation at 12,000× *g* for 10 minutes. The pellets were dried at least an hour inside the laminar flow cabinet. The dried DNA pellets were resuspended in 13 µL sterile distilled water and 2 µL loading dye. Digests were separated by gel electrophoresis using 0.8% agarose gel containing 5% ethidium bromide, and run at 25 volts overnight. Two DNA markers, *Hind III* cut lambda and 1,000 kb markers were loaded at each end of the digests. The samples were visualised over the UV light, and the image was captured with rulers adjacent to the marker lanes.

3.8.2 Southern blotting

The target separated digests of DNA in the gel were denatured by submerging the agarose gel in denaturation buffer (1.5 M NaCl and 0.5 M NaOH) for 45 minutes at room temperature over a shaker. This process denatured the digested DNA in the gel to single stranded hybridisation templates. The denaturation buffer was poured off and replaced with neutralisation buffer (0.5 M Tris at pH 8, 1.5 M NaCl and 2.5 mM) enough to submerged the gels, gentle shaking for 45 minutes to neutralised the gel. A Southern blot (Southern, 1975) was carried out by allowing the transfer of DNA from the gel to a positively charged nylon membrane (Hybond™ – N+, Amersham International plc, Amersham, UK) by capillary transfer using 20X sodium chloride and sodium citrate (SSC) overnight to ensure efficient transfer. The membranes were placed in a hot oven at 120°C for 30 minutes to dry and fix the DNA, and stored at 4°C until used.

3.8.3 Labelling of RNA probe

Two sterile Eppendorf tubes were pre-cooled on ice for approximately 15 minutes before they were used for the reaction. A total of 30 µL reaction mixture per tube contained 6 µL 5X cDNA buffer (250 mM Tris, pH 8.5, 40 mM magnesium chloride, 150 mM dithiothreitol), 1.5 µL RNase inhibitor (Boehringer Mannheim Corporation, Indianapolis, USA), 3 µL dNTP mixture

(mixture of 5 mM each of dATP, dCTP and dGTP), 6 µL 0.65 mM dTTP, 6 µL 0.35 mM Digoxigenin (DIG)-dUTP (Boehringer Mannheim) and 3 µL oligo primer (Boehringer Mannheim). One µg/µL of 16S and 23S *E coli* RNA (Boehringer Mannheim) after heated to 65°C for 15 minutes was placed immediately on ice for five minutes before adding 1.5 µL into the reaction mixture. Two µL (25 units/µL) AMV reverse transcriptase (Boehringer Mannheim) and 1 µL of sterile distilled water were added last. The mixture was incubated at 42°C in a waterbath for 90 minutes, and reaction was terminated by adding 1 µL of 200 mM EDTA. The RNA probe was mixed gently and stored in –20°C until use. This reaction synthesised cDNA from the RNA template and incorporated a dTTP analogue (DIG-dUTP) in the place of some of the dTTP bases to serve as label for detection.

A spot test was used to estimate the yield of labelled probe. Control Dig-labelled DNA (Boehringer Mannheim) was used to compare the signal of the probe. Two dilution series from the control DNA (5 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, and 0.1 pg/µL) and the probe (N, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were prepared. An aliquot of 1 µL of the diluted controls and probes were spotted onto a positively charged nylon membrane.

The membranes were placed between two sheets of 3 mm filter paper, and incubated at 120°C for 30 minutes to fix nucleic acids onto the membrane. Briefly, the membranes were washed for two minutes in a washing buffer1 (0.1M maleic acid, 0.15M NaCl pH 7.5) and incubated with a blocking solution (washing buffer containing 1% blocking agent) for 30 minutes at room temperature over a shaker. Used blocking solution was poured off and replaced with fresh blocking solution containing a diluted anti-DIG-alkaline phosphatase antibody (1:5,000) (Boeringer Mannheim). After incubation at room temperature for 30 minutes, the antibody solution was poured off and the membrane was washed twice with washing buffer 2 for 15 minutes at each wash. The membranes were incubated in detection buffer 3 (100 mM Tris pH9.5, 100 mM NaCl) for two minutes.

Each membrane was separately placed between two plastic page-protector sheets. At least 1 mL per 100 cm^2 membrane was evenly spotted with a diluted Disodium 3-(4-methoxyspiro {1,2-dioxetane-3-2'-(5'-chloro) tricyclo [3.3.1.$1^{3,7}$] decan}-4-yl) phenyl phospate (CSPD) (Boeringer Mannheim) at 1:100 in detection buffer. Drops of substrate were scattered over and distributed on the surface of the membranes by gently wiping the top plastic sheet with a tissue paper. A liquid seal should formed and ensure that no air bubbles or creases appeared on the membrane. The semi-dry membrane was placed between two sheets of page-protector and

wrapped with a cling-film. The membranes were incubated at room temperature for five minutes and a further 15 minutes at 37°C to ensure the reaction would reach to a steady state.

The membranes were exposed to a lumifilm (Boehringer Mannheim) in a dark room at room temperature. The exposure times of one, two, four, six, eight and 10 minutes were evaluated to obtain the optimum result. The film from the X-ray cassette was immediately placed in three solutions, developer (Ilford Australia Pty Ltd., Mt. Waverley, Victoria) for two minutes, acetic acid for 30 seconds and in fixative (Ilford Australia Pty Ltd.) for two minutes. It was necessary to continuously swirl the tray each time the film was transferred from one solution to the other. After the film had been fixed, it was rinsed in water for one minute and air dried. The concentration of the unknown probe was compared with that of the control labelled DNA through the intensity of signals as displayed on the developed x-ray film

3.8.4 Hybridisation

The prehybridisation buffer (5X SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 10% blocking stock solution) was preheated to 68°C. The hybridisation oven was preheated to 68°C. The fixed membrane was rolled with a hybridisation mesh and placed into a clean hybridisation bottle. Approximately 25 mL of preheated prehybridisation buffer was added to the hybridisation bottle and rolled gently to form a liquid seal between the membrane and the bottle sides. The bottles were placed in an oven so that when the rotisserie turned the rolled membrane it did not collapse. The membrane was subjected to prehybridisation at 68°C at least four hours. Longer prehybridisation times blocked all the non-specific binding sites on the membrane and gave a reduced background signal.

The Eppendorf tube containing the labelled RNA probe was boiled in a waterbath for 10 minutes and placed immediately on ice for five minutes before use. After prehybridisation in oven, the prehybridisation buffer was discarded and replaced with 25 mL of fresh prehybridisation buffer preheated to 68°C. Approximately 20 ng/mL of denatured probe was added into the bottle, immediately replaced into the rotisserie oven and left to hybridise overnight. To remove low stringency bound or unbound probe from the membrane that can produced high background, the membranes were washed twice in 2X wash solution (2X SSC plus 0.1% SDS) for five minutes on a rocker at 68°C. The membranes were further washed twice in 0.1X wash solution (0.1X SSC, 0.1% SDS) for 25 minutes at 68°C. The membranes were removed from the bottles and briefly washed in washing buffer 1 for two minutes. The used washing buffer 1 was poured off, replaced with blocking solution and incubated for 30 minutes on a shaker at room

temperature. After the blocking solution was discarded a fresh blocking solution containing anti-DIG-alkaline phosphatase antibody was added, and incubated for 30 minutes at room temperature. The blocking solution was poured off and the membranes washed twice with washing buffer 2. Following incubation for two minutes in detection buffer, the membranes were incubated for five minutes in CSPD diluted 1:100 in detection buffer. The membranes were removed from CSPD solution and placed between two sheets of plastic page protector. After ensuring no air bubbles and creases were present and a liquid seal was formed evenly, the page protector sheets containing the membrane was wrapped in a cling film, and incubated at room temperature for five minutes. An optimum result was achieved after further incubation at 37°C for 15 minutes. Hybridisation of rDNA probe was visualised with exposure to lumifilm as described in section 3.7.3. The hybridised ribotype fragments were measured with a ruler relative to the site of the original gel loading wells. The migration (in mm) against fragment size of the DNA markers on the original digest well were plotted and joined as a curve. This curve was used as basis to estimate the sizes of hybridised ribotype fragments.

CHAPTER 4

PATHOLOGY OF SKIN DISEASES IN CROCODILES

4.1 Introduction

Crocodiles are farmed mainly for their skin, and any disease that leads to downgrading of hides results in financial loss. Even a single blemish downgrades the hide to second grade and a further two to three blemishes results in a third to fourth grade hide, with a reduction in value of up to 50%. A variety of aetiological agents, such as parasites, viruses and bacteria, including a *Dermatophilus*-like organism have been implicated in such skin lesions. In addition to skin damage *per se*, reduced growth performance and high mortality of young crocodiles are common sequelae to severe skin infections.

To date, no study has addressed the broad spectrum of skin diseases of crocodilians. Most of the relevant information on such diseases is in reports of spontaneous outbreaks in animals held captive in zoos or on farms (Huchzermeyer *et al.*, 1991; Hibberd and Harrower, 1993). In addition, except for poxvirus infection, most descriptions of skin lesions are incomplete or inadequate regarding histopathological findings.

The main objective of this study was to establish which skin diseases occur in crocodiles, particularly those on farms, to indicate the relative frequency of each particular disease and to provide information on pathogenesis, especially in regard to lesions with two or more pathogens present.

4.2 Materials and Methods

4.2.1 Source of samples

Skin samples were obtained from crocodiles on nine farms (four in Queensland and five in the Northern Territory), and from a group of experimental animals and one crocodile found dead in the wild. One affected crocodile in the Northern Territory had recently been introduced to a farm after capture in the wild. Six of the farms, three in Queensland and three in the Northern Territory were visited between May and August 1996. When representative samples of lesions were obtained, data on morbidity and mortality, possible aetiological agents and other likely predisposing factors, were also collected. All data gathered on recent (1996) cases, especially the gross and microscopic findings, were combined for analysis with data on retrospectively

examined cases on file in the Australian Institute of Tropical Veterinary and Animal Science (AITVAS), James Cook University of North Queensland.

4.2.2 Retrospective study

All microslides of crocodile skin lesions on file were re-examined and each lesion was classified according to the suspected or known aetiological agents. For each case the origin, type of skin sample (skin biopsy or from either dead or euthanased animal), gross and microscopic findings, results of microbiological culture, species, age, length (snout to tail tip) and sex was recorded. A total of 119 skin samples from 109 cases initially received and processed at the AITVAS during the period 1989 to 1995, was included.

4.2.3 Pathological examination

In addition to the retrospective cases, 84 skin lesions from 71 recent (1996 to1997) cases were examined. These lesions were mostly on emaciated crocodiles that died (28 cases), or were killed by barbiturate overdose for necropsy (34) to ascertain the cause of illness; nine were obtained by biopsy (the retrospective cases on file). More than one skin sample was collected from 23 crocodiles with multiple lesions and in some animals more than one diagnosis was made. Collectively (both retrospective and recent cases), 135 of the crocodiles were saltwater (*Crocodylus porosus*), 44 were freshwater (*Crocodylus johnstoni*) and one was of unrecorded species. Their mean length was 82.9 cm (range 25 to 110 cm, n=121) and ages were between two to 36 months (average 15 months, n=89). Eighty-seven were male, 32 were female and in 84 sex was not ascertained or recorded.

Necropsies were performed either on the crocodile farm, at James Cook University or at the Department of Primary Industry and Fisheries – Berrimah Veterinary Laboratories (DPIF-BVL), Northern Territory. From each crocodile, three samples of skin of at least 2 cm^2, and including representative lesions were fixed in 10% buffered neutral formalin and embedded in paraffin wax. For histological examination, sections were cut at 6 µm and stained with haematoxylin and eosin, or other stains such as Gram-Twort for bacteria, Gomori methenamine silver and periodic acid-Schiff for fungi, and Ziehl-Neelsen for mycobacteria.

Duplicate specimens of some suspected 'brown spot' lesions were excised and placed in sterile 5 mL plastic tubes for bacteriological examination, which was commenced at the BVL or AITVAS, then continued at the latter, where microbiological procedures used were aimed primarily at

isolating the filamentous organism, which was presumed to be the aetiological agent of 'brown-spot' disease (Buenviaje, Hirst, Ladds and Millan, 1997). No attempt was made to culture other bacteria.

4.3 Results

Table 4.1 compares the relative frequency of crocodile skin diseases within our collected cases, based on their aetiology and source for the period 1989 to 1997. Five specific diseases in uncomplicated form, namely dermatophilosis, mycotic dermatitis, poxvirus infection, probable mycobacterial dermatitis and capillariasis, were identified. Two other skin disease categories were mixed infections, and those of undetermined other cause, but with bacteria present superficially. Of the four major skin diseases, dermatophilosis was the most prevalent (57 cases, 28.1%), followed by mycotic dermatitis (30, 14.8%), pox (7, 3.4%), and probable mycobacterial dermatitis (5, 2.5%). Lesions with dual or multiple pathogens present, such as concurrent infections with poxvirus, fungi and dermatophilosis or other bacteria were common, being present in 66 cases. The 38 lesions classified as 'other dermatitis' included a single case of capillariasis (presumably *Capillaria crocodilus* infection); in the other 37 lesions a mixture of both Gram-positive and Gram-negative, but mostly Gram-positive, bacteria was present.

Dermatophilosis

Dermatophilosis was present in crocodiles on six farms. Most affected animals were emaciated and weak, with stunted growth. There were discrete, focal, 1 to 4 mm diameter, 'brown spots' of variable prominence on the skin (Figure 4.1), predominantly on the ventral abdomen but also elsewhere on the abdomen, tail or head. The lesions were situated mostly at the centre of scales (24, 42.1%) or along the 'hinge joint' between scales (Table 4.2). Lesions on the skin of the lower jaws (19 cases, 33.3%) however, usually presented as linear erosions up to 5 cm in length. In severe cases lesions were large, with irregular ulceration up to 2 cm^2 diameter on the abdomen (Figure 4.2). A *Dermatophilus* sp that resembled *Dermatophilus congolensis* both biochemically and morphologically was isolated from five such skin lesions in crocodiles from two farms in Queensland and the Northern Territory (Buenviaje *et al.*, 1997). Except in some cases of mixed infection, the *Dermatophilus* sp filaments were easily seen in sections stained with haematoxylin and eosin or particularly with periodic acid-Schiff stain (PAS).

Table 4.1 Frequency of occurrence of skin diseases in 203 lesions from 180 crocodiles, based on histological examination.

Source Farm[a]	Dermatophilosis	Poxvirus infection	Mycotic dermatitis	Probable mycobacterial dermatitis	Other causes (mostly bacterial)	Mixed infections[b]					Total
						A	B	C	D	E	
1	7	0	1	2	3	1	5	1	0	0	20
2	22	3	8	3	17	2	16	12	2	3	88
3	6	2	0	0	3	3	5	0	0	0	19
4	0	0	2	0	1	0	0	0	0	0	3
5	1	0	0	0	1	0	0	0	0	0	2
6	6	0	1	0	3[b]	0	2	0	0	0	12
7	15	2	1	0	6	7	3	0	0	1	35
8	0	0	3	0	3	0	0	0	0	0	6
9	0	0	0	0	0	0	1	0	0	0	1
10	0	0	14	0	1	1	0	1	0	0	17
						14	32	14	2	4	
Total	57 (28.1 %)	7 (3.4%)	30 (14.8%)	5 (2.5%)	38 (18.7%)	66 (32.5%)					203 (100)

[a] 1 to 4, farms in Queensland; 5 to 9, farms in the Northern Territory; 10, experimental crocodiles including one adult from the wild

[b] Presumed *Capillaria crocodilus* infection

A: Dermatophilosis with other infections, mostly bacteria
B: Dermatophilosis and fungal infection
C: Dermatophilosis and poxvirus infection
D: Concurrent dermatophilosis, poxvirus and fungal infections
E: Poxvirus and fungal infections only

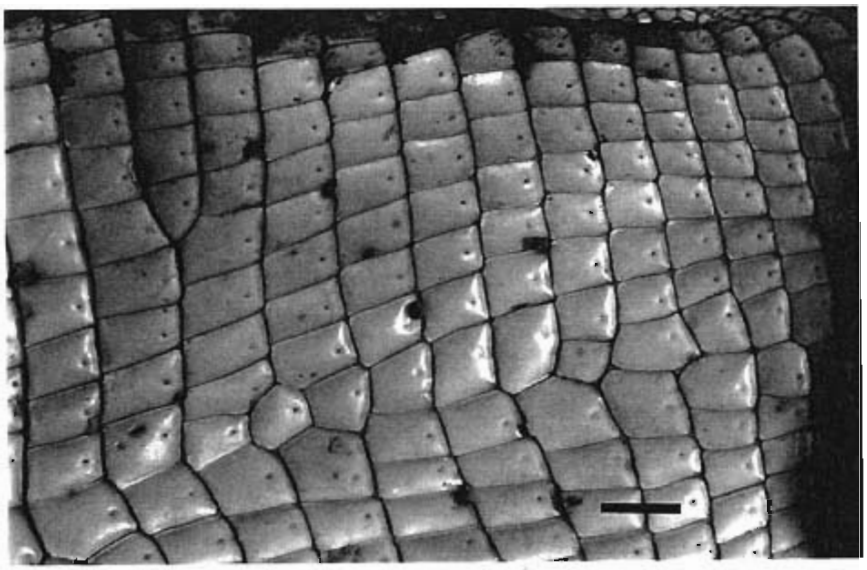

Figure 4.1 Dermatophilosis ('brown spot'), showing focal, usually discrete, lesions located mostly between scales of the abdomen of a hatchling crocodile. Bar = 4 mm.

Table 4.2 The location of 'brown spot' on the skin in 57 crocodiles with dermatophilosis.

Location	Number of cases	Percentage
a) centre of scales and along the hinge joints	35	61.4
b) jaws	19	33.3
c) feet	3	5.3

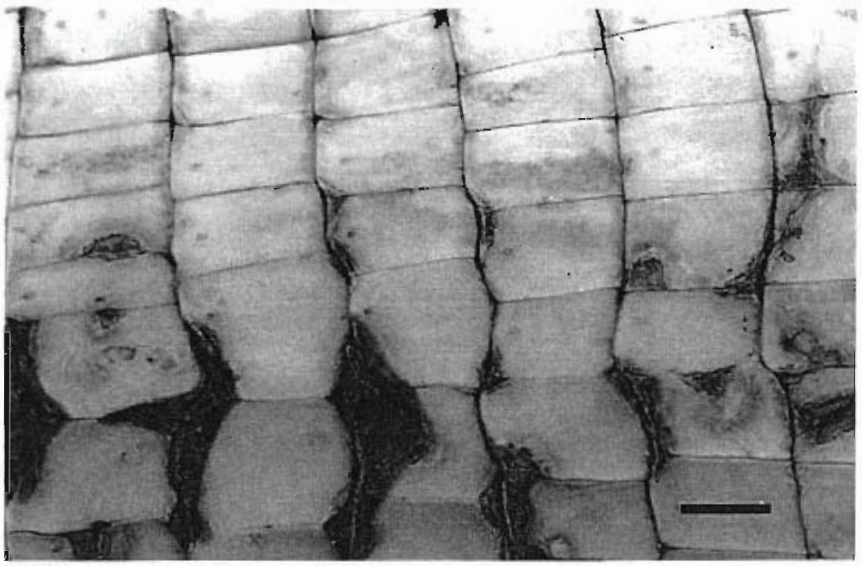

Figure 4.2 Dermatophilosis. More advanced lesion than in Figure 4.1. Note irregular ulceration of the skin of the abdomen. Bar = 1 cm

On the basis of histopathological examination, lesions of dermatophilosis were categorised into three, presumed progressive, stages of 'brown spot' development. The initial lesion was a focal lifting of the keratin accompanied by some accumulation of debris (Figure 4.3). *Dermatophilus* sp filaments and occasional Gram-positive coccoid organisms (1 µm diameter), possibly zoospores, were situated in the superficial layer of the epidermis (19 lesions) (Table 4.3), mostly in the keratinised 'periderm' (Sengel, 1976), but often deeper within intact epidermis (14 lesions).

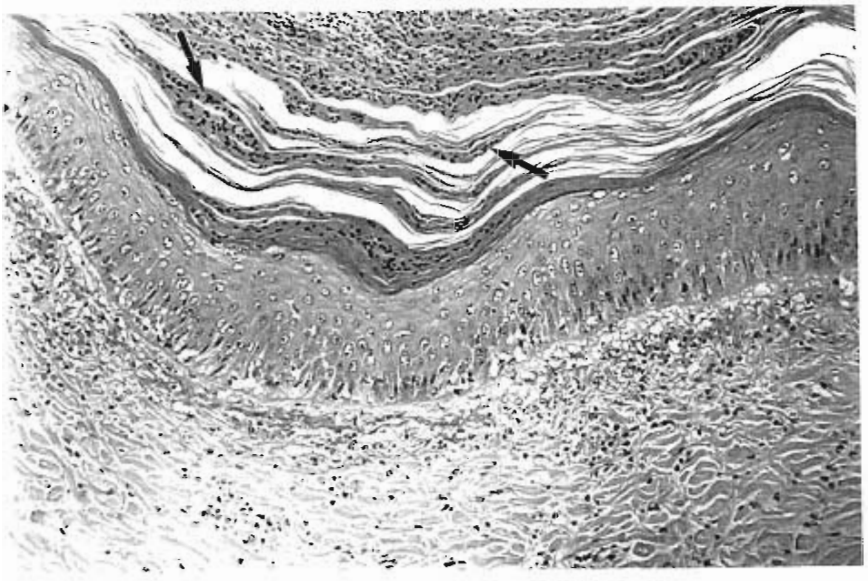

Figure 4.3 Early dermatophilosis lesion. Note lifting of laminated keratin, accompanied by debris between layers of keratin (arrows). Haematoxylin and eosin ×123.

Table 4.3 Location of *Dermatophilus* filaments on the skin in 57 crocodiles affected with dermatophilosis.

Location	Number of cases	Percentage
Superficial	19	33.3
Within intact epidermis	14	24.6
Extension into dermis from eroded or ulcerated epidermis	15	26.3
Extension to subcutis or muscle layer from eroded or ulcerated epidermis	9	15.8

In the next stage of lesion development the epidermis was indented, but still intact, presumably because of continuous replacement of the necrotic cells by hyperplasia of cells of the stratum basale (Figure 4.4). However, eventual erosion of the epidermis frequently seemed to result in the third stage ulceration (15 lesions, 26.3%) which was accompanied by increased debris, keratin and extension of the filamentous organism into the subcutis, either directly or across remaining eroded epidermis (Figure 4.5). At this stage, much debris and nuclei, presumably those of parakeratotic cells, were apparent as layers within keratin in 32 of 57 lesions (Table 4.4). The debris was composed of necrotic inflammatory cells, keratin and proteinaceous exudate, and was extensively infiltrated at all levels by filamentous organism (Figure 4.6). Cholesterol clefts were sometimes present within this debris. Heterophils were scattered between cells of the epidermis and in the dermis. Other changes were acanthosis, spongiosis and exaggerated formation of rete ridges. Apoptosis and dyskeratosis were also observed in association with acanthosis. The presence of cell debris of affected epidermis, associated with lifting of keratinised periderm, occurred early (Figure 4.3), but was an almost consistent change, being present in 86% of lesions. Inflammatory infiltrates in the dermis were mostly (75% of lesions) of lymphohistiocytic type, surrounding the lesion, but specifically around blood vessels. Multinucleate giant cells were present in two lesions associated with the filamentous organisms. In one, they were immediately beneath damaged epidermis, while in the second they were deeper within the dermis. Late in the third stage of development of lesions, ulceration extended to involve a larger area of the dermis and often also the underlying muscle (Figure 4.7), which in nine cases (15.8%) was infiltrated by *Dermatophilus* sp. Other bacteria were present superficially in all dermatophilosis lesions, but were never observed in deeper tissue.

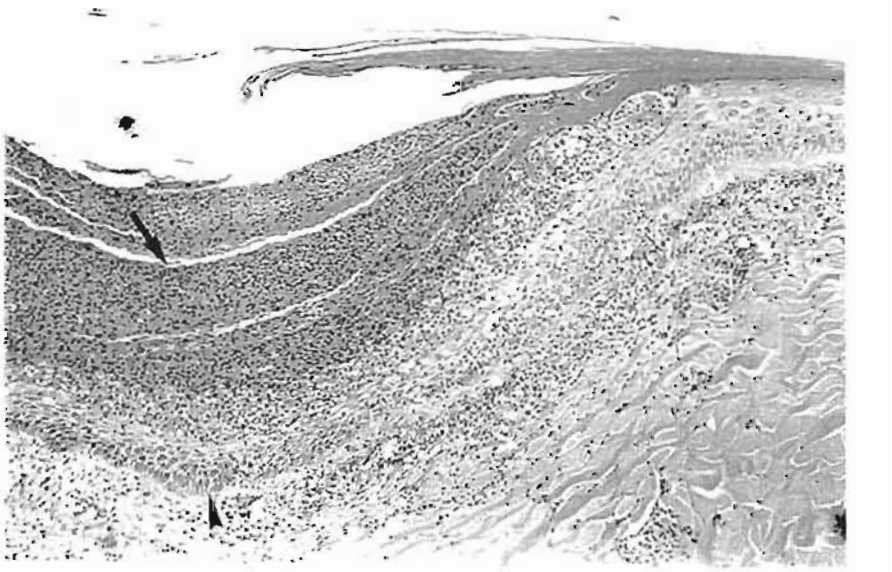

Figure 4.4 Dermatophilosis lesion at a more advanced stage than shown in Figure 4.3. Note excessive superficial debris (arrow), hyperplasia of cells of the basal layer (arrow head), and increased inflammatory cell infiltration of the dermis. Haematoxylin and eosin ×86.

Figure 4.5 Dermatophilosis, showing numerous thin filaments across the ulcerated epidermis (arrow head). Haematoxylin and eosin ×235.

Table 4.4 Histopathological changes of the skin in 57 crocodiles affected with dermatophilosis.

Microscopic lesions	Number of cases	Percentage
Accumulation of debris	49	85.9
Acanthosis	40	70.2
Parakeratosis/hyperkeratosis	32	56.1
Spongiosis	37	64.9
Rete ridges formation	13	22.8
Migration of heterophils	20	35.1
Apoptosis	5	8.7
Lymphohistiocytic perivasculitis	43	75.9
Cholesterol clefts	3	5.3

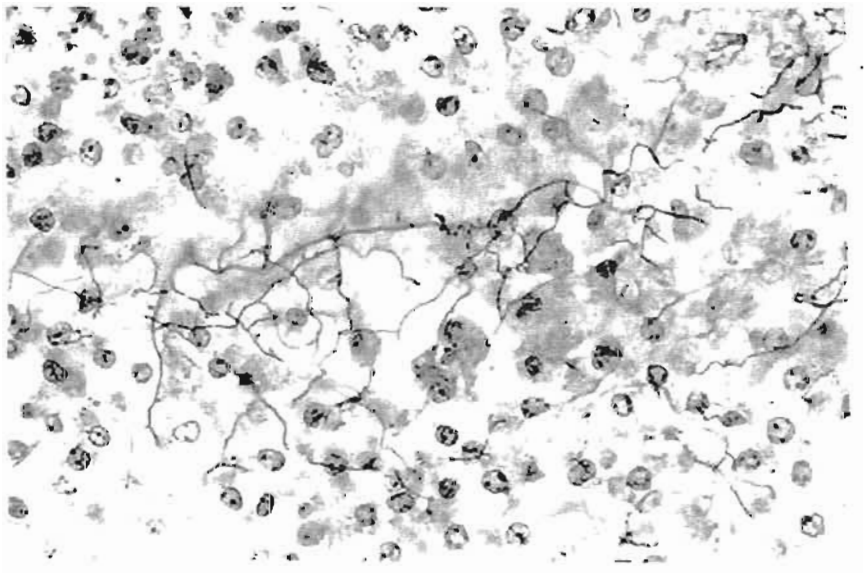

Figure 4.6 Higher magnification of the lesion in Figure 4.5, showing irregularly branching filamentous organism on debris composed of cellular infiltrates and degenerating inflammatory cells. Haematoxylin and eosin ×700.

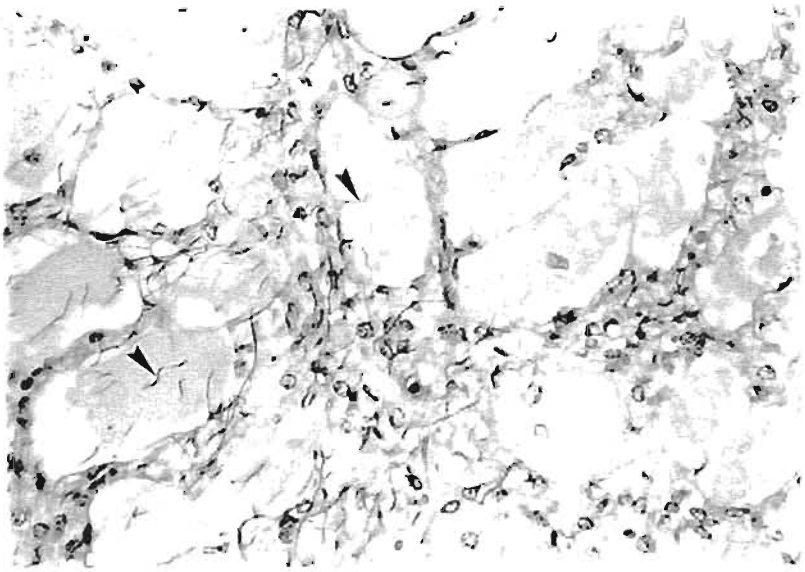

Figure 4.7 Dermatophilosis, showing degenerating muscle fibres infiltrated with thin filamentous organisms (arrow heads). Haematoxylin and eosin ×515.

Mycotic dermatitis

Although mycotic dermatitis as a primary disease was the second most frequently seen, only three lesions, in a total of 30 diagnosed, were recent, and the prevalence of mycotic dermatitis extrapolated from the data seemed to have declined between 1993 and 1996, with a large number of cases in 1992 and previously (Appendix Table 2). A grey, gelatinous appearance of affected skin was a consistent gross characteristic. Such lesions, which developed over a period of one week or so, were present on any location of the body, but especially on the dorsal skin of the head (Figure 4.8).

Figure 4.8 Mycotic dermatitis. Grey, gelatinous appearance on the dorsal skin of the head and the shoulder (arrow heads).

Microscopically, affected skin was mostly ulcerated (16 lesions) or eroded (12 lesions) and in two lesions a subcutaneous granuloma was below an intact epidermis, probably indicating that fungi had persisted in this (deep) location after re-epithelialisation of ulcers. Mats of fungal hyphae were on the surface (Figure 4.9) but they often (18 lesions) infiltrated down to the dermis or muscle. Affected epidermis was acanthotic, with heterophilic infiltration. Other consistent microscopic changes were spongiosis, formation of rete ridges and accumulation of debris. Histiocytes, other mononuclear cells and heterophils were widely scattered, but were more densely accumulated near the ulcers. In the subcutaneous granulomas, centrally located fungal elements were surrounded by multinucleate giant cells, histiocytes, and other mononuclear cells, few heterophils and fibrous tissue (Figure 4.10 and Figure 4.11).

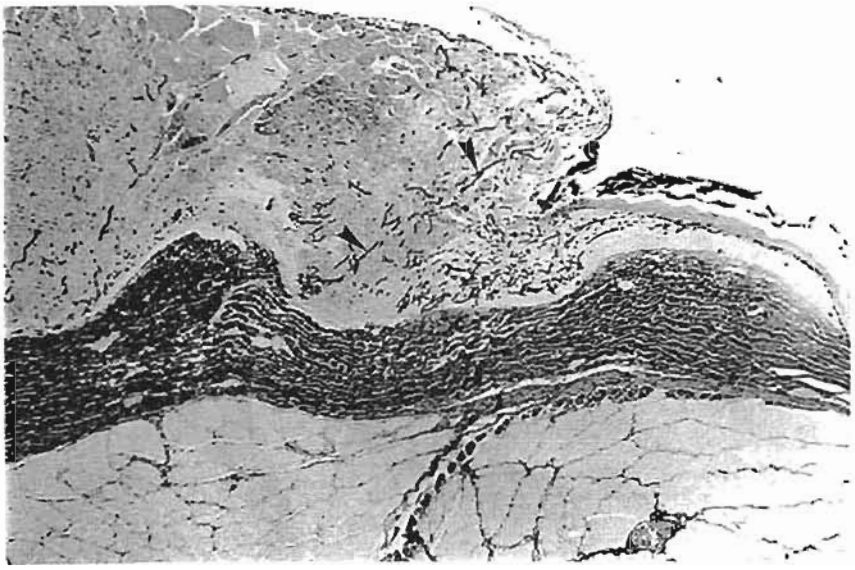

Figure 4.9 Photomicrograph of mycotic dermatitis showing fungal hyphae (arrow heads) infiltrating into the dermis. Gomori methenamine silver ×69

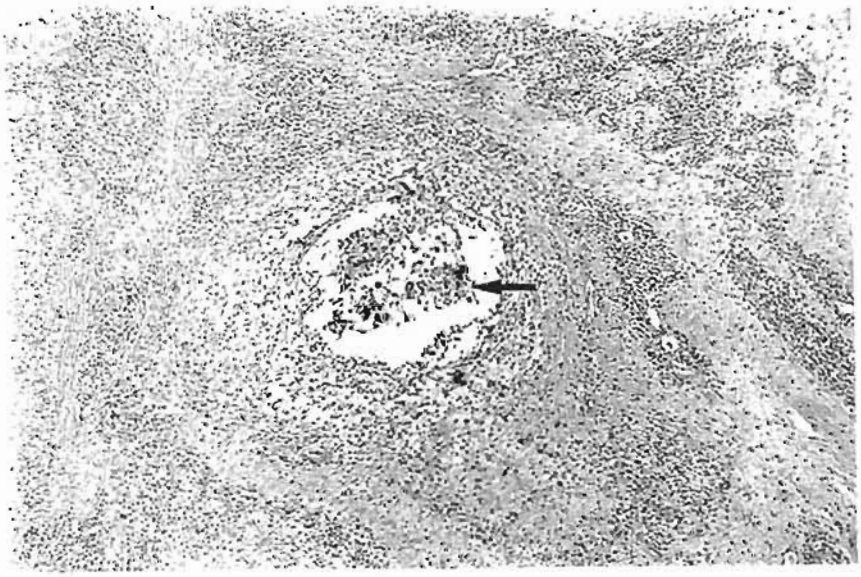

Figure 4.10 Mycotic dermatitis. Fungal granuloma showing cellular aggregates at the centre and surrounded by inflammatory cells (arrow). Haematoxylin and eosin ×75.

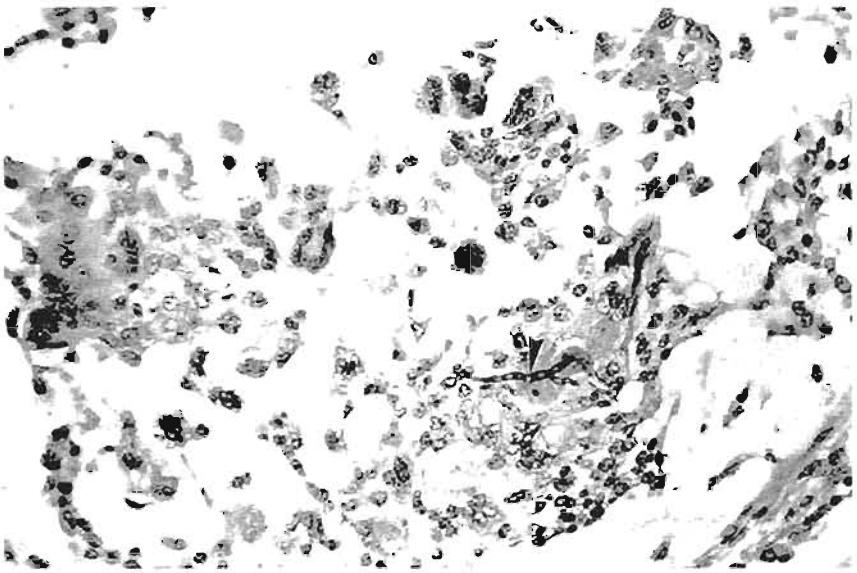

Figure 4.11 Higher magnification photomicrograph of the same section as in Figure 4.10, showing fungal hyphae (arrow head) surrounded by mostly histiocytes and few giant cells. Periodic acid-Schiff ×353

Fungal hyphae were stained strongly by the PAS method. In most lesions they were septate, had a relatively uniform diameter of 2.5 - 3 µm and sometimes branched in a dichotomous manner. This morphology was consistent with their being *Fusarium* sp (Chandler *et al.*, 1980). In several crocodiles mixed accumulations of fungal hyphae, yeast, algae and bacteria were also present superficially on intact skin; as they had not infiltrated the epidermis, and as there was no inflammatory response, these organisms were not judged to be pathogenic and were therefore discarded. Fungi isolated from retrospectively examined cases revealed *Fusarium* sp as the most common isolate (8 of 12 lesions). Other fungi identified as reported in retrospectively examined cases were *Candida* sp (2), *Syncephalastrum* sp (2) and a single isolate of *Candida parasilosis*, *Aspergillus niger*, *A flavus* and *Trichosporon cutaneum*.

Poxvirus infection

Seven primary poxvirus lesions were seen in five crocodiles from two farms (farms 2 and 3) in Queensland and a further two lesions were on crocodiles from one farm (farm 6) in the Northern Territory. Of the seven poxvirus lesions, four were diagnosed in 1996 while three were retrospective cases. The gross and microscopic lesions were in all cases typical to those infections already reported. Two lesions on the foot were adjacent to the gas filled spaces of

concurrent interdigital subcutaneous emphysema (Turton *et al.*, 1990). Ulceration of a further single poxvirus lesion however had extended to the deep dermis.

Mycobacterial dermatitis

Five lesions of presumed mycobacterial dermatitis were diagnosed in crocodiles from two farms in Queensland. Three were recent cases from farm 2 and two were retrospective (1992) cases from Farm 1. Gross examination of affected skin revealed individually raised, red to grey nodules, 2 to 5 mm in diameter, on the snout, conjunctiva, jaws and along the ventral side of the neck and medial thigh. Histological examination of two retrospective cases showed a well-circumscribed granuloma in the dermis, beneath an intact epidermis. The other three lesions had erosion of the epidermis and accumulations of multinucleate giant cells, histiocytes, lymphocytes and heterophils. Acid-fast organisms in Ziehl-Neelsen stained sections were scattered throughout the granuloma, with some organisms present inside the giant cells (Figure 4.12).

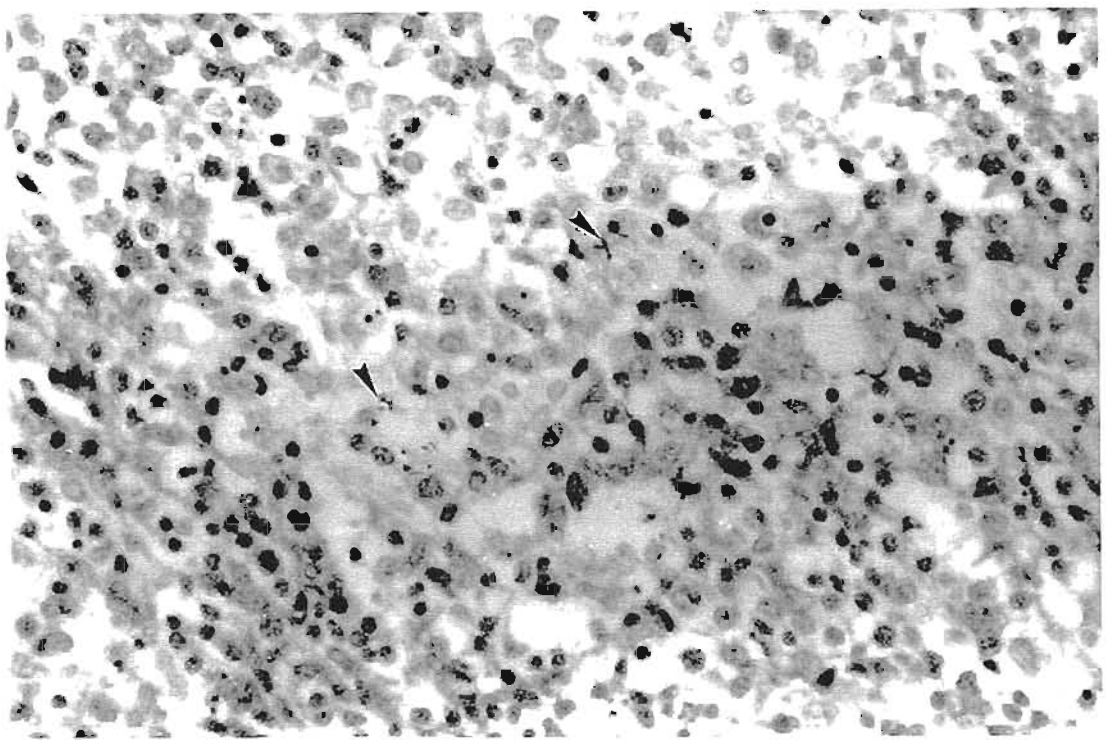

Figure 4.12 Photomicrograph of mycobacterial dermatitis. Note rod-shaped bacterial cells (arrowheads) associated with mostly histiocytic cells. Ziehl-Neelsen ×504

Mixed infections

Of the 66 lesions with demonstrable dual or multiple infections, 62 (94%) contained *Dermatophilus* sp. Most of these lesions were deep ulcers that involved a wide area of the dermis and subcutis. Occasionally, along the edges of the ulcers, there were scattered multinucleate giant cells associated with Gram-positive bacteria (Figure 4.13). In this particular type of dual infection only a few, usually superficial, *Dermatophilus* sp filaments were present while the bacteria were located deeper within the ulcers. The affected skin was oedematous, congested and sometimes haemorrhagic, and was infiltrated with predominantly heterophils and lymphohistiocytic cells.

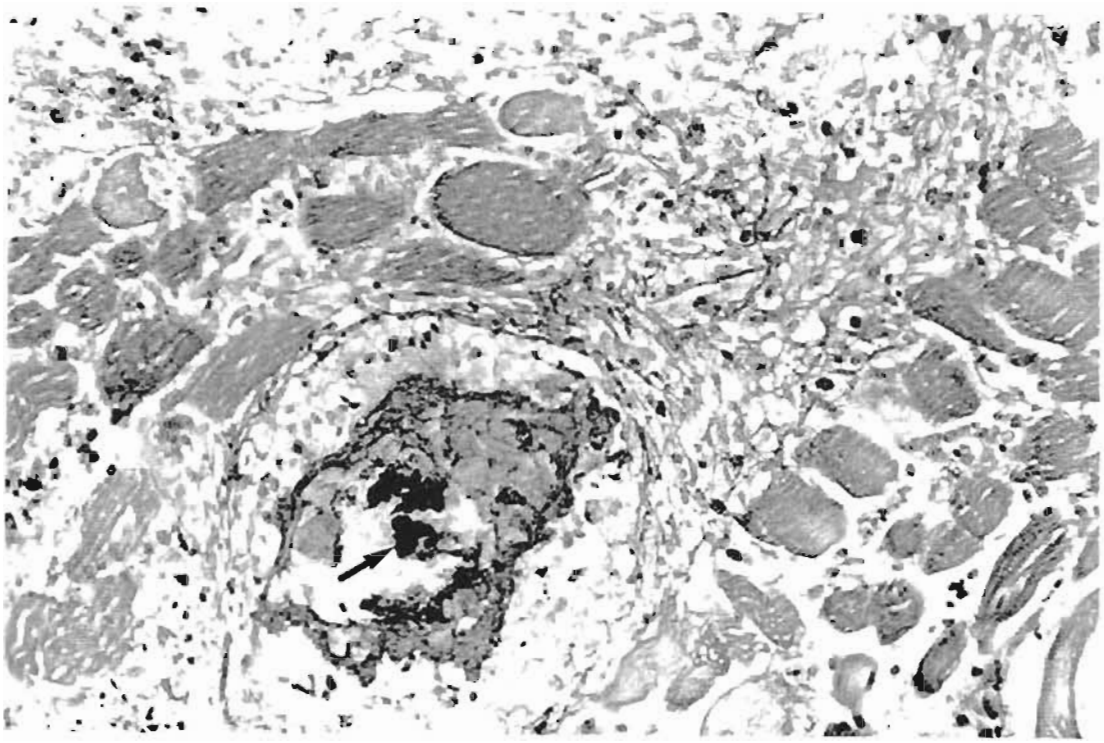

Figure 4.13 Bacterial dermatitis. Note colonies of bacteria (arrow) surrounded with histiocytes. Gram's stain ×258

Dermatophilosis was also diagnosed as a mixed infection with other bacteria in the adult crocodile that was found dead in the wild. This animal had multiple ulcers up to 40 mm in diameter, which sometimes extended to underlying bone, on both rami of the mandible. Trauma, perhaps from fighting, was the likely primary cause, with *Dermatophilus* sp and other bacterial infection being secondary. Microscopically, lesions in this case were similar to those in dermatophilosis in farmed crocodiles but, in addition, the filaments were seen inside blood vessels of the deep dermis where they were associated with other Gram-positive bacteria.

Other causes of dermatitis

In 20 recently collected lesions there were raised ulcers up to 6 mm in diameter, filled with debris. These lesions were mostly located on the skin of the back and tail. The irregular shape of the ulcers often suggested recent puncture wounds, probably bites. Microscopically, accumulated debris within the ulcers was associated with numerous bacteria. There was oedema, congestion and inflammatory infiltration of the dermis composed of heterophils, lymphocytes, macrophages, lymphohistiocytes and some multinucleate giant cells. Adjacent to ulcers were colonies of Gram-positive cocci encircled by multinucleate giant cells and histiocytes. However, on the surface of the lesions there was a mixture of numerous pleomorphic, Gram-positive and Gram-negative bacteria. In addition, intradermal inclusion cysts filled with debris, proteinaceous material, inflammatory infiltrates and bacteria, were present close to these lesions.

In a single, 18-month-old *C porosus* that measured about 150 cm in length (snout to tail tip), a striking, dark, serpentine pattern was present on a number of adjacent scales near the cloaca (Figure 4.14). The crocodile, which was clinically normal and had no other lesions on the skin, had recently been introduced to the farm (farm 6) after its capture in the wild. Microscopically, both the keratinised and cellular layers of the epidermis had numerous, slightly irregular, cystic spaces (93 × 175 µm) that contained three to five operculated eggs measuring from 27 µm up to 73 µm (Figure 4.15) or, in some cases, two to three eggs as well as transverse sections of what was presumed to be a single adult female parasite (Figure 4.16) with ova in its uterus. Approximate diameter of this nematode as measured in histological sections was 138 µm. The clear cystic spaces in the periderm were smaller than those in the cellular layer of the epidermis, possibly because the absence of adult parasites in the keratin layer permitted greater contraction of keratin during fixation and histological processing. Eggs present in the periderm had scanty basophilic granules, and were presumably those of an embryo in its first to second stage of development. Epidermal cells adjacent to the parasite-containing cystic spaces were flattened.

In one *C porosus* juvenile that died after not eating and being listless for several days, there was extensive necrosis with lifting of entire scales that were easily detached (Figure 4.17). Necropsy revealed extensive subcutaneous oedema, myolysis and numerous large colonies of Gram-positive bacteria, but few infiltrating inflammatory cells, immediately beneath the detached scales. In addition to the skin lesions, there was a disseminated focal hepatitis and splenitis with bacteria present in the liver. A fulminating bacterial septicaemia was diagnosed.

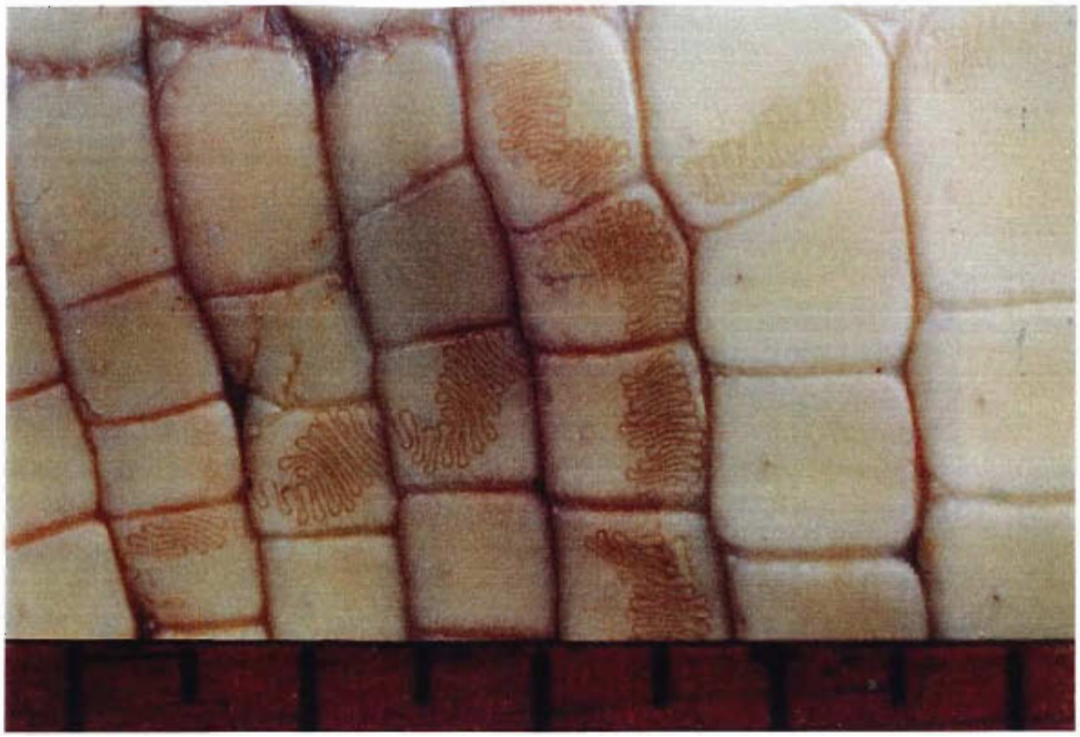

Figure 4.14 Skin infected with presumed *Capillaria crocodylus*. Note striking black serpentine pattern on the scales. Scale = cm

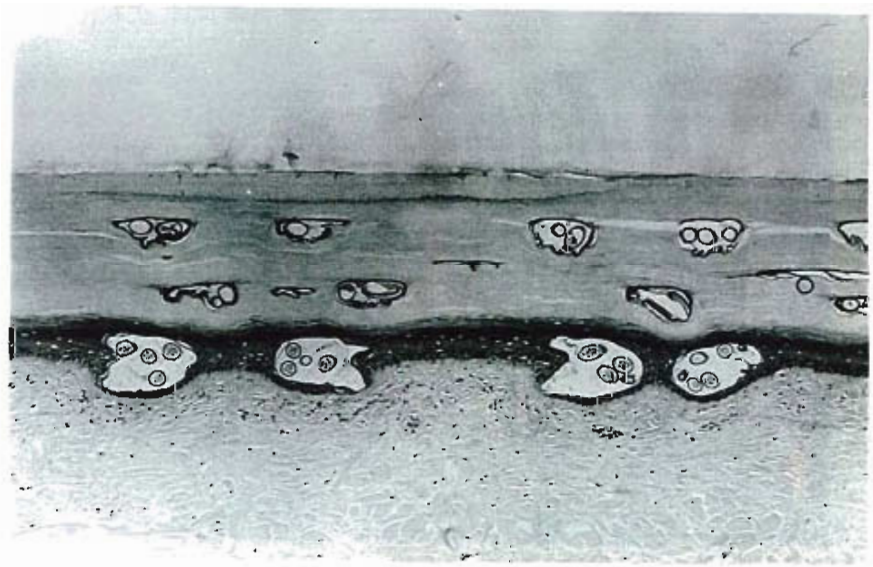

Figure 4.15 Same section as in Figure 4.7, showing eggs inside clear cysts in both the keratin and cellular layers of the epidermis. Haematoxylin and eosin ×79.

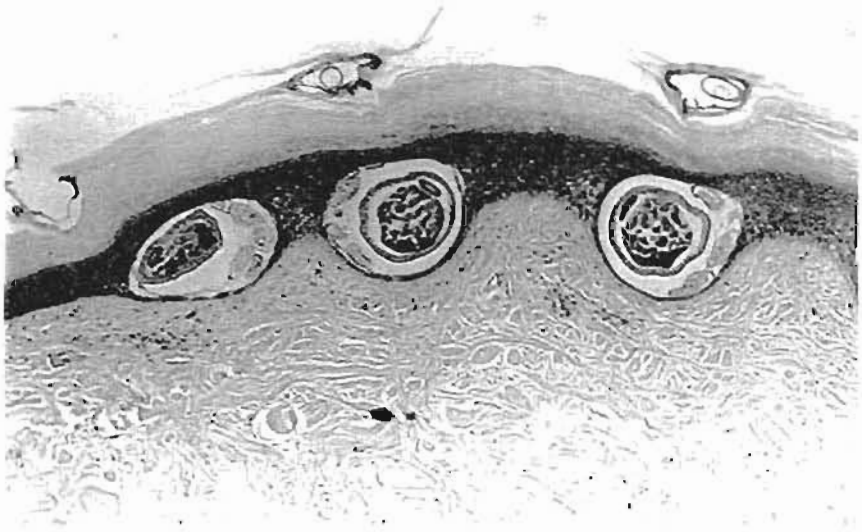

Figure 4.16 Adult presumed *Capillaria crocodylus* with some eggs, inside the clear cystic spaces of the same section as in Figure 4.7. Haematoxylin and eosin ×79.

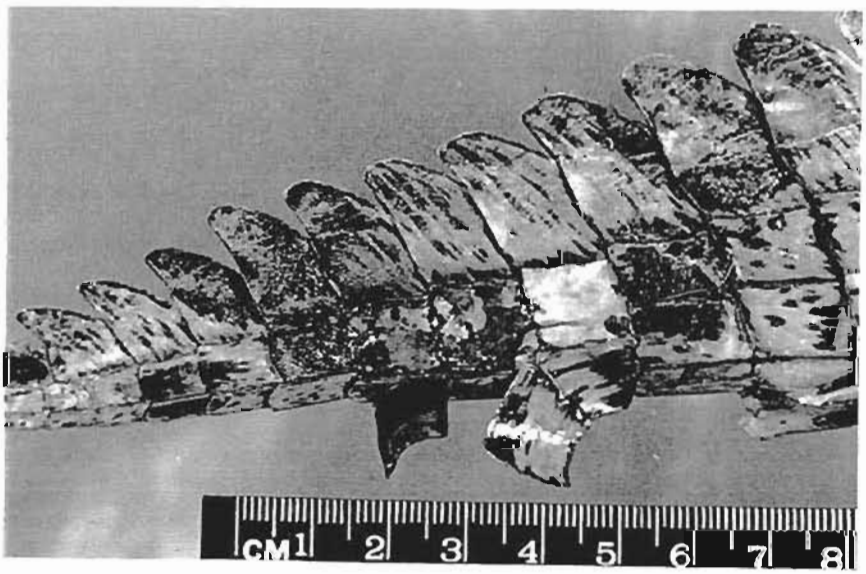

Figure 4.17 Necrotising lesion, with lifting of entire scales of the skin of the tail. Microscopically there was diffuse necrosis of the subcutis and contiguous muscle in this crocodile, which died as a consequence of fulminating bacterial septicaemia.

4.4 Discussion

Although we recognised that there were some limitations and problems in this study as far as the standard epidemiological procedure is concern, the main purpose in this study was to identify the different skin diseases present in farmed crocodiles in Australia. The results of this study served as benchmark information, which were useful for further research described in Chapters 5 to 9.

Based on the overall results of pathological examination of the 203 lesions, dermatophilosis was the most frequently diagnosed, and probably the most important skin disease in farmed crocodiles in Australia. The 'typical brown' spot in this study pitted the scales to various depths, especially on the abdomen, and sometimes extended to involve the underlying tissues, including muscles, as does 'brown spot' in alligators (Newton, 1992). An investigation into skin lesions in farmed Nile crocodiles (*C niloticus*) in Zimbabwe (Stuart, 1993) also identified the 'brown spot' lesion as an important skin problem. An organism that culturally resembled *D congolensis* and was isolated from farmed alligators (Newton, 1992) and crocodiles (Buenviaje *et al.*, 1997) was considered the causative agent.

Dermatophilosis in other reptiles has not been characterised by typical 'brown spot' lesions; instead, a subcutaneous abscess was seen in Australian bearded lizards (*Amphibolurus barbatus*) (Simmons *et al.*, 1972) and turtles (Masters *et al.*, 1995). Microscopic study in Australian bearded lizards (Montali *et al.*, 1975), however, did reveal changes comparable to those in the present study.

Although caution is needed when using spontaneous lesions to interpret pathogenesis, several infected skins in the present study had Gram-positive coccoid organisms (about 1 µm in diameter) associated with a few filaments on the periderm. These organisms were considered likely to be zoospores of *Dermatophilus* sp in their initial stage of attachment.

As the infective zoospores of *D congolensis* are chemotactically attracted to CO_2 (Roberts, 1962) its normal release from the skin may facilitate the establishment of infection in reptiles or its spread to other areas of the body. The volume of CO_2 released through the skin varies among species of reptiles, aquatic turtles such as *Trionyx spiniferous* losing 64% CO_2 in this manner (Gans and Pough, 1982).

In cattle, under appropriate conditions such as during periods of heavy rainfall or after minor damage to the epidermis (Lloyd and Jenkinson McEwan, 1980) the zoospores of *Dermatophilus* sp are stimulated to germinate and form filaments that invade the epidermis and sometimes extend to the dermis or underlying tissues, as was observed in crocodiles in the present study. The initial damage to the keratin layer of the epidermis led in turn to necrosis of the underlying cells with ulceration and build up of debris. Such necrosis was presumably due to the keratolytic enzymes produced by *D congolensis* (Hermoso de Mendoza, Arenas, Alonso, Rey, Gil, Anton and Hermoso de Mendoza, 1993). In addition, a haemolytic substance (Skalka and Pospisil, 1993) produced by *D congolensis* may exacerbate the condition, although its role in the pathogenesis of dermatophilosis is uncertain. In crocodiles in the present study, as in cattle, it was presumed that *Dermatophilus* sp had invaded uninjured epidermis. This presumption is in accord with experimental observation of the rapid development of 'brown spot' lesions in the skin of normal *C porosus* juveniles, the aquatic environment perhaps facilitating spread of infection.

The presence of *Dermatophilus* sp filaments in 94% of lesions with multiple infections clearly suggests that this organism plays a significant role in causing damage to the skin. Histopathological study of the lesions with multiple infections consistently revealed changes typical of those in 'uncomplicated', *Dermatophilus* sp infection. Presumably in such cases, *Dermatophilus* sp initiates the damage to the epidermis, then further invasions by other opportunistic microorganisms follow, particularly in crocodiles whose immune and/or inflammatory responses are compromised by stress. Because *Dermatophilus* sp often grow slowly, other fast-growing microorganisms may aggressively cause ulceration, then invade deeply into the dermis. Some bacteria, especially *Bacillus* spp are able to produce a substance that inhibits the growth of *Dermatophilus* sp (Kingali, Heron and Morrow, 1990) so that, in such mixed infections, as observed in 65 lesions in the present study, the filaments were scanty and mostly superficial.

The finding of typical histological lesions of dermatophilosis in the single crocodile that died in the wild is of particular interest. The present and previous studies suggest a high prevalence of *Dermatophilus* sp in farming environment; its occurrence in the wild emphasises its ubiquitous nature and clearly suggests a likely route of its introduction to farms.

Uncomplicated mycotic dermatitis seems to be no longer a serious problem on Australian crocodile farms considering that it was the cause of only three lesions diagnosed recently in this study. Retrospectively, however, mycotic dermatitis was a serious disease problem in all farms in Queensland and the Northern Territory (Buenviaje *et al.*, 1994) but, with improved husbandry,

especially in regard to provision of a water temperature of 32°C for hatchlings, the occurrence of mycosis in this study seemed to have decreased.

The typical grey, gelatinous appearance of affected skin is a consistent clinical and gross pathological finding in mycotic dermatitis, which can be readily diagnosed by the microscopic demonstration of fungal hyphae, especially in ulcerated or eroded skin. The subcutaneous fungal granulomas in which hyphae were surrounded predominantly by lymphohistiocytic cells and some multinucleate giant cells have also been observed by others (Hibberd *et al.*, 1996) and it appears that such lesions result from re-epithelialisation over deep lesions, essentially 'burying' fungal elements in the dermis and subcutis.

The occurrence of poxvirus infection was relatively low on crocodile farms in Australia compared with severe outbreaks with high morbidity in Africa (Huchzermeyer *et al.*, 1991). In this present study, the absence of an inflammatory response in many lesions may have been related to a poor immunological response (Huchzermeyer *et al.*, 1991).

Although mycobacterial infections in alligators (Shotts, 1981) and other crocodilians (Cooper, 1981) have been reported, details of histopathological findings have not been described. The ulcerative cutaneous granulomas as found in five lesions in both *C porosus* and *C johnstoni* were morphologically similar to cutaneous mycobacteriosis in other reptiles (Marcus, 1971). Bacteriological studies were not done in this case, but recent polymerase chain reaction studies on disseminated mycobacterial granulomas in *C johnstoni* confirmed the presence of *Mycobacterium* sp (Ariel *et al.*, 1997b).

The sizes of the operculated eggs and adult worm inside 'serpentine tunnels' of one crocodile in this study were consistent with the parasite being *Paratrichosoma crocodilus* or *Capillaria crocodilus*, as described in infected skin of the New Guinean crocodile (*C novaeguineae*) (Asford and Muller, 1978; Spratt, 1985). As the larvae within the eggs in the keratin layer were more developed than those in the epidermis, it seems likely that they mature in the periderm, and are shed with the keratin into the environment. Infection of other crocodiles in the same pen however, was not observed, indicating that this infection is maybe restricted to crocodiles in the wild.

CHAPTER 5

ISOLATION OF *DERMATOPHILUS* SP FROM SKIN LESIONS IN FARMED SALTWATER CROCODILES (*CROCODYLUS POROSUS*)

5.1 Introduction

The skin is the most valuable product of crocodile farming and any disease affecting it diminishes the quality of the leather and hence its market value. So called 'brown spot' disease, characterised by multiple, small, tan to brown lesions on skin in most body locations, has been recognised by farmers as an important disease problem because affected hides are downgraded.

Several reports on crocodilian skin lesions have described the presence of a branching, filamentous organism and on this basis a tentative diagnosis of probable dermatophilosis has been made (Ladds and Donovan, 1989; Ladds and Sims, 1990; Foggin, 1992; Turton, 1993). Similar lesions have been noted in alligators (Newton, 1992) and a filamentous organism resembling *Dermatophilus* was isolated from several animals with 'brown spot' lesions on one farm in Louisiana (Bounds and Normand, 1991).

Dermatophilus congolensis has been isolated from lizards (Simmons *et al.*, 1972; Montali *et al.*, 1975) and chelonids and genetic analysis of isolates from chelonids justifies their designation as *Dermatophilus chelonae* (Masters *et al.*, 1995; Trott *et al.*, 1995). *Dermatophilus congolensis* is a recognised cause of skin diseases in mammals and other animals (Radostits, Blood and Gay, 1994).

The objective of this study is to investigate the cause of 'brown spot' disease affecting farmed crocodiles in the Northern Territory and Queensland. Furthermore, isolation of the aetiological agent from the skin lesions was necessary so that further studies on transmission (see Chapter 6) and nucleic acid characterisation (see Chapter 7) could be undertaken.

5.2 Materials and Methods

5.2.1 Laboratory preparation

Thirty-three farmed saltwater crocodiles (*Crocodylus porosus*) with various skin lesions were examined clinically. Twenty were males, five were females and eight were unsexed, and their

lengths ranged from 64 to 147 cm. On one farm in the Northern Territory, the owner reported that more than 85% of crocodiles one to three years old had 'brown spot' disease; a lower prevalence was observed on other farms. Twenty-three crocodiles were killed by barbiturate overdose and these, and a further 10 that died naturally, were necropsied. At least three skin samples, each about 2 cm^2 and representative of different types of lesions, were collected from each affected crocodile, fixed in 10% buffered neutral formalin and processed for histopathological examination in the routine manner. Duplicate specimens excised for bacteriological examination were placed immediately in 5 mL sterile plastic tubes and stored at –20°C. Blood agar (see Appendix 3.2) was prepared with polymyxin B (1,000 unit/mL of medium). At least 24 hours after collection the skin samples were ground with a mortar and pestle and 1 mL of sterile pH 7.0 PBS (see Appendix 3.21) was added. At least three drops of each suspension were then spread on five blood agar plates with polymyxin B, and incubated at 37°C in an atmosphere of 5% CO_2. The cultural, morphological and the biochemical examinations were performed on the isolates (Chapter 7).

5.2.2 Clinical examination

Gross examination of the skins of affected crocodiles showed 'brown spot' lesions of varying size, sometimes accompanied by subcutaneous nodules up to 3 cm in diameter. The lesions were mostly scattered over the abdomen and neck, but were occasionally also on the tail and feet. Four different types of lesions were observed, namely, brown or red spots, 1 to 4 mm in diameter located either on the 'hinge joints' between the scales or at the centre of them (Figure 5.1); irregular brown ulcerations up to 2 cm in diameter; extensive skin erosions, up to 5 cm in length particularly on the jaw; and subcutaneous granulomas, which were sometimes beneath ulcers.

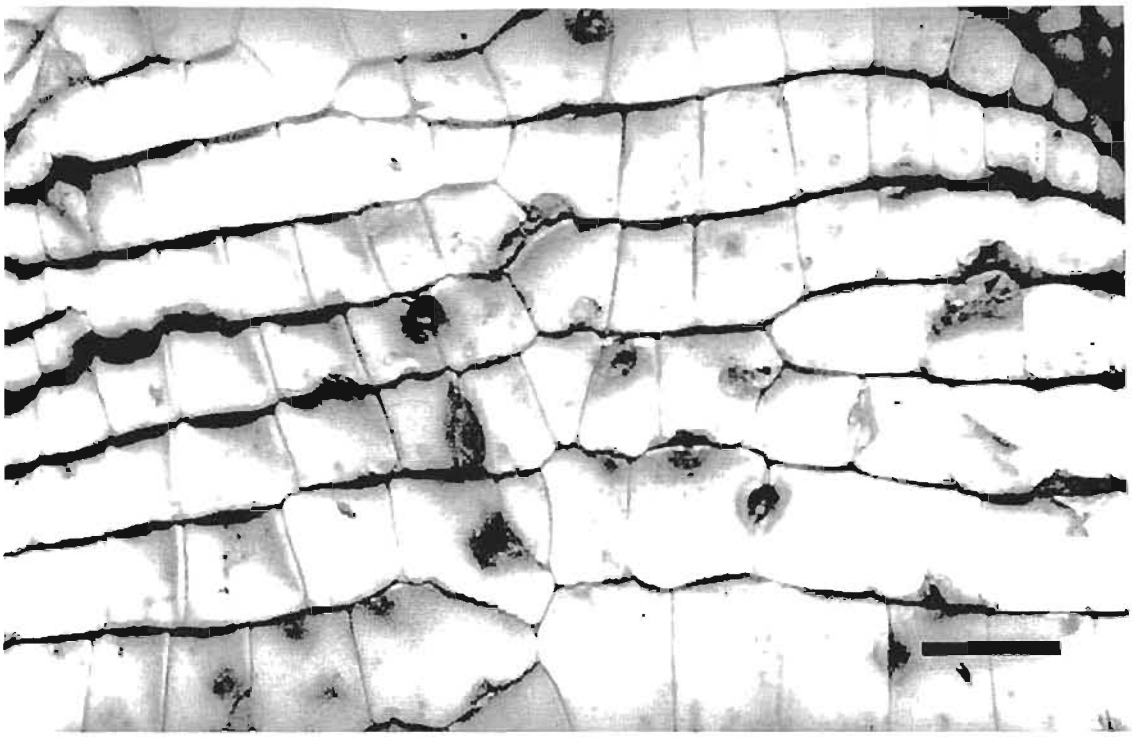

Figure 5.1 Lesions of 'brown spot' disease, on and between scales of the belly of a crocodile. Bar = 2 cm

5.3 Results

Histopathological examination in 22 of 27 (82%) skin samples, revealed a branched, filamentous organism in association with debris composed of occasional inflammatory cells, proteinaceous exudates, superficial laminated keratin and degenerating or necrotic epidermal cells. Other lesions present in the epidermis were spongiosis, formation of rete ridges, acanthosis and exocytosis. In severe cases the epidermis was ulcerated, with invasions of filamentous organisms into the dermis. The most consistent lesion in the dermis was lymphohistiocytic perivasculitis.

After culture for 48 hours, a few white to grey colonies, up to 3 mm in diameter and with pitting into the medium, were observed in three isolates (TVS 96-367-15A, TVS 96-490-7B and TVS 96-490-9B). After one week the colour of colonies changed to orange. The organisms were filamentous and branching, Gram-positive, non-acid-fast, catalase positive and oxidase negative. Comparative bacteriological studies were performed using three different *D congolensis* type cultures obtained from the University of Queensland and one clinical isolate from cattle from the Queensland Department of Primary Industries, Oonoonba Veterinary Laboratory. The

D congolensis type culture (ACM No 532 from the University of Queensland) was compared with four isolates of *Dermatophilus* sp from crocodiles.

Culturally, all the isolates were identical and were indistinguishable from the type culture. All *Dermatophilus* sp isolates and the type culture, when grown at 37°C in an atmosphere of 5% CO_2, produced grey to yellow colonies up to 3mm in diameter (Figure 5.2) and with pitting into the medium. A zone of beta haemolysis developed between one and five days and the colonies were raised and rugose to crateriform. Two of the four isolates produced occasional, white, aerial hyphae similar to the type culture. Furthermore, all isolates cultured on 10% ovine serum in tryptose phosphate broth at 37°C produced, after 72 hours a clear supernatant with granular clumps at the bottom of the tubes. Cellular morphology of all organisms at 48 hours was similar, namely, a Gram-positive filamentous organism (Gordon, 1976) with hyphae branched at right angles (Figure 5.3). The width of hyphae ranged from 0.5 to 1.5 µm, while the zoospores were 1 µm in diameter. All crocodile isolates differed from the type culture in regard to the following: variable hydrolysis of Loeffler's coagulated serum and casein, and nitrate and indole production. In addition, all crocodile isolates had filaments divided by transverse septa only and produced very few zoospores in contrast to *Dermatophilus congolensis*. Other biochemical test results of all isolates and the type culture was similar, namely, fermentative, catalase and urease positive; negative for oxidase, methyl red, Voges-Proskauer and tyrosine; no acid produced from lactose, sorbitol, xylose, dulcitol, mannitol and salicin; acid produced from glucose (Chapter 7).

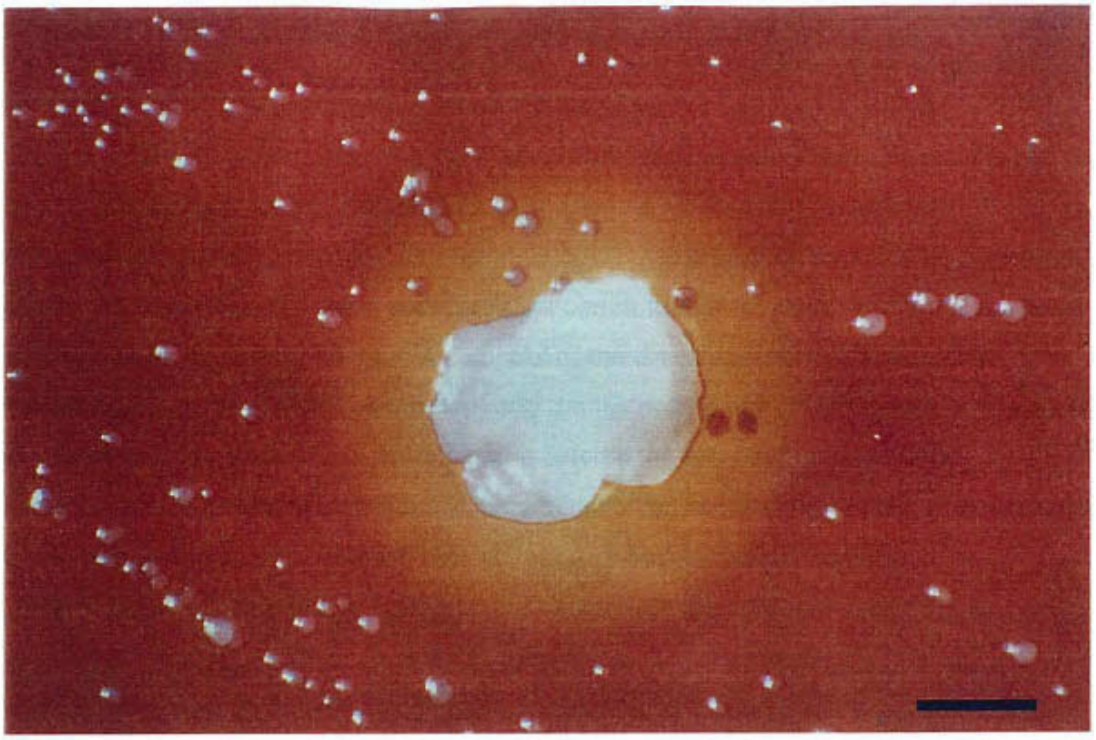

Figure 5.2 *Dermatophilus* sp from a 'brown spot' lesion; 48 h culture on blood agar. Note a single large rugose colony surrounded by a zone of beta haemolysis, and smaller colonies. Bar = 15 cm

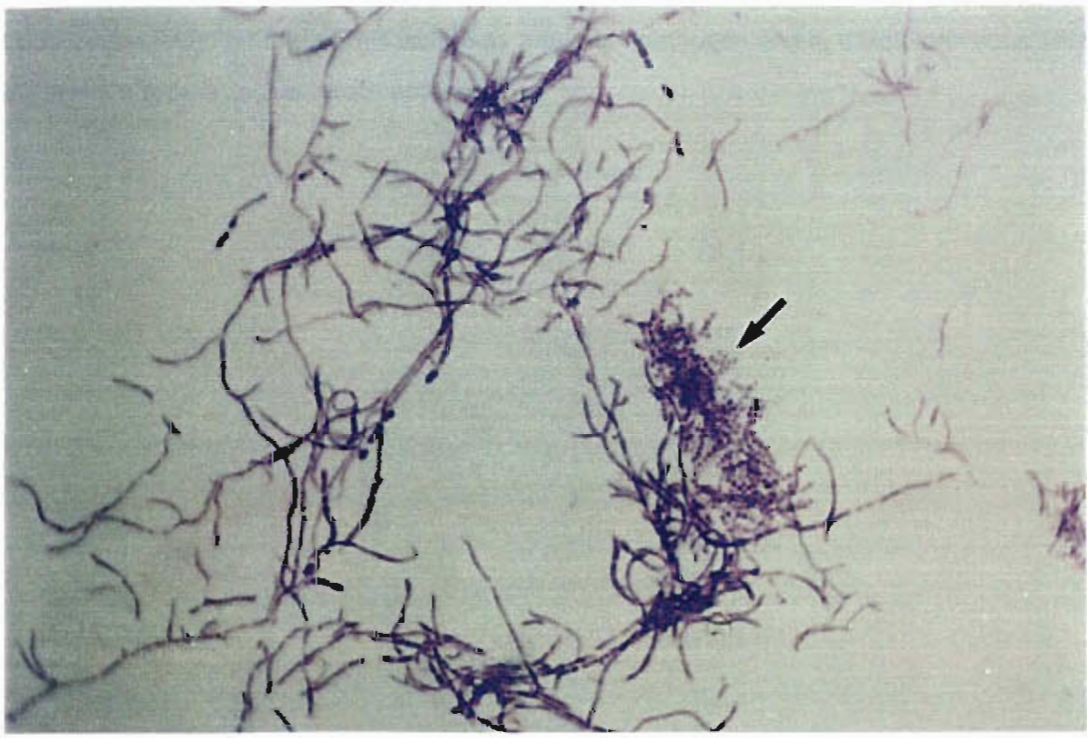

Figure 5.3 *Dermatophilus* sp from a 'brown spot' lesion on the belly skin; 48 h culture on blood agar. Note filaments branching at right angles, and zoospores (arrow). Gram stain ×1440

5.4 Discussion

Although a filamentous organism has been previously noted in crocodilian skin lesions and has been isolated from alligators, this appears to be the first report of the identification of *Dermatophilus* sp from crocodiles. The isolation of *Dermatophilus* sp in crocodiles affected with 'brown spot' disease is significant because it will permit further research on the pathogenesis, pathology, epidemiology, treatment and control of the disease. Most of the results from the biochemical tests, the cellular morphology and the histopathology of skin lesions were consistent with *Dermatophilus congolensis*. However, the differences observed in terms of the morphological, cultural and biochemical characteristic between *Dermatophilus* sp in crocodiles from the *Dermatophilus congolensis* type culture may suggest another species. DNA characterisation will be reported in a separate chapter (Chapter 7).

The aquatic habitat of crocodiles is probably a major factor favouring the occurrence of this disease, as constant wetting of the skin reduces the shedding of keratin and favours a build up of this and debris suitable for the growth of microorganisms such as *Dermatophilus*. A similar high prevalence of dermatophilosis in domestic animals may occur during the heavy rainfall season in the tropics (Radostits *et al.*, 1994). Studies on the experimental transmission in crocodiles to clarify the role of this isolate as a primary pathogen and in mixed infections will be reported in a separate chapter (Chapter 6).

CHAPTER 6

ATTEMPTED TRANSMISSION OF DERMATOPHILOSIS IN SALTWATER CROCODILES (*CROCODYLUS POROSUS*)

6.1 Source of Animals

So called 'brown-spot' disease as reported in alligators (Bounds and Normand, 1991) and crocodiles (Ladds and Donovan, 1989) is now recognised as an economically important skin disease of farmed crocodiles and alligators in Australia (Buenviaje *et al.*, 1997), Africa (Stuart, 1993) and America (Newton, 1992). *Dermatophilus* sp culturally resembling *Dermatophilus congolensis* has been isolated from 'brown spot' lesions in both alligators (Bounds and Normand, 1991) and crocodiles (Buenviaje *et al.*, 1997). In this chapter, experimental transmission of this disease in *Crocodylus porosus* hatchlings is described.

6.2 Materials and Methods

Three separate experiments were conducted. In an initial pilot study, 16 'runt' crocodiles approximately one year of age individually identified by metal tags inserted into the webbing of the feet were divided into experimental and control groups containing 12 and four animals, respectively. Animals in each group were placed together in a single oval tank made of recycled plastic (Reln Pty Ltd, Ingleburn, New South Wales), with a floor area of 2.35 × 1.1 m (Figure 6.1). The floor of the tank was slightly inclined so that approximately two thirds of the area was covered with water up to 10 cm deep while one third was dry. An epoxy-painted marine ply lid with a central hinge joint completely covered the tank except during cleaning, feeding and examining the crocodiles. An automatic immersion heater (Rena Corporation, Charlotte, NC, USA) was used to maintain the water temperature at 31° to 32°C. All materials used were disinfected with a 1% chlorine solution and allowed to air dry for at least 12 hours before the experiment. The tank was refilled with warm tap water daily after cleaning. Crocodiles were offered minced chicken heads with a vitamin premix (22 to 25 g /crocodile/day) immediately after cleaning and refilling the tank.

Figure 6.1 A plastic oval tank covered with a marine ply lid was used to keep the animals in the pilot transmission experiment and in the third transmission study. The power point on the pole was connected to two immersion heaters, which provided a temperature of 32°C.

Two isolates of *Dermatophilus* sp (Strains TVS 96-366-5A and TVS 96-490-7B), both from cases of dermatophilosis in farmed crocodiles in Queensland and the Northern Territory (Buenviaje *et al.*, 1997) were used. The isolates were initially grown on blood agar plates; and after four days all colonies were harvested into 10% bovine serum in tryptose broth (see Appendix 3.4), then incubated for a further 24 hours at 37°C in an atmosphere of 5% CO_2 in air. On the following day the inoculum was prepared by homogenizing the broth using a dounce homogenizer, then collecting the fluid into 5 mL Bijoux bottles.

After a settling period of one week, infection of 12 crocodiles was attempted by directly applying broth culture of one of the two isolates of *Dermatophilus* sp to marked areas of normal skin or skin lightly scarified with a scalpel. Six crocodiles were inoculated with strain TVS 96-366-5A and six with strain TVS 96-490-7B. To prevent the inoculum being washed from the skin, hatchlings were kept out of the water for one to two hours after inoculation. Sterile broth only was similarly applied to the four in-contact control hatchlings.

Within several days of inoculation, typical 'brown spot' lesions were apparent, not only at inoculation sites, but also at other locations in both infected and in-contact control animals,

indicating direct spread of infection, presumably via organisms in the tank water. Microscopic examination of the lesions in hatchlings killed at five and 20 days PI revealed changes characteristic of spontaneous dermatophilosis (Buenviaje *et al.*, 1997; Buenviaje, Ladds and Martin, 1998a), and *Dermatophilus* sp was isolated from skin lesions on day five PI and from tank water collected at day 30 PI. Bacteriological and biochemical characterisation of the isolates indicated that on each occasion it was strain TVS 96-490-7B; strain TVS 96-366-5A was never reisolated.

Anticipating, in view of the pilot study, that transmission could be readily reproduced, the protocol for the second transmission experiment was changed to evaluate possible control and treatment procedures; therefore following inoculation as above with strain TVS 96-490-7B, four groups of six-week-old *C porosus* hatchlings (an inoculated but untreated control group of 10 crocodiles plus three inoculated then treated groups of 19 crocodiles per group). Each group was held in adjoining but separate concrete tanks in which the water was non-medicated or contained chlorine (4 ppm), salt (500 g/100 L of water) or formalin (0.04%). Tank water was maintained at 32°C by automatic, thermostatically controlled intermittent flow of warm water into the tank. Again both dry and wet areas were available to hatchlings.

Although occasional minute skin lesions developed in crocodiles in both treatment and control groups but mostly in the non-medicated water (control) group, these quickly regressed so effective transmission was not achieved.

A third experiment was conducted using facilities and procedures as in the pilot study but with control and inoculated hatchlings maintained in separate tanks in separate buildings 60 metres apart. On this occasion only strain TVS 96-490-7B was used for inoculation. Sixteen six-month-old *C porosus* hatchlings were divided into a sham-inoculated control group of six and an experimental group of 10, which in turn was divided into two subgroups of five animals each; the skin of crocodiles in one subgroup (A) was scarified then inoculated while those in the other subgroup (B) served as non-inoculated but in-contact controls. Sterile 10% bovine serum in tryptose broth was used to inoculate scarified sites on the six animals in the control group. All crocodiles were caught and individually examined on days three, six, nine and 20 PI, and at termination of the experiment on day 36 PI when all remaining crocodiles were killed by barbiturate overdose.

6.3 Results

In the final transmission experiment (third experiment), on day three PI focal lesions up to 1 mm in diameter (Figure 6.2) were present in eight crocodiles – five inoculated and three in-contact. Microscopic examination of skin lesions from two emaciated animals at this time (one died and one killed – both from subgroup B) revealed branching filaments in superficial debris. A *Dermatophilus* sp that was culturally and biochemically identical to isolate TVS 96-490-7B, was grown from lesions in the crocodile that was killed.

On day six PI, skin lesions were present in all crocodiles in the experimental group. Microscopically the skin lesions from one animal killed at this time were confirmed as ulcers, and a filamentous organism was found associated with hypertrophic epidermal cells that contained characteristic poxvirus inclusions. A *Dermatophilus* sp identical to strain TVS 96-490-7B was again isolated from these lesions. On day nine PI, linear ulcers up to 10 mm were on present on one crocodile, but there was little change in either the number or size of lesions present and no attempt was made to reisolate *Dermatophilus* sp. On day 20 PI, however, there were frequent focal lesions of both of the above types on all crocodiles in the experimental group. Some linear ulcers were also present. Microscopic examination of skin lesions from one crocodile in subgroup B revealed both dermatophilosis and poxvirus infection. *Dermatophilus* sp was isolated from the lesions.

At the termination of the experiment on day 36 PI, lesions up to 2 mm in diameter were present on all animals (Figure 6.3) but whereas some initial lesions had resolved, other new ones had developed. Microscopy confirmed the presence of both dermatophilosis and poxvirus infection and again a *Dermatophilus* sp identical to strain TVS 96-490-7B was isolated.

In control crocodiles no skin lesions were observed until day 20 PI when a few minute and slightly depressed grey circular lesions in one animal were confirmed microscopically as poxvirus infection. On day 36 PI, three focal lesions up to 1 mm in diameter and confirmed by microscopy as dermatophilosis, were detected on two crocodiles; one of these also had poxvirus lesions. Similar translucent to grey circular lesions observed in four of five control crocodiles killed on day 36 PI were confirmed microscopically as poxvirus infection. Overall, a total of 11 animals six in the treatment group and five in the control group had concurrent poxvirus infection. *Dermatophilus* sp was again isolated from one animal with dermatophilosis only.

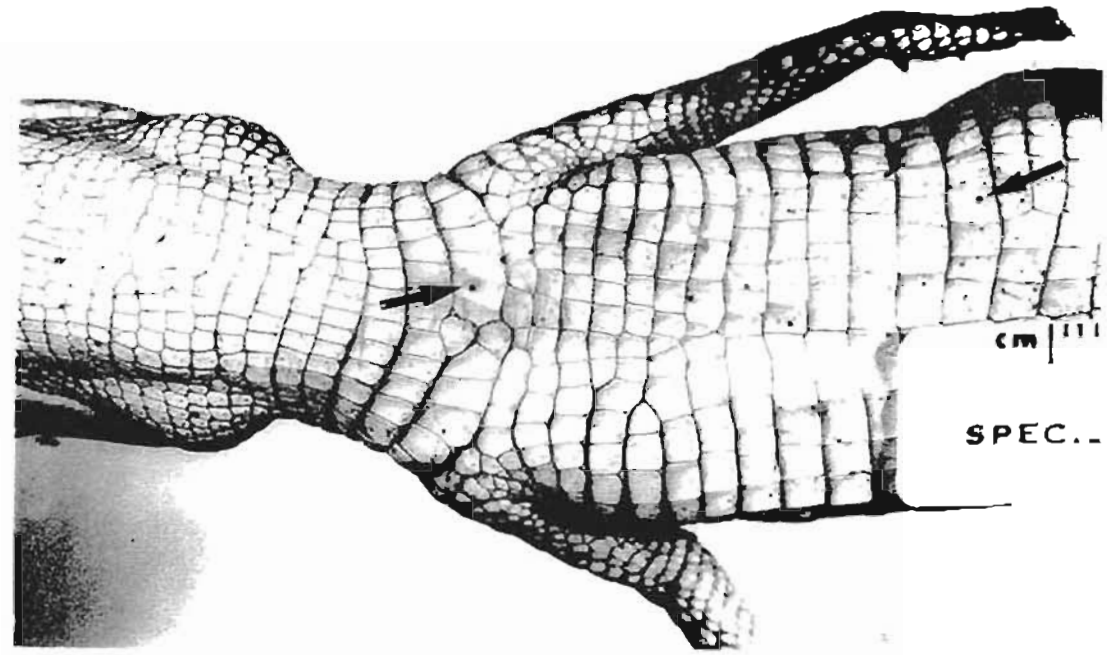

Figure 6.2 'Brown spot' lesions on the skin of a non-scarified and non-inoculated in-contact control hatchling at three days post-inoculation. The lesions were less than a millimetre in size and were scattered mostly on the skin of the abdomen (arrows).

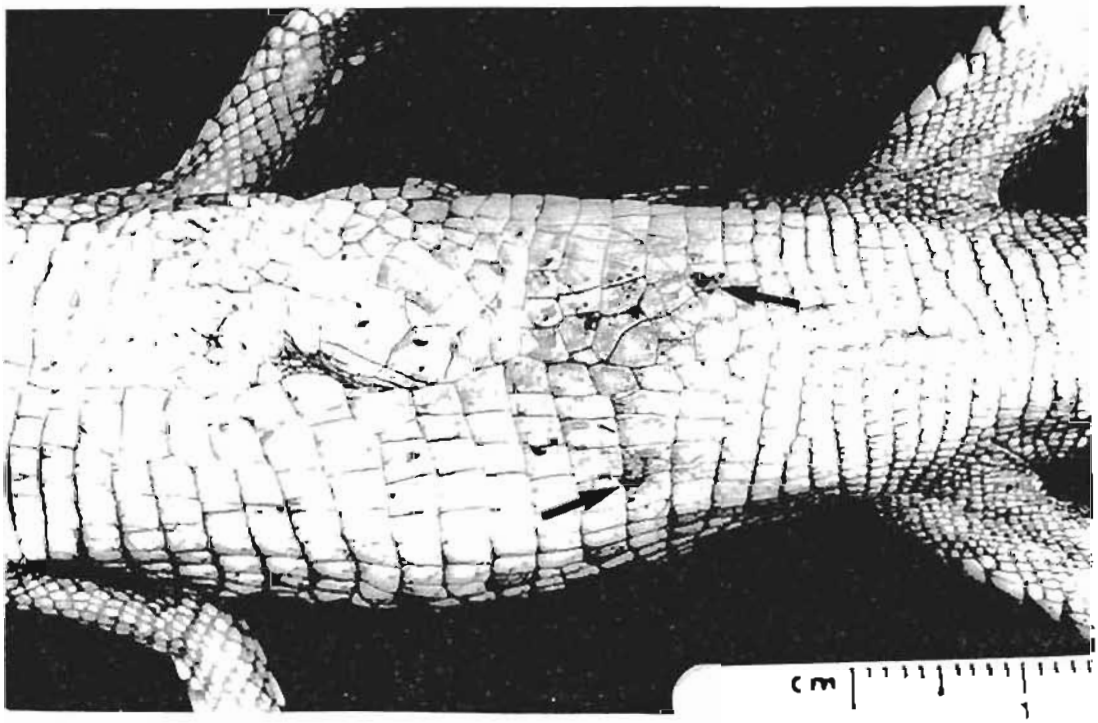

Figure 6.3 More advanced 'brown spot' lesions in a non-scarified, non-inoculated in-contact control hatchling at 36 days post-inoculation (arrows).

6.4 Discussion

Collectively, the results of these experiments demonstrate that *Dermatophilus* sp is the cause of the multifocal dermatitis (so called 'brown spot') in young crocodiles, but that the development, structure and prevalence of lesions may be influenced by concurrent poxvirus infection. Following inoculation, the rapid development of characteristic lesions containing a branching filamentous organism, and re-isolation of the inoculated organism from such lesions on five occasions, supports this claim.

Comment on several outcomes of these experiments is needed. In the pilot study it is unclear why *Dermatophilus* sp strain TVS 96-366-5A was not reisolated. Possibly its *in vitro* culture was associated with diminished virulence. Decreased virulence may likewise explain failure to effect transmission in the second experiment although dilution of the inoculum both on the skin and within tank water by its frequent replenishment also seems likely. Isolation of *Dermatophilus* sp from tank water at day 30 PI in experiment 1 emphasises the likely importance of water-borne spread. Therefore it is relevant that continually changing tank water for hatchlings is increasingly becoming routine on commercial crocodile farms.

Results of transmission in experiment 3 were confounded first by concurrent poxvirus infection in both experimental and control hatchlings, and by the occurrence of dermatophilosis in non-inoculated controls at day 36 PI. The emergence of poxvirus lesions, not observed in the pilot study and not previously observed in this group of six-month-old hatchlings, seemed likely to be related to activation of dormant infection by the stress of handling, and/or experimental *Dermatophilus* sp infection. Concurrent poxvirus infection and dermatophilosis in crocodiles have been observed previously (Buenviaje, Ladds, Hirst, Summers and Millan, 1998b), and such synergism is also recognised in cattle (Isitor, Kazeem, Njoku, Adegboye and Dellman, 1988). In planning experiment 3, it was expected that examination of lesions at different PI times would clarify their sequential development. Unfortunately, the appearance of poxvirus infection compromised this aspect of the study.

The development of typical dermatophilosis lesions in two non-infected control hatchlings may have resulted from inadvertent transfer of infection from experimental crocodiles (though these were housed 60 metres away) but, as with poxvirus, it is also possible that dormant *Dermatophilus* sp zoospores may have been present in these controls at the commencement of the experiment and their activation and growth may have been due to the stress of handling and concurrent poxvirus infection. Previous studies of skin lesions in crocodiles emphasise the

ubiquitous nature of *Dermatophilus* sp (Buenviaje *et al.*, 1998b). Importantly, however, dermatophilosis was confirmed on PI days three and six and two subsequent intervals in crocodiles in the experimental group but only on day 36 PI in the controls.

Because of the likelihood of water-borne transmission of *Dermatophilus* sp in crocodiles, studies on medication and other appropriate treatments of water to limit the spread of infection are reported in Chapter 8. Findings in the third experiment described here underline the importance of synergism between poxvirus and dermatophilosis in young farmed crocodiles.

CHAPTER 7

PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF *DERMATOPHILUS* SP ISOLATED FROM 'BROWN SPOT' LESIONS IN FARMED CROCODILES

7.1 Introduction

The actinomycete *Dermatophilus congolensis* is known to cause a variety of skin diseases from a wide range of animal species including man. The disease produced by *D congolensis* has resulted in significant economic loss especially in cattle and sheep where skin was produced primarily for leather. Initially, three species of the genus *Dermatophilus* were identified as the cause of several skin lesions in animals; these were *D congolensis*, the aetiological agent of cutaneous streptothricosis, *Dermatophilus dermatonomus* the aetiological agent of mycotic dermatitis and *Dermatophilus pedis* the aetiological agent of strawberry foot rot. These three species were later considered congeneric (Austwick, 1958). A comparison of all *Dermatophilus* isolates based upon the source of isolates from various animals and their respective clinical manifestations, and the geographical locations of the disease have led to the conclusion that all the species of *Dermatophilus* could be accommodated by a single species called *D congolensis* (Gordon, 1964).

Recently, a new species called *Dermatophilus chelonae*, isolated from the turtle and tortoise, was proposed by Masters *et al.* (1995) based on its differences from *D congolensis* with respect to the morphological and biochemical characteristics, DNA restriction enzyme digestion and protein electrophoresis patterns.

A Gram-positive filamentous organism resembling *D congolensis* has been isolated from alligators in USA (Newton, 1992) and crocodiles in Australia (Buenviaje *et al.*, 1997) suffering from skin disease called 'brown spot'. This disease has been reported on a number of occasions, and is considered the most important skin disease in farmed crocodiles in Australia (*Buenviaje et al.*, 1998a). Although initial microbiological examination of crocodile isolates showed a close resemblance to *D congolensis* (see Chapter 5), there were some significant differences in the morphological, cultural and biochemical characteristics.

Further studies were undertaken to compare *Dermatophilus* spp isolated from crocodiles with other species of *Dermatophilus* using the standard microbiological laboratory tests and molecular tests based on the 16S rDNA sequences and ribotyping. What is most important, these analyses were used to confirm whether the isolate used to inoculate the animals in the

experimental transmission (Buenviaje *et al.*, 1998b) was identical to those recovered from infected crocodiles (see Chapter 6).

7.2 Materials and Methods

7.2.1 Strains investigated

The study group consisted of 19 isolates as listed in Table 7.1. Isolates DCD, DCS1 and DCS2 were supplied by the Australian Collection of Microorganisms, Centre for Bacterial Diversity and Identification, Department of Microbiology, University of Queensland, Brisbane. The Animal Health Laboratories, Department of Agriculture, South Perth, Western Australia supplied two strains of *D chelonae* (DCH1and DCH2). The Department of Primary Industries, Oonoonba Veterinary Laboratory, Townsville supplied a strain of *D congolensis* (DCC1) isolated from cattle.

Table 7.1 *Dermatophilus* isolates used in this study.

Code	Strains	Accession No.	Source
Fi 1	field isolate	TVS 96-490-7B	saltwater crocodile (Qld)
Fi 2	field isolate	TVS 96-490-9B	saltwater crocodile (Qld)
Fi 3	field isolate	TVS 96-367-15A	saltwater crocodile (Qld)
Fi 4	field isolate	TVS 96-366-5A	saltwater crocodile (NT)
Fi 5	field isolate	TVS 96-700-1C	saltwater crocodile (Qld)
WS	water isolate	TVS 97-124-ws	water sample (pilot study)
Ri 1	recovered isolate	TVS 97-124	saltwater crocodile (pilot study)
Ri 2	recovered isolate	TVS 97-405	saltwater crocodile (transmission study)
Ri 3	recovered isolate	TVS 97-412	saltwater crocodile (transmission study)
Ri 4	recovered isolate	TVS 97-427-1	saltwater crocodile (transmission study)
Ri 5	recovered isolate	TVS 97-472	saltwater crocodile (transmission study)
Ri 6	recovered isolate	TVS 97-474	saltwater crocodile (transmission study)
DCC1	*D congolensis*	OVLDPI No. 22204	cattle
DCD	*D congolensis*	ACM 530	deer
DCS1	*D congolensis*	ACM 531	sheep
DCS2	*D congolensis*	ACM 532	sheep
DCH1	*D chelonae*	WA 430	turtle
DCH2	*D chelonae*	WA 1305	snapping turtle
ATCC	*D congolensis*	ATCC 14637	cattle

Qld – Queensland NT – Northern Territory

7.2.2 Morphological, cultural and biochemical characterisation

Five crocodile field isolates, a *Dermatophilus congolensis* type culture (ATCC 14637) and a strain of *Dermatophilus chelonae* (DCH 2) were compared with each other phenotypically. Six additional isolates recovered from infected animals during the transmission study and one isolated from water sampled during the pilot transmission experiment (see Chapter 6), were also included for comparison. The bacterial culture media and the reagents used in this study were described in detailed in Appendix 3. The isolates were initially grown on blood agar containing 5% sheep blood, and incubated in two different temperature settings at 37°C in the presence of 5% CO_2 and at 28°C in the ambient atmosphere. All isolates were inoculated in tryptose phosphate broth containing 10% bovine serum, brain heart infusion broth, Mueller-Hinton agar and Mueller-Hinton agar supplemented with 9% sheep blood, and incubated at 37°C in an atmosphere of 5% CO_2, except *D chelonae*, which was found to grow best at 28°C. The bacterial cultures were examined daily for two weeks.

All isolates were grown in blood agar for antibiotic sensitivity testing. *D congolensis* isolates were harvested from blood agar after three days whereas both the crocodile isolates and *D chelonae* isolate were harvested after five days of incubation. Bacterial colonies were gently homogenised using a sterile dounce homogeniser. The homogenised suspension was diluted with a sterile pH 7.0 PBS until it matched the turbidity of a McFarland 0.5 standard. Approximately 20 µL of homogenised suspension was uniformly spread onto three Mueller-Hinton agar plates supplemented with 9% sheep blood. A disk diffusion susceptibility method was used as described by Isenberg (1992). Eight antibiotic disks (Oxoid) of ciprofloxacin (5 µg), cefotaxim (30 µg), cloxacillin (5 µg), penicillin (10 units), ampicillin (10 µg), streptomycin (10 µg), erythromycin (15 µg) and tetracycline (30 µg) were dispensed in two Petri plates, each plate containing four disks. The remaining plate was used as a control. All plates were incubated at 37°C in the atmosphere of 5% CO_2 except *D chelonae* which requires incubation at 28°C. The zone of inhibition obtained with the isolates was measured after 48 hours (Masters *et al.*, 1995). Another disk diffusion susceptibility test was performed following a similar procedure to verify the initial results. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as reference organisms for comparison on the antibiotic sensitivity of test isolates.

Gram-staining was performed to observe the morphological features of mature filaments and zoospores. Kinyoun modified Ziehl Neelsen staining method was also used to examine for acid fastness. Nigrosin methylene blue staining and a hanging drop method (Baker and Silverton,

1976) were used to determine the presence of a capsule and the motility of all *Dermatophilus* isolates, respectively. Biochemical tests used included catalase, oxidase, urease, indole production, methyl red, Voges-Proskauer, and nitrate reduction (Baron and Finegold, 1990). Acid production was recorded after inoculating each isolate into carbohydrate fermentation broth base containing 5% of either lactose, sorbitol, xylose, dulcitol, mannitol, salicin, sucrose, fructose or glucose. The proteolytic activity of the isolates was also recorded after inoculation onto Loeffler's serum medium, casein, tyrosine and xanthine agars.

7.2.3 16S rDNA sequence determination and phylogenetic analysis

Four methods were used to extract the DNA from the 19 isolates. The preparation of the reagents used in DNA extractions were described in Appendix 4. DNA extraction was initially carried out using the method described for *Aeromonas hydrophila* (Oakey, 1997). Other methods used were (1) Qiagen protocol for DNA extraction (Qiagen Pty. Ltd., Clifton Hill, Victoria); (2) caesium chloride DNA extraction; (3) modified DNA extraction protocol based upon the *A hydrophila* method (see Chapter 3, Section 3.6).

Two PCR protocols were carried out for the amplification of 16S rDNA in *Dermatophilus* spp isolates from crocodiles. The PCR amplification protocol as described by Marchesi *et al.* (1998) consisted of a forward primer (63 f) of 5'–CAG GCC TAA CAC ATG CAA GGC–3' and a reverse primer (1387r) of 5'–GGG CGG WGT GTA CAA GGC –3'. These primers were designed to amplify approximately 1,300 base pairs of the consensus 16S rDNA genes of Gram-positive bacteria that contained a high percentage G-C base sequence. The PCR reaction described by Marchesi *et al.* (1998) was modified to consist of 10 ng of template DNA, 1 µM of each primer, 200 µM dNTP, 2 mM $MgCl_2$, 1.1 unit *Taq* polymerase, 5 µL 10× buffer and sterile distilled water to a final volume of 50 µL reaction mixture. The cycles were programmed (see Chapter 3, Section 3.7.2) and the tubes were placed in the PTC-100™ thermal cycler (Bresatec Ltd., Therbaton South Australia) preheated to 70°C.

In the second PCR protocol, three primers were designed as described in Chapter 3, Section 3.7.1. One forward primer (fwd1 of 5'–AGA GTT TGA TCC TGG CTC AG–3') and two reverse primers (rvs1 of 5'–CGC TCG TTG GAC TTA ACC–3' and rvs2 of 5'–CG GCT ACC TTG TTA CGA CTT–3') were tested for amplification abilities. Optimisation of the PCR reaction (see Section 3.7.2) was carried out. The final reaction consisted of 2.5 mM each dNTP (1.75 mM total final concentration); 50 pmols of each primer; 6.25 mM (final concentration) magnesium chloride; 2.5 ng template DNA; 1.5 units *Taq* polymerase; 4 µL 10× reaction buffer; sterile distilled water

to 40 µL. A PCR product of approximately 1,000 bp was obtained by using fwd1 and rvs1 primers. Amplicons were visualised and size estimated as described in Chapter 3, Section 3.7.2.

All PCR products were purified following the procedure in QIAquick PCR purification kit protocol (Qiagen Pty Ltd, Clifton Hill, Victoria). The 'cleaned PCR' product was used in subsequent cycle sequencing reactions (see Section 3.7.4) and purification of extension products (see Section 3.7.5).

Sequence reaction products were electrophoresed with a model ABI prism 310 genetic analyser (Perkin-Elmer Corporation, USA). The 16S rDNA sequences were verified and corrected for any ambiguous sequence, and the percentage similarities of sequence between two isolates were obtained using the Sequencher 3.0 computer program. In addition to the 16S rDNA sequence generated in this study, previously published sequences of *Streptomyces* sp (Genbank accession number AF 012741), *Frankia* sp (AF 034776) and *Geodermatophilus obscurus* (X 92359) obtained from the Genbank sequence database were analysed. Complete multiple alignment of 16S rDNA sequences were examined by using the computer program Clustal X (Thompson, Gibson, Plewniak, Jeanmougin and Higgins, 1997). The Tamura-Nei neighbour joining complete deletion method of the computer program, Molecular genetic analysis (MEGA) version 1.01, was used to obtain a phylogenetic tree. Bootstrap confidence values were obtained with 1,000 resamplings.

7.2.4 Ribotyping

DNA samples from 13 isolates excluding DCD, DCS1, DCS2, DCC1, Fi 4 and Fi 5 (see Table 7.1) were digested using *Eco* R1 and *Sca* 1. The reagents used in ribotyping were described in Appendix 5. The agarose gel containing digested DNA with rulers next to the marker lanes of *Hind* III cut Lambda DNA and 1,000 kb DNA markers loaded at each end of the digests was visualised over UV light (see Chapter 3, Section 3.8.1). The transfer of fragmented DNA from the gel to a positively charged nylon membrane was achieved through a Southern blotting technique (see Chapter 3, Section 3.8.2). The 16S and 23S RNA from *E coli* was labelled with digoxigenin (DIG), and the concentration was estimated (see Section 3.8.3). The labelled RNA was used as a probe for high stringency hybridisation to the Southern blot membrane. Hybridisation was detected with chemiluminescence. The migration (mm) of hybridised ribotype restriction fragments against known fragment sizes of DNA markers were determined and analysed as described in Section 3.8.4.

7.3 Results

7.3.1 Phenotypic characterisation

The morphological, cultural and biochemical characteristics of the crocodile isolates, *Dermatophilus congolensis* type strain (ATCC 14637) and *Dermatophilus chelonae* (DCH 2) are summarised in Table 7.2. All crocodile isolates were Gram-positive and non-acid fast filamentous bacteria. The hyphae branched at right angles, and the width was measured from 0.5 to 1 µm. The zoospores were motile, and measured 1 µm in diameter. Except for the two field isolates and one isolate recovered from water, all isolates shared similar phenotypical characteristics. The colonies were initially a grey colour on blood agar containing 5% sheep blood. In addition, all colonies were pitting and exhibited haemolysis between two and five days after inoculation. In tryptose phosphate broth and brain heart infusion broth, all isolates produced granular flocculent sediments (Appendix Table 1.3). Biochemical tests showed that all isolates were catalase positive and oxidase negative. Acid was always produced from glucose but not from sorbitol, xylose, dulcitol, mannitol or salicin (Appendix Table 1.7). The results for methyl red and Voges-Proskauer, xanthine and tyrosine tests were negative (Appendix Table 1.5 and Table 1.6). Two of five field isolates (Fi 4 and Fi 5) produced a clear zone around the colonies in xanthine agars and no hydrolysis on tyrosine agars except for the presence of dark pigments. Also, the colonies of field isolates Fi 4 and Fi 5 had different cultural characteristics compared with the three field isolates (Fi 1, Fi 2 and Fi 3) (see Appendix Table 1.1). The size of the colonies was much bigger (1 to 3 mm after 48 hours of incubation), and became dark in colour after five days compared with the other three field crocodile isolates. After five days incubation, the colonies were 3 to 5 mm in diameter, and white aerial hyphae were prominent on the surface of the colonies. The isolate from water (TVS 97-124 ws) was different from the other six recovered isolates as the colonies were much bigger (2 to 3 mm) and excessively folded or rugose, and haemolysis was not always observed after five days of incubation.

Table 7.2 Comparison of crocodile isolates with *D congolensis* type strain (ATCC 14637) and *D chelonae* (DCH 2).

Characteristics	Crocodile isolates (Fi1, Fi2, Fi3 and recovered isolates)	*Dermatophilus congolensis* (type strain ATCC 14637)	*Dermatophilus chelonae* (DCH 2)
Morphological	Hyphae divided by transverse septa. The colonies produced less zoospores after five days.	Hyphae divided by transverse and longitudinal septa. The colonies were predominantly zoospores after five days.	Hyphae divided by transverse and longitudinal septa. Produced less zoospores after five days.
Cultural	Very tiny and barely visible colonies after 48 hours. The colonies were pitting into the medium. The colonies were pale yellow colour and rugose sometimes crateriform. Poor growth at 28°C. No clumps formed around the tubes in brain heart infusion broth and no thin membrane flocculents or microbodies in tryptose phosphate broth. No growth on Mueller-Hinton agar.	The colonies ranged from 1 to 2 mm in diameter after 48 hours incubation and deeply pitted into the medium. The colonies were also golden yellow in colour, more rugose and crateriform. Granular flocculents present at the bottom and occasionally around the tubes in brain heart infusion broth. Thin membrane flocculents on the surface and granular sediments at the bottom of tryptose phosphate broth. Growth on Mueller-Hinton agar.	No growth after 48 hours incubation. Grew best at 28°C. The colonies were pale colour and sticky, pitted and strongly attached to the medium. No growth both brain heart infusion broth and tryptose phosphate broth at 37°C in the atmosphere of 5% CO_2. No growth on Mueller-Hinton agar.
Biochemical	Variable results for indole and nitrate. Hydrolysed Loeffler's serum (LS). Variable hydrolysis for casein. Urease positive. No acid produced from lactose. Resistant to streptomycin. Susceptible to ampicillin, penicillin, cloxacillin, cefotaxim, ceprofloxacin, erythromycin and tetracycline.	Negative for nitrate and indole. Hydrolysed LS and casein. Urease positive. Susceptible to streptomycin, ampicillin, penicillin, cloxacillin, ceprofloxacin, cefotaxim, erythromycin and tetracycline.	Variable results for indole and nitrate. Hydrolysed casein but not LS. Urease positive and acid was not always produced from lactose. No acid produced from fructose. Resistant to streptomycin. Susceptible to ampicillin, penicillin, cloxacillin, cefotaxim, ceprofloxacin, erythromycin and tetracycline.

Field isolates Fi1, Fi 2 and Fi 3 and all recovered isolates grew better at 37°C in an atmosphere of 5% CO_2 than at 28°C. The colonies were moist and slightly pitted on blood agar incubated at 37°C in the atmosphere of 5% CO_2, whereas at 28°C the colonies were sticky and caseous. At 37°C incubation *D congolensis* grew faster (1 to 2 mm in diameter) than the crocodile isolates (less than 0.5 mm in diameter) and no growth of *D chelonae* was seen after 48 hours of incubation. It was also observed that *D congolensis* had a golden yellow colour and was more rugose and crateriform, than the crocodile isolates (Figures 7.1, Figure 7.2, Figure 7.3 and Figure 7.4) after three days incubation (Appendix Table 1.2). After five days incubation, three field isolates (Fi1, Fi2, Fi 3) and six recovered isolates (Ri 1 to Ri 6) had formed tough and leathery colonies compared with *D congolensis* type strain (ATCC 14637), which was soft and friable. In addition, the colonies of crocodile isolates (1 to 2 mm in diameter) were slightly rugose, occasionally crateriform and had a white to pale yellow colour compared with *D chelonae*. Characteristic white and smooth umbonate colonies (less than 2 mm in diameter) with a slightly depressed area at the centre was observed for *D chelonae* but not for the crocodile isolates after 10 days incubation (Figure 7.5, Figure 7.6, Figure 7.7 and Figure 7.8). Interestingly, it was found that the crocodile isolates did not grow on straight Mueller-Hinton agar. Instead the colonies grew faster when Mueller-Hinton agar supplemented with 9% sheep blood was used. An important observation particularly with crocodile isolates was their ability to grow bigger colonies (1 to 2 mm in diameter) on Mueller-Hinton agar supplemented with 9% sheep blood than on blood agar with only 5% sheep blood (less than 0.5 mm) after 48 hours incubation. All crocodile isolates produced fewer zoospores even after several days of incubation. In contrast, *D congolensis* formed into packets of zoospores in three days of incubation and *D chelonae* after five to seven days of incubation (Appendix Table 1.4). The mature hyphae of crocodile isolates were divided by transverse septa whereas with *D chelonae* and *D congolensis*, the hyphae divided into both transverse and longitudinal septa.

The susceptibility result must be interpreted with caution because the procedure was modified and has not been standardised for *Dermatophilus* species. In reference to the published zone of inhibition (Isenberg, 1992), the crocodile isolates had a zone of inhibition ranging from 28 to 38 mm indicate susceptibility to ampicillin (10 µg), tetracycline (30 µg), cloxacillin (5 µg), erythromycin (15 µg) ciprofloxacine (5 µg), cefotaxim (30 µg) and penicillin (10 units) (see Appendix Table 1.8). All crocodile isolates were resistant to streptomycin (10 µg). The isolate from water sample (WS) and field isolates (Fi 4 and Fi 5) had different resistance and susceptibility to the same antibiotics used.

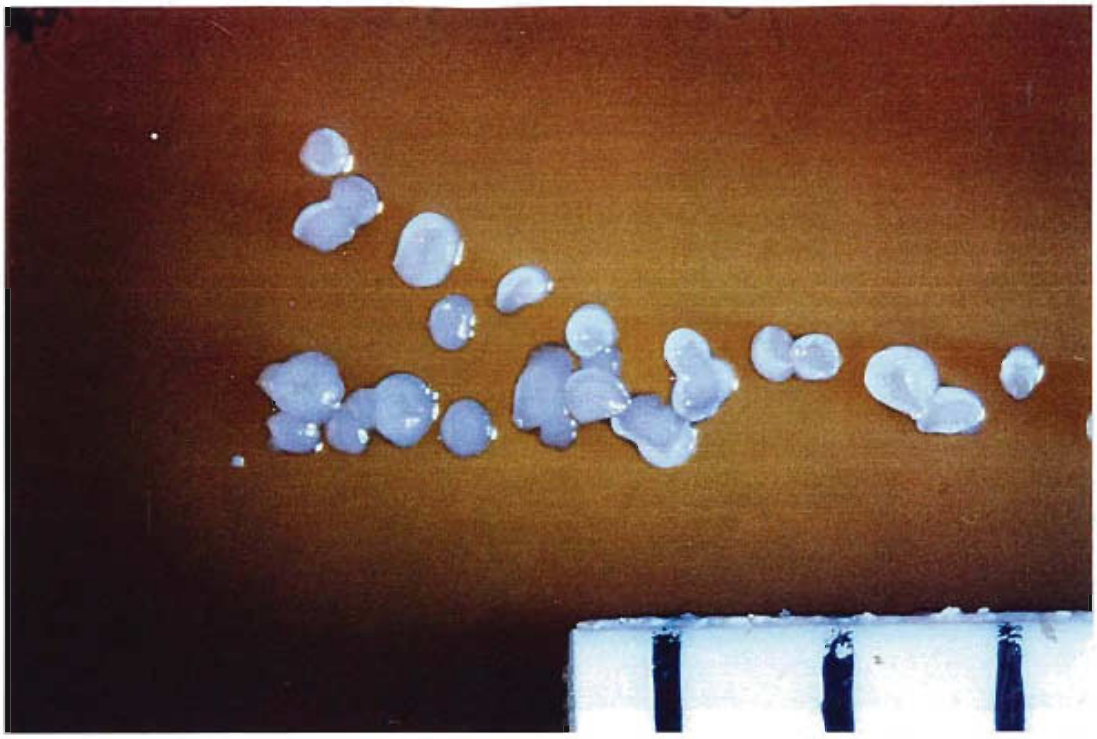

Figure 7.1 A five-day-old culture of field isolate TVS 97-490-7B. This isolate was used to inoculate crocodiles in several transmission experiments. Scale = mm.

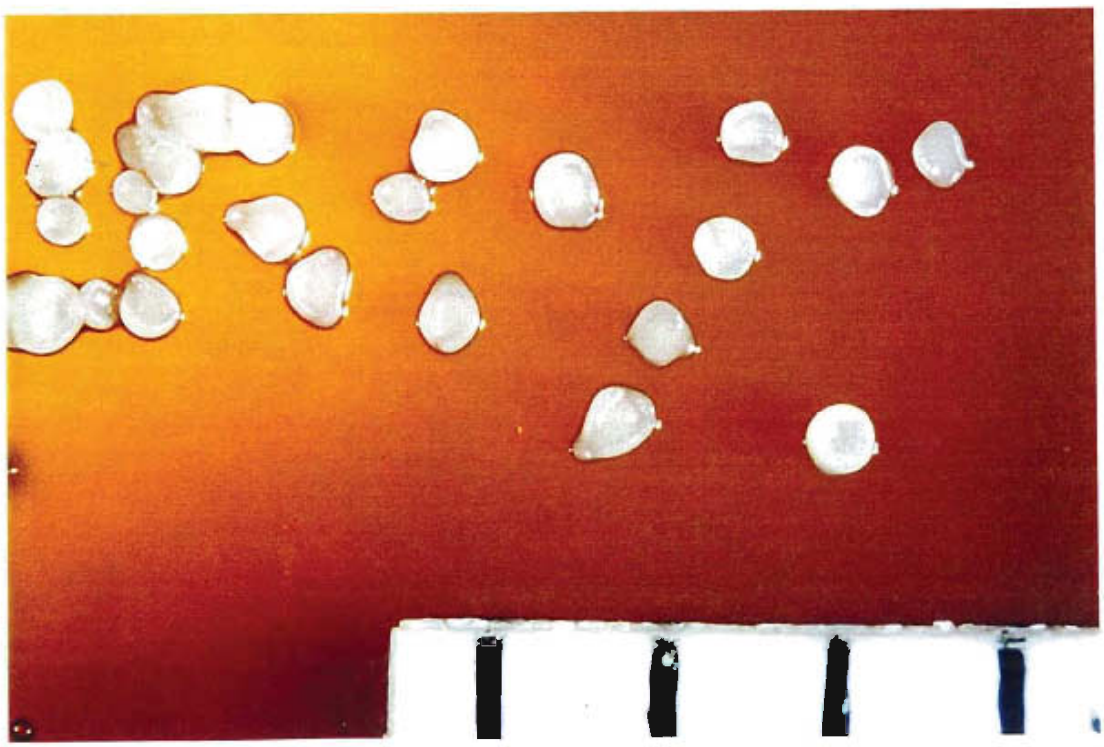

Figure 7.2 A five-day-old culture of recovered isolate TVS 97-472. Scale = mm

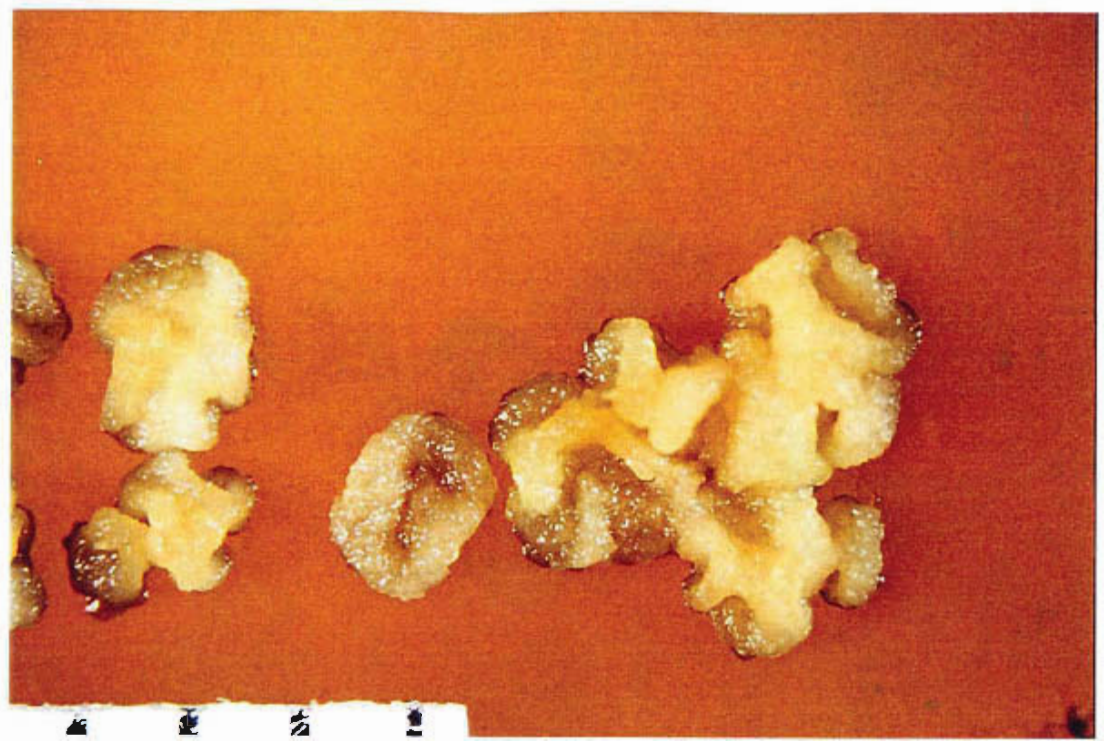

Figure 7.3 A five-day-old culture of *Dermatophilus congolensis* type strain (ATCC 14637). Note the colonies were golden yellow in colour and more rugose and crateriform. Scale = mm

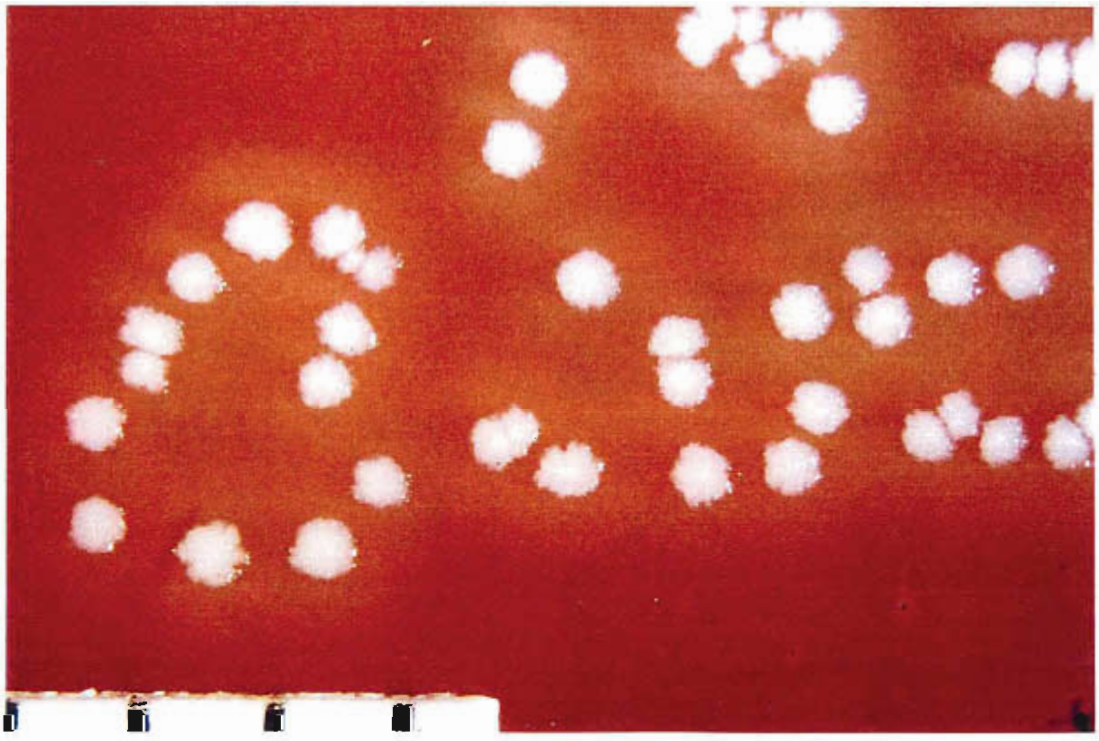

Figure 7.4 *Dermatophilus chelonae* (DCH2). The colonies were white in colour and flat after five days incubation. Note the haemolysis around the colonies. Scale = mm

Figure 7.5 Field isolate TVS 96-490-7B. The colonies were more rugose and surrounded by a clear zone of haemolysis after 10 days inoculation. Scale = mm

Figure 7.6 Ten-day-old culture of recovered isolate TVS 97-472. Note a similar appearance in Figure 7.5 of field isolate TVS 97-490-7B. Scale = mm

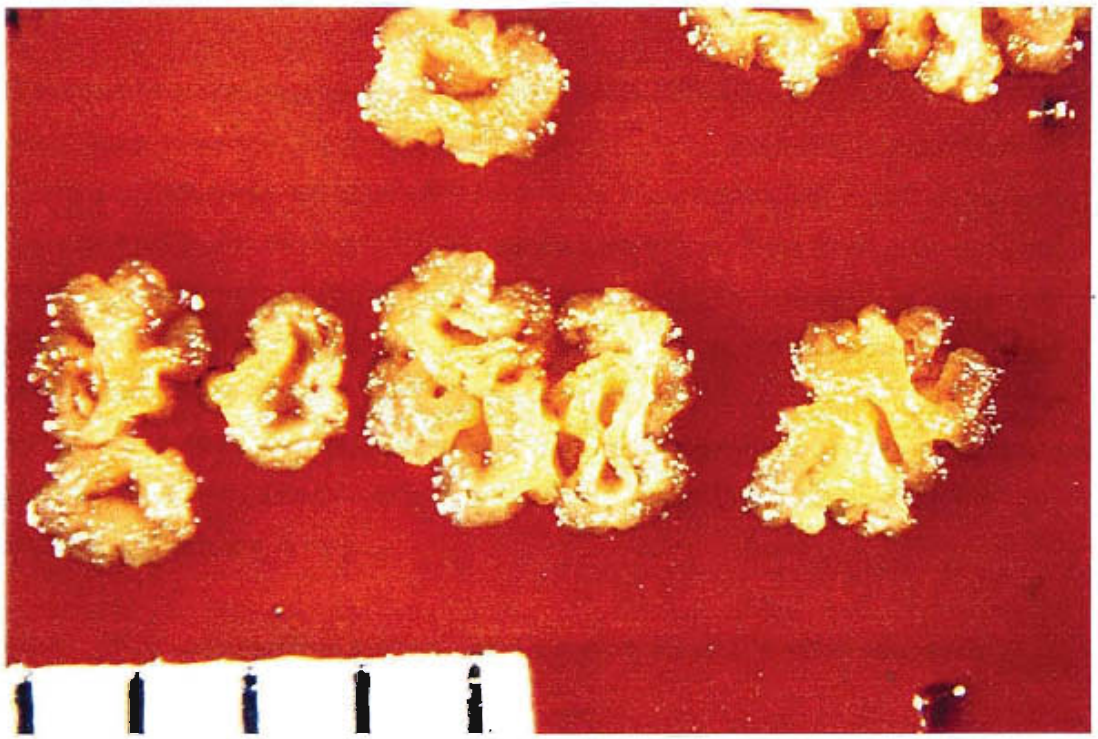

Figure 7.7 Ten-day-old culture of *Dermatophilus congolensis* type strain (ATCC 14367). Scale = mm

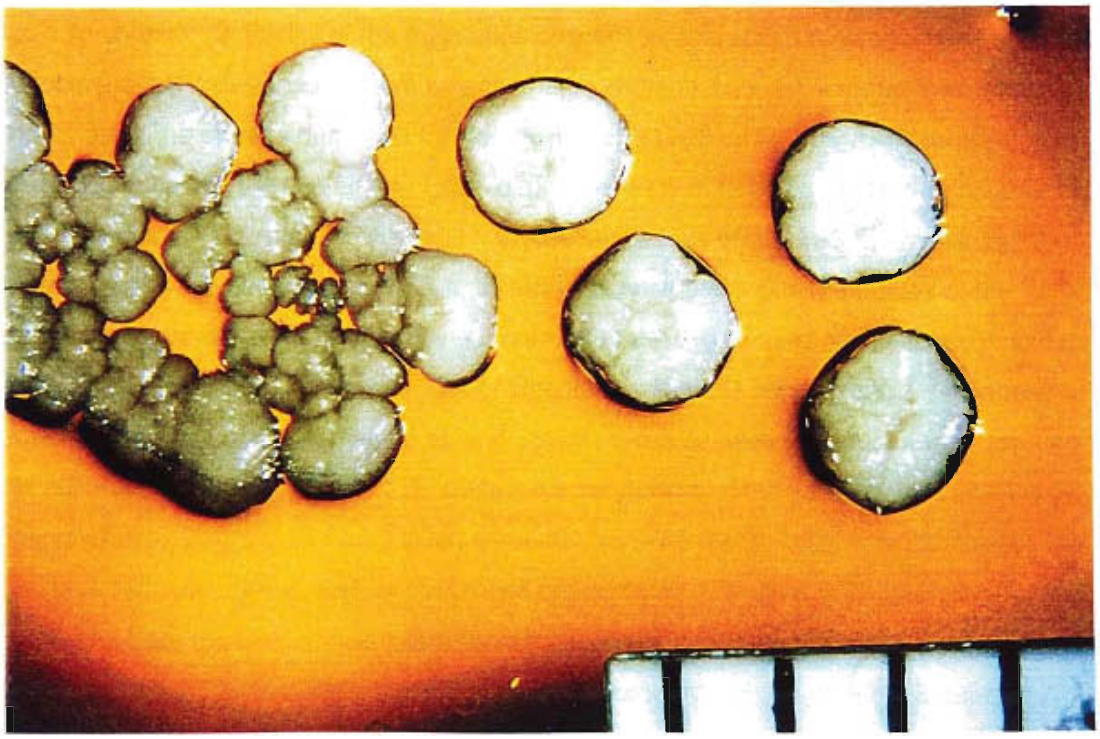

Figure 7.8 Ten-day-old culture of *Dermatophilus chelonae*. Note the colonies were rounded with a slightly depressed central area and occasional two or three grooves across each colony. Scale = mm

7.3.2 DNA extraction

Of the four DNA extraction protocols, only the modified DNA extraction protocol for *A hydrophila* (see Chapter 3, Section 3.6.1) produced DNA extracts from all isolates. Approximately 250 ng/µL of DNA were extracted after the protocol was modified. The modification included repeated freezing and thawing of the pelleted bacteria at least three times, increasing the lysozyme (from 1 mg/mL to 5 mg/mL) in the lysis buffer and an increase in incubation period of the bacterial suspension in ice (from 5 to 15 minutes). Adding 2 mL of hexadecyltrimethyl ammonium bromide (CTAB) to the mixture and incubation in a water bath at 55°C overnight was another modification used for DNA extraction. DNA was also extracted from only five isolates after the caesium chloride DNA extraction technique was modified, however, the amount extracted was very low (5 ng/µL).

7.3.3 PCR amplification and phylogenetic analysis of 16S rDNA sequence

Two of 19 isolates (Fi 4 and Fi 5) did not produce PCR products. Prior to the direct sequencing of the PCR products, the reproducibility of PCR from the 15 isolates was tested at least three times. Isolates DCD and CDS2 did not produce a quality sequence and were removed from the study. To confirm the fidelity of the nucleotide sequences, the 16S rDNA sequence from the PCR products using the designed primers was compared with the 16S rDNA sequence from the PCR product using the primers described by Marchesi *et al*. (1998). The 16S rDNA sequences of isolates produced by the two different sets of primers were matched and carefully reviewed for possible sequencing errors. The size of nucleotide sequences of the 16S rDNA genes of the isolates in this study ranged from 965 to 1,345 total base pairs. Since the sizes of the nucleotide sequences of all isolates varied, only 965 bases were used for comparison to avoid biased results. The 16S rDNA sequences of crocodile isolates *D congolensis*, *D chelonae*, *Deodermatophilus obscurus*, *Frankia* sp and *Streptomyces* sp were used for comparison (see Appendix Tables 2.1 - 2.17). The 16S rDNA gene sequences of the six recovered isolates (see Appendix Tables 2.4 to 2.9) from the experimental transmission studies were identical to the 16S rDNA sequence of the original challenged organism (Fi 1) (Figure 7.9). Interestingly, the recovered isolate (Ri 6) from one infected crocodile in the control group of the transmission experiment had a 100% sequence similarity with the other recovered isolates. Except for Fi 2, the sequences of two field isolates Fi 1 and Fi 3 from the spontaneous outbreaks of 'brown spot' disease in Queensland and in the Northern Territory respectively, exhibited a 100% similarity despite the geographical distance between the sources (Table 7.3). The 16S rDNA sequence of field isolate 2 (Fi 2) had 87.1% and 91.3% sequence similarity to Fi 1 and Fi 3, respectively.

Figure 7.9 16S rDNA sequence of field isolate (Fi 1) with a total of 1,263 nucleotide bases.

```
GGTGGATTAG  TGGCGAACGG  GTGAGTAACA  CGTGAGTAAC  CTGCCCTTCA
CTCTGGGATA  ACCACGGGAA  ACCGGGGCTA  ATACTGGATA  TGACACACTG
GCGCATGATG  GTGTGTGGAA  AGATTTATTG  GTGGAGGATG  GACTCGCGGC
CTATCAGCTT  GTTGGTGAGG  TAATGGCTCA  CCAAGGCGAC  GACGGGTAGC
CGGCCTGAGA  GGGTGACCGG  CCACACTGGG  ACTGAGACAC  GGCCCAGACT

CCTGCGGGAG  GCAGCAGTGG  GGAATATTGC  ACAATGGGCG  AAAGCCTGAT
GCAGCGACGC  CGCGTGAGGG  ATGAAGGCCT  TCGGGTTGTA  AACCTCTTTC
GCCAGGGAAG  AAGCGAAAGT  GACGGTACCT  GGAGAAGAAG  CACCGGCTAA
CTACGTGCCA  GCAGCCGCGG  TAATACGTAG  GGTGCGAGCG  TTGTCCGGAA
TTATTGGGCG  TAAAGAGCTT  GTAGGCGGTT  TGTCGCGTCT  GCCGTGAAAA

TCCAAGGCTT  AACTTTGGAC  GTGCGGTGGG  TACGGGCAGG  CTAGAGTGTG
GTAGGGGAGA  CTGGAATTCC  TGGTGTAGCG  GTGAAATGCG  CAGATATCAG
GAGGAACACC  GATGGCGAAG  GCAGGTCTCT  GGGCCATAAC  TGACGCTGAG
AAGCGAAAGC  ATGGGGAGCG  AACAGGATTA  GATACCCTGG  TAGTCCATGC
CGTAAACGTT  GGGCGCTAGG  TGTGGGGCTC  ATTCCACGAG  CTCTGTGCCG

CAGCTAACGC  ATTAAGCGCC  CCGCCTGGGG  AGTACGGCCG  CAAGGCTAAA
ACTCAAAGGA  ATTGACGGGG  GCCCGCACAA  GCGGCGGAGC  ATGCGGATTA
ATTCGATGCA  ACGCGAAGAA  CCTTACCAAG  GCTTGACATA  CACCGGAAAA
GTGCAGAGAT  GTGCTCCCCG  TAAGGTCGGT  GTACAGGTGG  TGCATGGTTG
TCGTCAGCTC  GTGTCGTGAG  ATGTTGGGTT  AAGTCCCGCA  ACGAGCGCAA

CCCTCGTTCC  ATGTTGCCAG  CACGTAATGG  TGGGGACTCA  TGGGAGACTG
CCGGGGTCAA  CTCGGAGGAA  GGTGGGGATG  ACGTCAAATC  ATCATGCCCC
TTATGTCTTG  GGCTTCACGC  ATGCTACAAT  GGCCGGTACA  AAGGGCTGCG
ATACCGTGAG  GTGGAGCGAA  TCCCATAAAG  CCGGTCTCAG  TTCGGATCGG
GGTCTGCAAC  TCGACCCCGT  GAAGTCGGAG  TCGCTAGTAA  TCGCAGATCA

GCAACGCTGC  GGT
```

Table 7.3 Levels of 16S rDNA sequence similarity between the *Dermatophilus* species isolated from crocodiles and other *Dermatophilus* species.

	% Sequence similarities				
Species	**Fi 3**	**Fi 1**	**Recovered isolates**	**Fi 2**	**WS**
Fi 3	–	100	100	91.3	92.1
Fi 1	100	–	100	–	88.3
Recovered isolates	100	100	–	87.3	88.3
Fi 2	91.3	87.7	87.7	–	82.5
WS	92.1	88.3	88.3	82.5	–
Dermatophilus chelonae (DCH2)	98.8	99.0	99.0	91.1	92.5
Dermatophilus congolensis (DCD)	95.7	94.8	94.8	85.3	88.9
Dermatophilus congolensis (DCS1)	93.7	93.1	93.1	83.7	87.7
Dermatophilus congolensis (ATCC)	96.0	95.1	95.1	86.3	89.1
Dermatophilus congolensis (DCC1)	69.5	57.3	57.3	57.7	55.7

A phylogenetic tree was determined using the Tamura-Nei neighbour joining complete deletion method (Figure 7.10). The crocodile isolates composed of two field isolates (Fi 1 and Fi 3) and all six recovered isolates (Ri 1 to Ri 6) were in one group separate from *D chelonae* (DCH 2) and the group of *D congolensis*. Crocodile isolates and *D chelonae* had a 12 base difference or 99% sequence similarity based upon 16S rDNA sequence. The isolate WS from water sample TV 97-124 WS collected at 30 days post inoculation during the pilot transmission experiment was closely related to *Streptomyces* sp. All the major clusters in the phylogenetic tree were supported with 100% bootstrap values.

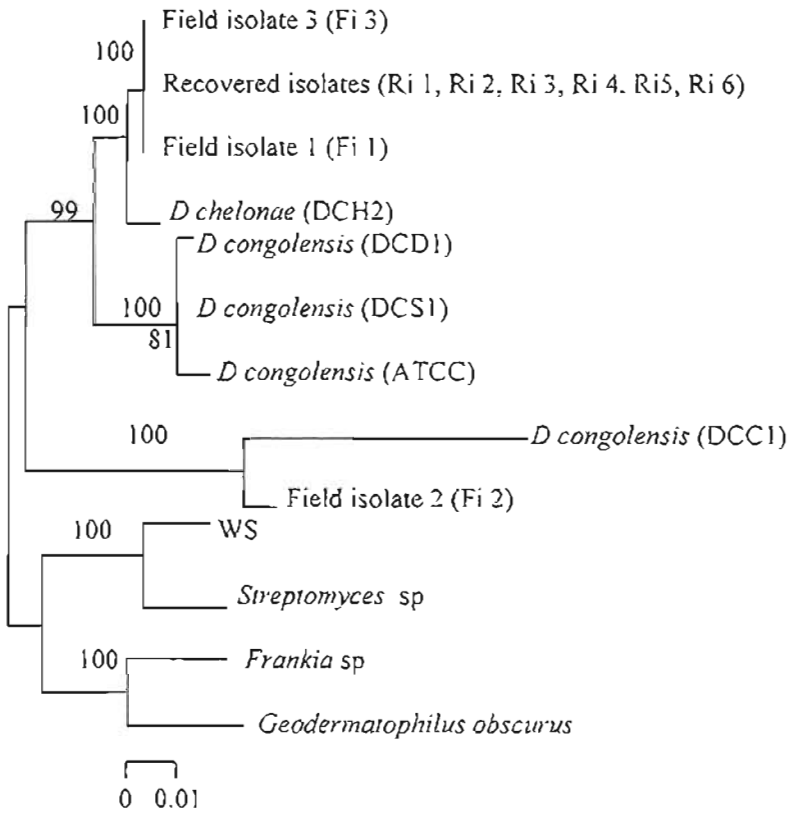

Figure 7.10 Phylogenetic tree for genus *Dermatophilus* and closest relative based on a sequence analysis of 16S rDNA gene. The tree was constructed using the Tamura-Nei neighbour joining complete deletion method. The number at the branch points are bootstrap values based on 1,000 replicates. The scale bar indicates a genetic distance of 0.01.

7.3.4 Ribotype identification

A total of 13 DNA extracts were digested with restriction enzymes *Eco* R1 and *Sca* 1, and this was followed by Southern blotting and hybridisation with labelled RNA probe containing 16S and 23S *E coli* RNA (Boehringer Mannheim). Both restriction enzymes *Eco* R1 and *Sca* 1 were found to provide ribotype fragments from nine isolates. As the ribotype bands were sometimes difficult to read, the X-ray films were read and interpreted independently by two researchers. This was done to help eliminate subjectivity.

Four isolates, of which two were recovered (Ri 1 and Ri 3) and two strains of *D chelonae* (DCH 1 and DCH 2) did not produce ribotype bands following digestion of chromosomal DNA with *Eco* R1. Likewise no ribotype bands were produced from one field isolate (Fi 3), two recovered isolates (Ri 1 and Ri 3) and one strain of *D chelonae* (DCH 1) with restriction enzyme *Sca* 1.

Eco R1 restriction enzyme revealed similar ribotype patterns of the recovered isolates (Ri 2, Ri 4, Ri 5 and Ri 6) and the original challenged isolate (Fi 1) used in transmission studies (Figure 7.11). The ribotype bands with fragment size 7,097 kb, 2,644 kb and 2,249 kb were present in all isolates tested. Isolate WS was different from the crocodile isolates by the presence of a ribotype band with fragment size of 1,514 kb.

Only a single ribotype fragment was produced by restriction enzyme *Sca*1 (Figure 7.12) of which six isolates (Fi 1, Fi 2, Ri 2, Ri 4, Ri 5 and Ri 6) had a fragment size of 5,385 kb. The ribotype fragment of isolates WS and *D chelonae* (DCH2) have the same ribotype fragment size of 4,352 kb.

Fragment size (kb)	Isolate								
	ATCC	Fi 4	Fi 2	Fi 1	Ri 2	Ri 4	Ri 5	Ri 6	WS
7,097	▬	▬	▬	▬	▬	▬	▬	▬	▬
4,510	▬	▬	▬						
3,863	▬	▬		▬	▬	▬	▬	▬	▬
2,644	▬	▬	▬	▬	▬	▬	▬	▬	▬
2,249	▬	▬	▬	▬	▬	▬	▬	▬	▬
1,752	▬	▬	▬						
1,654	▬								
1,514	▬								▬
1,319	▬								

Figure 7.11 Ribotype patterns observed following digestion of DNA of 9 out of 13 isolates with restriction enzyme *Eco* R1. *Dermatophilus congolensis* type strain (ATCC) was used to compare ribotype fragments with the test isolates.

Fragment size (kb)	Isolate								
	ATCC	Fi 2	Fi 1	Ri 2	Ri 4	Ri 5	Ri 6	WS	DCH2
11,110	▬								
5,385	▬	▬	▬	▬	▬	▬	▬		
4,352	▬							▬	▬
2,644	▬								
2,249	▬								
1,752	▬								
1,654	▬								
1,514	▬								
1,319	▬								

Figure 7.12 A single ribotype band was observed following digestion of chromosomal DNA with a restriction Sca1.

7.4 Discussion

Of the methods described and tested for DNA extraction, a modified method described for *A hydrophila* by Oakey (1997) proved successful. The cell wall of the Gram-positive bacteria is made up of a thick layer of peptidoglycan, forming a rigid structure (Tortora, Funke and Case, 1998). In this investigation the success of DNA extraction could be due to the action of lysozyme, which catalyses hydrolysis of the bonds between the sugars in the repeating disaccharides of the peptidoglycan. Repeated thawing and freezing of the pelleted bacterial cells initially weakened the cell walls thus making them vulnerable to lysis. CTAB enhanced the lytic process by precipitating the polysaccharide slime capsule that covers the outer cell wall. CTAB was not used in DNA extraction as described for *A hydrophila* (Oakey, 1997) and the concentration of lysozyme was lower because *A hydrophila* is Gram-negative and therefore has less peptidoglycan.

Although the crocodile isolates shared some morphological, cultural and biochemical characteristics with *D congolensis* and *D chelonae*, the significant differences strongly suggest that the crocodile isolates were phenotypically distinct from *D congolensis* and *D chelonae*. The appearance of colonies, the rate of growth, the temperature requirements during incubation and the division of septa differentiate crocodile isolates from other *Dermatophilus* species. Based on the result of the growth of crocodile isolates on Mueller-Hinton agar, it strongly suggests that the crocodile isolates are fastidious organisms, which require blood to grow. Furthermore, the transverse and longitudinal septation observed in histological sections of the skin infected with *D congolensis* (Simon *et al.*, 1972; Montali *et al.*, 1975) and *D chelonae* (Masters *et al.*, 1995) was not seen in 'brown spot' disease in crocodiles (Buenviaje *et al.*, 1998a). According to Gordon (1976), Roberts (1981) and Lennette, Spaulding and Truant (1974), the transverse and longitudinal septation, which formed a 'train track' appearance is a typical characteristic of *D congolensis*.

The reason for the failure of field isolates Fi 4 and Fi 5 to produce PCR products despite several attempts is not known. It may be they were incompatible to the primers. Furthermore, the phenotypical differences especially the cultural characteristics between field isolates Fi 4 and Fi 5 from other field isolates were considered very significant. It was therefore concluded that Fi 4 and Fi 5 could be another species of *Actinomycete*. Field isolate Fi 4 used in the pilot transmission experiment (see Chapter 6) could not be recovered from the skin lesions further indicating that this organism was not capable of causing 'brown spot' disease.

The 100% similarity of the 16S rDNA sequence of the isolate used for inoculation (Fi 1) with the recovered isolates, confirmed that these isolates were the same organism. Likewise, the 100% sequence similarity with the isolate recovered from the control animals after 36 days also proved that these were the same organisms, suggesting that cross infection had occurred. Although the control group was kept in separate buildings, we believed the organisms were carried over from the animals in the treatment group by students or staff members using both facilities in the Department. This finding repudiates an earlier claim (Buenviaje *et al.*, 1998b) that the control group of animals may have harboured a dormant infection, and that 'brown spot' disease was activated by stress of handling and concurrent poxvirus infection.

The 100% sequence similarity of the two field isolates Fi 1 and Fi 3 isolated from spontaneous outbreaks of 'brown spot' disease in farmed crocodiles from the Northern Territory and Queensland, respectively, provide strong evidence that the same organism was responsible for both outbreaks. Since early 1990 replacement stocks of crocodile hatchlings have been purchased by the Queensland farms from crocodile farms in Darwin (P. Freeman, 1999, personal communication), which may have had a dormant infection or the bacteria may have been transferred between farms through farm equipments. *Dermatophilus* is a soil-borne bacterium, an important consideration regarding the possible source of vertical transmission in crocodiles. It was presumed that after the initial invasion of *Dermatophilus* into the skin of crocodiles, it became pathogenic and thus caused the horizontal spread of infection. According to Martinez and Prior (1991), *D congolensis* can survive in clay soil for long periods, retaining its pathogenicity and thus providing a potential source of infection.

The phylogenetic analysis showed that the two field isolates (Fi 1 and Fi 3) and all six recovered isolates were distinct from *D congolensis* and *D chelonae*. Whilst the crocodile isolates were closely related to *D chelonae* having a 99% sequence similarity, they differed phenotypically. This finding is similar to a study on 16S rDNA sequences of all *Xanthomonas* species in which two species differed phenotypically but the 16S rDNA sequence similarity was 99.9% (Hauben, Vauterin, Swings and Moore, 1997). The differences in morphological, cultural and biochemical characteristics between crocodile isolates and *D congolensis* type strain (ATCC 14367) is reinforced by the differences in 16S rDNA sequence (95.1 to 96% sequence similarity), thus strongly supporting the conclusion that the crocodile isolates are distinct from *D congolensis*.

Although field isolate Fi 2 is phenotypically similar to field isolates Fi 1 and Fi 3, they differed in 16S rDNA sequence, perhaps indicating a different species of *Dermatophilus* (Rainey, Nobre, Schumann, Stackebrandt and Da Costa, 1997). A further investigation on the ability of Fi 2 to

cause 'brown spot' disease should be carried out. A similar finding was noted between *D congolensis* (DCC1) isolated from cattle suffering from dermatophilosis (A. Thomas, 1996, personal communication) and *D congolensis* type strain (ATCC 14637); the difference in 16S rDNA (59% sequence similarity) appeared to suggest the isolate DCC1 might fall into a different genus.

The ribotype patterns differed in all crocodile isolates. The heterogeneity of the ribotype patterns among *D congolensis* isolates is consistent with the findings of Faibra (1993). In this study it is acknowledged that as the procedure was not repeated due to time constraints, the validity of the results maybe questioned. However, ribotype fragments of all the recovered isolates and the isolate used in transmission studies were identical, which is consistent with the result of 16S rDNA sequence analysis.

Overall, the results of these studies have confirmed that the isolates recovered from infected animals in the transmission experiment on 'brown spot' disease, were identical to the challenged strain. This has strengthened considerably the claim that a *Dermatophilus* species is the agent of 'brown spot' disease in crocodiles. The phenotypic and molecular differences are sufficient to propose a new species of *Dermatophilus* – *Dermatophilus crocodyli* sp nov.

CHAPTER 8

TREATMENT AND CONTROL OF DERMATOPHILOSIS ('BROWN SPOT') DISEASE IN FARMED CROCODILES

8.1 Introduction

Dermatophilosis in domestic animals can cause considerable economic loss (Edwards, 1985; 1988). The major economic losses due to dermatophilosis of cattle in Africa include reduced milk production, lose of weight, reduced working efficiency of draught cattle and downgrading of hides (Lloyd, 1976). Because of the economic importance of this disease, a number of studies into the treatment and control of the disease have been undertaken.

Chemotherapy, such as a single intramuscular injection of either long-acting oxytetracyline or lincomycin-spectinomycin mixture was shown to be an effective treatment for *Dermatophilus*-infected sheep (Jordan and Venning, 1995). Dipping *Dermatophilus*-infected animals in 0.2% copper sulphate is also considered effective (Hart and Tyszkiewicz, 1968). Direct spraying on the infected skin with 0.3 to 1% cresols and quaternary ammonium compounds or by application of lotions containing iodine, 5% tar and gentian violet have also been reported to have a good therapeutic effect on the skin lesions caused by *Dermatophilus congolensis* (Blancou, 1976). Several studies to develop an effective immunological control of dermatophilosis (Makinde, Molokwu and Ezeh, 1986; Ellis, Robertson, Sutherland and Gregory, 1987; Sutherland, Ellis, Robertson and Gregory, 1987; Sutherland and Robertson, 1988) have been carried out, however, no vaccine has been developed to date.

Despite a significant boom in the growth of the crocodile industry and the increasing demand for skins, very little research has been directed at the control and treatment of diseases affecting the skin, particularly dermatophilosis. In this chapter, two studies describe the possible strategies for the control and treatment of dermatophilosis. The first study compared the effect of flowing water and static water on the development of 'brown spot' skin lesions. The second study described the efficacy of the therapeutic agents used to treat dermatophilosis.

8.2 Materials and Methods

8.2.1 Evaluation on the effects of flowing and static water on dermatophilosis

Animal inoculation

Forty-two *Crocodylus porosus* hatchlings from the Berrimah Farm, Department of Primary Industry and Fisheries, Darwin, Northern Territory were used in this study. The hatchlings were seven-week-old with an average length of 35 cm from the snout to the tip of the tail. The hatchlings were kept in a concrete pen with a sloping floor filled with approximately 200 L of water, while two-thirds of the area was dry. Randomly selected hatchlings individually identified by a marked cut on the scutes were allocated to three groups consisting of 15 in each group A and group B, and a further 12 hatchlings in group C (sham-inoculated control group). All animals in groups A and B were inoculated with a homogenised six-day-old broth culture of *Dermatophilus* sp (TVS 96-490-7B) as was described in Chapter 6. A cotton swab dipped in the inoculum was gently rubbed on at least 10 sites on the skin of the abdomen, chest, neck, jaws and tail. Animals in the sham-inoculated control group C were rubbed with sterile distilled water. Immediately after inoculation, the animals were kept in plastic crates for an hour before being returned to their respective pens, to avoid the inoculum being washed off.

Animal management and maintenance

The animals were housed in two enclosed sheds approximately one metre apart, and each shed has two adjoining pens. The two inoculated groups (A and B), were placed in two separate pens in the first shed (Figure 8.1 and Figure 8.2) whereas the sham-inoculated control (group C) was in the second shed. Group A was placed in a pen with static water (fixed volume of water for 24 hours) heated with automatic immersion heaters (Rena Corporation, Charlotte, NC, USA) maintained at 32°C. Group B and the sham-inoculated control (group C) were in flowing water, which was supplied with intermittent inflow of warm water from a hot water tank whenever the water temperature dropped below 32°C. In pens provided with flowing water, the water is totally replenished approximately every three hours during the daytime. The development of 'brown spot' lesions was recorded to compare the effect of flowing water against static water.

Figure 8.1 Two experimental sheds about a metre apart.

Figure 8.2 Concrete pen divided into two partitions. On the left far end of the pen is a heater tank, which automatically supplies inflow of warm water to maintain the temperature of water at 32°C.

The pens were emptied and cleaned each morning, and the crocodiles were fed approximately 27 g/crocodile/day of minced red meat with a vitamin supplement each afternoon. To avoid possible cross-infection, each shed was provided with a set of cleaning instruments. In addition, the pen of the control group was cleaned first and all materials used in cleaning were scrubbed thoroughly and disinfected with 1% chlorine solution. Surgical gloves were discarded after handling the experimental animals in each group. The feet and hands were washed with 2% Savlon solution (Pharmedica Laboratories Pty Ltd, London) before and after working with the animals. There was only one handler in charge in the management of the experimental animals throughout the duration of the study.

Clinical examination

The challenged animals were restrained and examined clinically on days 1, 3, 7 and 10 post-inoculation (PI). The 'brown spot' lesions in each animal were counted and measured. Two animals with the most lesions were euthanased by barbiturate overdose injected directly into the heart. A post-mortem examination was performed, and two skin samples (2 cm^2) with 'brown spot' lesions were fixed in 10% buffered formalin. The samples were processed for histopathological examination to confirm if effective transmission of 'brown spot' disease was achieved.

8.2.2 *In vitro* antibacterial sensitivity testing of *Dermatophilus* sp to copper sulphate, formalin and salt

Preparation of test organism

Prior to the clinical treatment trial of 'brown spot' disease, an *in vitro* sensitivity testing using copper sulphate, salt and formalin was undertaken. The desired colony forming units (CFU) were determined initially before the *in vitro* study was carried out. *Dermatophilus* sp isolate with accession number TVS 96-470-7B used previously in several transmission experiments (Buenviaje *et al.*, 1998b) was tested for its sensitivity to antibacterial agents. Two loopsful of a six-day-old bacterial culture from a blood agar plate were placed into a dounce homogeniser tube and 1.5 mL of sterile distilled water was added. The clumps of bacterial colonies were homogenised until the mixture became uniformly cloudy. Serial ten-fold dilutions were performed in six Eppendorf tubes from neat to 10^{-6}. Approximately 0.5 mL from each tube containing 1.5 mL of homogenised bacterial suspension was dispensed and uniformly spread onto three blood agar plates containing 5% sheep blood. The plates were incubated at 37°C in the presence of 5% CO_2 in air, and the colonies counted after five days. The 10^{-5} dilution provided 150 to 250 CFU, and was selected as the standard dilution for the sensitivity testing.

Preparation of antibacterial agents

Copper sulphate was prepared at concentrations of 0.1, 0.25, 0.5 and 1 ppm, salt at 1 mg/mL, 2, 3 and 5 mg/mL and formalin at 0.02, 0.03, 0.05 and 0.10%. Each antibacterial solution was prepared in aliquots of 1.35 mL in Eppendorf tubes. To each tube, 150 µL of homogenised bacterial suspension at 10^{-4} dilution was added to achieve a final concentration of 150 to 250 CFU. The bacteria were exposed to different concentrations of the antibacterial solutions for 30 minutes, one hour, three hours, and six hours. Untreated control tubes containing sterile distilled water was prepared for every group of antibacterial solution and the same protocol was carried out as described above. Each antibacterial solution containing the homogenised *Dermatophilus* sp suspension, and the untreated controls were plated onto three blood agar plates after exposure times mentioned above. The number of CFU in each plate was counted after five days of incubation.

8.2.3 Comparative study on the efficacy of copper sulphate and formalin on dermatophilosis

A total of 29 hatchlings with 'brown spot' lesions (22 hatchlings from the study on the evaluation of flowing water and seven hatchlings from the pilot experiment on poxvirus, see Chapter 9) were randomly allocated to three groups. Group 1 (12 hatchlings) was treated with 1 ppm copper sulphate, group 2 (seven hatchlings) with 0.1% formalin and 10 hatchlings were used as the untreated control group. Copper sulphate and formalin were used as therapeutic agents based upon the results of the *in vitro* antibacterial sensitivity testing. The copper sulphate solution was prepared by dissolving 100 mg of copper sulphate in approximately 4 L of water, and 100 mL of formalin were added to 4 L of water. The solutions were mixed thoroughly in approximately 96 L of water in each pen to achieve the desired final concentration of 1 ppm and 0.1% for copper sulphate and formalin, respectively. The solutions were prepared each day, and added to the pen immediately after cleaning. To maintain the desired concentrations of the antibacterial solutions, both the copper sulphate- and formalin-treated groups were kept in pens provided with static water. The untreated control group was placed in a pen provided with flowing water. Individual animals in the copper sulphate-treated group, formalin-treated group and the untreated control group, were restrained on clinical examination days 4, 7, and 11 post-treatment (PT). Data on the number of recovered animals and the number and size of 'brown spot' lesions in remaining infected animals were recorded.

8.3 Results

8.3.1 Gross and microscopic findings

Gross examination of the skin from the two hatchlings (each from a group provided with static water and the sham-inoculated control group) revealed the presence of 'brown spot' lesions up to 1 mm in diameter scattered mainly on the belly, chest and neck. 'Brown spot' lesions were present in both inoculated and non-inoculated sites of the skin. Samples of skin sectioned and examined microscopically revealed histopathological changes typical of dermatophilosis. Changes included the presence of indentations or ulcerations of the epidermis and accumulations of debris composed of dead epidermal cells, keratin and inflammatory cells, sometimes associated with laminated keratin. The layer of epidermis on the indented portion of the skin was thinner compared with the adjacent epidermis of the unaffected skin. Numerous filamentous bacteria infiltrated the debris in deeply ulcerated sections of the skin. These filamentous bacteria were similar in morphology, and consistently present in skin samples infected with 'brown spot' disease in previous experiments (Buenviaje *et al.*, 1998b).

Effect of flowing water on the development of 'brown spot' lesions

Sixty percent (9 out of 15) of the hatchlings in static water (group A) developed one to two 'brown spot' lesions less than a millimetre in size on day one PI (Table 8.1). All hatchlings were infected with dermatophilosis during the 10-day-period after inoculation, and the number of 'brown spot' lesions had increased up to 10 lesions. The 'brown spot' lesions were up to 2 mm in diameter but mostly less than a millimetre in size, located predominantly on the belly, neck and chest except a few spots on the tail. On the skin of the jaws in three animals were either 'brown spot' or linear lesions up to 1 cm in length. After clinical examination on day 10 PI, the lesions on the jaws and neck in three animals had resolved. In addition, the hatchlings from the two groups (group B and the sham-inoculated control group) in flowing water started eating on the 3rd day after inoculation, and ate approximately 40 of 405 grams of food per day until the 5th day, whereas, the hatchlings in static water (group B) commenced to eat approximately 20 to 30 grams of food per day on the 5th day. All animals resumed normal feeding two weeks after inoculation, however the animals in all groups stopped feeding for at least a day every after clinical examination.

Table 8.1 Comparison of static water and flowing water on the development of 'brown spot' lesions.

Experimental groups	Number of Animals	Number of animals with 'brown spot' lesions at PI days			
		PI day 1	PI day 3	PI day 7	PI day10
Group A (static water)	15	9 (60%)	10 (67%)	14 (93%)	12 (80%)
Group B (flowing water)	15	3 (20%)	1 (7%)	4 (27%)	3 (20%)
Group C (control)	12	0	4 (33%)	5 (42%)	9 (75%)

Three (20%) hatchlings from a group in flowing water developed 'brown spot' lesions on day one PI. Affected hatchlings had one or two lesions located either on the belly or tail. The lesions from two infected hatchlings had resolved after clinical examination on day three PI. However, new lesions developed at other sites of the skin on day 10 PI. A total of four animals from the group provided with flowing water developed 'brown spot' lesions during the 10-day-period after inoculation. The sham-inoculated control group of 12 hatchlings had not developed any 'brown spot' lesions on day one PI. However, small 'brown spot' lesions up to 1mm were found in four animals on clinical examination day three PI. Likewise, some original lesions had resolved, but reappeared at other sites on day seven and 10 PI. A total of nine hatchlings developed 'brown spot' lesions during the 10-day-period after inoculation. Two hatchlings, one each from static water (group A) and flowing water (group B) had pale and gelatinous discolouration on the skin at the right lateral side of the abdomen, typical of superficial fungal infection (Buenviaje *et al.*, 1994).

8.3.2 *In-vitro* antibacterial sensitivity

The average number of CFU from the three blood agar plates inoculated with *Dermatophilus* sp (96-490-78) after exposure to each concentration of antibacterial solution at a given time are presented in Table 8.2. No colonies were present in any of the blood agar plates inoculated with *Dermatophilus* sp exposed to different concentrations of copper sulphate at 30 minutes, one hour, three hours and six hours after five days incubation. Further incubation of plates for another week or so yielded the same results with no colonies present in any blood agar plates. In contrast, an average range of 117 to 248 CFU was present in all control blood agar plates after five days incubation. The cultural and morphological characteristics of these colonies were consistent in all plates. The colonies were initially grey in colour, haemolytic and slightly pitting into the blood agar after three days incubation, and became white and more rugose, sometimes crateriform after five days.

Table 8.2 *In vitro* sensitivity test. Number of colony forming units (CFU) of *Dermatophilus* sp (TVS 96-490-7B) after exposure for various times to different concentrations of antibacterial solutions.

	Number of CFU at exposure times			
Antibacterial solutions	**30 minutes**	**1 hour**	**3 hours**	**6 hours**
$CuSO_4$ (ppm)				
0 (control)	248	126	176	117
0.1	0	0	0	0
0.25	0	0	0	0
0.5	0	0	0	0
1	0	0	0	0
$NaCl_2$ (mg/mL)				
0 (control)	220	137	141	191
1	208	123	134	180
2	206	135	141	108
3	215	112	128	160
5	216	118	121	78
Formalin (%)				
0 (control)	208	154	151	155
0.02	210	103	90	94
0.03	153	103	98	75
0.05	171	95	137	23
0.1	188	196	113	24

The effects of time and the different concentrations of salt and formalin on the number of CFU were analysed using the Tukey HSD statistical method. No significant difference in the number of CFU was found between concentrations of salt including the control. However, there was a reduction of the number of CFU after exposure of *Dermatophilus* sp at one hour, three hours and six hours ($P<0.05$). A reduction in the number of CFU was observed between zero concentration (control) and 0.1% formalin ($P<0.05$). All other concentrations of formalin (0.02, 0.03 and 0.05%) did not differ significantly ($P>0.05$) on the mean number of CFU. In addition, there was no significant effect ($P<0.05$) of the exposure times (30 minutes, one hour, three hours and six hours) on the number of CFU.

8.3.3 Treatment of dermatophilosis

As a result of husbandry problems, the treatment of dermatophilosis was modified to four protocols. The comparative assessment on the efficacy of copper sulphate and formalin on 'brown spot' lesions was terminated on day 11 PT due to the increased severity of 'brown spot' lesions.

Copper sulphate and formalin treatment

The 'brown spot' lesions resolved in eight of 12 (67%) hatchlings from the copper sulphate-treated group on day four PT. However, there was a recurrence of 'brown spot' lesions in five of eight hatchlings on clinical examination day 11 PT. The 'brown spot' lesions present in all infected hatchlings from the copper sulphate-treated group were very small (<0.5 mm), and were apparently resolving. One of seven hatchlings in the formalin-treated group had lesions resolved on day four PT, but became reinfected after day seven PT. More lesions were present in infected hatchlings from the formalin-treated group compared with the hatchlings from the copper sulphate-treated group on day four PT. In addition, one animal from the formalin-treated group had 16 lesions (up to 2 mm in diameter) scattered mostly on the belly. On clinical examinations days 7 and 11 PT, all animals developed severe lesions with an average of 19 lesions per animal. One severely affected hatchling had 30 'brown spot' lesions scattered on the belly, neck, chest and tail, but mostly on the belly. The severity of 'brown spot' lesions in the control group was similar to the infected animals in the formalin-treated group. In addition, two 'brown spot' infected hatchlings in the copper sulphate-treated group were complicated with superficial fungal infection.

Copper sulphate treatment in pen provided with static water

Due to the increased severity of 'brown spot' lesions on hatchlings both in the formalin-treated group and the control group, the formalin treatment was discontinued. The total number of animals with skin lesions included 22 hatchlings with 'brown spot' lesions and two hatchlings with dual skin infections ('brown spot' and superficial fungal skin lesions) were from the treatment groups. An additional 11 infected hatchlings from the pilot study on poxvirus (five hatchlings with 'brown spot' lesions, four superficial fungal skin infections, one with dual infection and one hatchling with an abscess on the right axilla) were also included. Overall, 27 hatchlings had 'brown spot' lesions, three with dual skin infections, four with a superficial fungal infection and a single case of an abscess.

All infected animals were placed in two separate pens in static water treated with copper sulphate. A group of non-infected and recovered hatchlings in a pen provided with flowing water also received copper sulphate medication. On the eighth day PT, the immersion heaters provided in static water failed, thus the flowing water was restored. The treatment of copper sulphate was continued for two weeks. On clinical examination after 14 days PT, 21 of the 27 (77.8%) hatchlings with 'brown spot' lesions, three of four hatchlings with superficial fungal infection and one hatchling with the abscess were resolved. Two of the three hatchlings with dual infections recovered from both skin infections, and the fungal lesions in one hatchling had also resolved

but not the 'brown spot' lesions. One hatchling recovered from a superficial fungal infection but developed 'brown spot' lesions. An additional four new cases of 'brown spot' infection from the non-infected group were included. The total of 12 hatchlings with skin infections included 11 with 'brown spot' lesions (seven from old cases and four new cases) and one dual infection.

Copper sulphate treatment in pen provided with flowing water

Copper sulphate medication was continued, but because of the problem of maintaining the desired concentration (1 ppm) in flowing water, the animals were treated twice. The first treatment was given in the morning after cleaning and the second treatment was given in the afternoon. All 12 infected animals including those recovered and non-infected animals were treated twice daily for 10 days. Clinical examination after ten days of copper sulphate medication showed no therapeutic response from all 12 infected hatchlings. Instead, there were additional four new cases and three recurrent cases of superficial fungal infection. Another two hatchlings (one new case from a non-infected group and one recurrent case) developed 'brown spot' lesions. A total of 21 infected animals included 14 with 'brown spot' lesions and seven with superficial fungal infection were recorded.

Immersion of animals in copper sulphate-medicated water for at least 15 minutes

The copper sulphate treatment was continued, but all animals were immersed in copper sulphate medicated water for at least 15 minutes twice daily, one in the morning after cleaning and another one in the afternoon after the animals were offered food. After six weeks of medication, 50% (7 of 14) of hatchlings with 'brown spot' lesions had recovered. The remaining seven infected hatchlings had one to four lesions, which were apparently resolving. All seven hatchlings infected with superficial fungal infection had also recovered. A final clinical examination after a further three weeks revealed all infected animals had completely recovered from 'brown spot' lesions. No recurrence of lesions resembling either 'brown spot' or superficial fungal infection was noted. All animals were perfectly healthy and not one scar from previous lesions was found.

8.4 Discussion

The experiment on the control of dermatophilosis was primarily designed to demonstrate the influence of flowing water and static water in regards to the infectivity rate or the protection time from *Dermatophilus* sp infection. Although the initial clinical examination on day one PI revealed more hatchlings were infected in static water compared with hatchlings in flowing water, the infection rate of both treatments increased until day seven PI. Had clinical observation been

continued for another week or so, all animals would probably have been infected. The reason for delayed infection in some animals presumably relates to the individual immunological response to infection. Although the percentage of infected hatchlings in static water was lower than in flowing water, the percentage of infected hatchlings in the control group, which was also provided with flowing water was similar for both treatments.

Despite every effort to prevent contamination, the infection that developed in the sham-inoculated control group may be attributed to the frequent movement of staff between sheds due to husbandry problems encountered on the second day after inoculation. In addition, the pen of the sham-inoculated control group in the second shed was only a metre away from the inoculated groups. Contamination may have been prevented if the sham-inoculated control was located far from the inoculated groups, and if the two groups were cared for separately (Buenviaje *et al.*, 1998b). These conditions however, could not be complied with given the existing infrastructures and staffing management on the farm. This investigation has confirmed earlier transmission experiments (Buenviaje *et al.*, 1998b) that the morbidity rate due to dermatophilosis can be very high and the disease can be easily transmitted. By not providing treatment to early developing 'brown spot' lesions, the disease can progress to severe or chronic lesions and spread rapidly between susceptible animals.

To date, there have been few published research reports on the treatment of skin diseases in crocodiles, especially on dermatophilosis, whereas a number of reports on the treatment of dermatophilosis in domestic animals are available. Apparently, effective and less expensive treatment of affected animals has been mainly considered, especially if therapeutic agents can be incorporated into the water (Franklin, Gibson, Caffrey, Wagner and Steffen, 1991). Treatment through the parenteral route is not possible if an outbreak of 'brown spot' disease is widespread. Medication through the water is clearly the best method in intensive crocodile farming. Frequent handling of crocodiles for parenteral medication would require more personnel and the stress this causes to the sick animals would probably exacerbate the condition.

Because crocodiles are semi-aquatic animals, the copper sulphate used for the *in vitro* sensitivity test was based upon the recommended therapeutic dose for the treatment of external parasitic infection in fish (Cross and Needham, 1988). Of the three antibacterial compounds used for the *in vitro* sensitivity test, copper sulphate was the most effective antibacterial agent against *Dermatophilus* sp. Although prolonging the exposure time of *Dermatophilus* sp to formalin and increasing the concentration to 1% significantly decreased the number of CFU, biologically this was not significant because the mere presence of a single bacterial colony under

favourable conditions could multiply exponentially. Salt was not included in the assessment of its efficacy against 'brown spot' disease *in vivo* because it did not affect the growth of bacteria in the *in vitro* sensitivity test. In contrast, Stuart (1993) had recommended its use as one of the disinfectants or therapeutic agents for crocodiles.

The cure rate of the antibacterial solutions indicates that copper sulphate was more effective than formalin. The recurrences of infection in some recovered animals may be due to incomplete recovery or as a result of stress by frequent handling on examination during the 11 days treatment period. Another reason could be attributed to the thermoregulatory function of crocodiles. This means that infected hatchlings may not stay in medicated water or may spend a short period once the body temperature has reached 32°C. Individual responses to infection or the time required for the lesions to resolve could also be another reason for partial recovery and reinfection in some hatchlings, but these hypotheses need to be investigated further. The possibility of *Dermatophilus* sp developing resistance to copper sulphate should be investigated further (Cervantes and Gutierez-Corona, 1994).

The recurrence of infection and emergence of new cases in non-infected animals in flowing water, despite treatment twice daily with copper sulphate, strongly suggests that the animals did not receive sufficient exposure to medication. Affected animals recovering from a sub-therapeutic dose could be the source of recurrent or prolonged infections. The results of the four treatment protocols used in this study strongly suggest that immersion of animals for at least 15 minutes in copper sulphate medicated water is the best method.

Copper sulphate is cheap and the amount required for the treatment of 'brown spot' disease is very low at 1 ppm or 100 mg/100 L of water. At the current price of $12 per kilogram of copper sulphate (Askern Farm and Garden Supplies Pty Ltd., Townsville), the treatment cost is 1.2 cents per 1,000 L. Besides, copper sulphate has been shown to be effective not only on 'brown spot' disease but also effective against superficial fungal infections and an abscess in this study. Although there is no explanation for the action of copper sulphate on *Dermatophilus* sp, copper sulphate was found to inhibit the respiratory electron transport system of the cytoplasmic membrane in *Paracoccus dentrificans* (Smit, Van Der Goot, Nauta, Pijper, Balt, De Bolster, Stouthamer, Verheul and Vis, 1980). Copper sulphate also inhibits the metabolism of lactate dehydrogenase and NADH oxidase in *Mycoplasma gallisepticum* (Gaisser, De Vries, Van Der Goot and Timmerman, 1987).

In conclusion, both *in vivo* and *in vitro* studies have demonstrated that copper sulphate is an effective antibacterial agent against *Dermatophilus* sp, and is an effective therapeutic agent in superficial mycotic dermatitis and abscess in crocodiles in this study. Despite some management problems encountered during the experimental treatment period, copper sulphate has prevented further development of severe lesions and other secondary infections. Pens provided with flowing water did not reduce or prevent further infection with *Dermatophilus* sp, therefore static water in the pen is recommended with total immersion for at least 15 minutes. Further studies on the use of dipping bath treatment of 'brown spot' disease are required.

CHAPTER 9

STUDIES ON POXVIRUS IN CROCODILES

9.1 Introduction

The occurrence and pathology of poxvirus has been documented and well described in both vertebrate and invertebrate hosts. The virus affects mainly the skin, sometimes the oral mucosal membranes and rarely the respiratory tract. It is usually a non-fatal type of disease, however the location of the lesions such as the mouth, eyelids and throat can cause discomfort and in severe cases lead to blindness and deprivation of food. This in turn leads to starvation, lower immune function, and death. Increased mortality in poxvirus infection is compounded with dual or multiple infections such as salmonellosis and chlamydiosis (Wernery, Wernery, Zachariah and Kinne, 1998) and neoplasia (Mayet, Sommer and Heenan, 1997).

The poxvirus can be readily identified morphologically through electron microscopy. Poxvirus is known as the largest virus measuring from 200 to 400 nanometre (nm). The oval shaped body of virions has a core that appears biconcave or 'dumb bell' shape typical of poxvirus. Six genera of vertebrate poxviruses have been documented, each consisting of different species of poxviruses identified and confirmed by nucleic acid hybridisation, restriction endonuclease and DNA sequence analysis (Moss, 1990). Recent techniques to confirm the species of poxvirus is based upon the polymerase chain reaction (PCR) technique (Thompson, 1997; Ireland and Binepal, 1998; Neubauer, Reischl, Ropp, Esposito, Wolf and Meyer, 1998) and DNA sequence analysis (Thompson, Yager and Van Rensburg, 1998; Tryland, Sandvik, Mehl, Bennett, Traavik and Olsvik, 1998).

Infection caused by poxvirus has been reported in a captive Hermann's tortoise (*Testodo hermanni*) (Oros, Rodriguez, Deniz, Fernandez and Fernandez, 1998) and in crocodiles (Buenviaje *et al.*, 1992). Poxvirus infection was one of the most important skin disease problems that had caused economic losses in the crocodile farming industry in South Africa (Horner, 1988). In addition, severe outbreaks of poxvirus infection have been reported in Zambia (Pandey *et al.*, 1990), Zimbabwe and Transvaal Lowveld, South Africa (Huchzermeyer *et al.*, 1991) and Kenya (Buoro, 1992). In contrast to severe outbreaks of poxvirus infection in South Africa, occasional cases of poxvirus infection, of which the majority were incidental findings, had been diagnosed in farmed crocodiles from Queensland and the Northern Territory (Buenviaje *et al.*, 1992; 1998a). In spite of the perceived significance of infection, no one has addressed comprehensibly poxvirus infection in crocodiles. This study was undertaken primarily because of

the increasing reports on poxvirus infection in farmed crocodiles in Australia and the high incidence of poxvirus infection in the previous study (Chapter 6).

9.2 Materials and Methods

9.2.1 Gross examination of skin lesions and preparation of samples for laboratory analysis

Eleven of 16 six-month-old *C porosus* hatchlings showing lesions typical of poxvirus during the transmission study of *Dermatophilus* sp (Chapter 6) were used in this study. All infected animals with pox lesions were euthanased by barbiturate overdose (1 mL) injected directly into the heart. On clinical examination, the gross appearance and size of the poxvirus lesions on the skin and their location were recorded. Skin lesions from infected animals were collected into sterile plastic bags at post mortem examination, and stored at –80°C. At least three representative skin samples from each animal were fixed in 10% buffered formalin for histopathological examination. The lesions on two skin samples were excised from the adjacent normal skin with a sterile scalpel blade, cut into 1 mm^2 and fixed in either 10% glutaraldehyde or formaldehyde. After fixation for one hour, the samples were transferred to Eppendorf tubes containing 1 mL of distilled water. The tissue sample was post-fixed in 1% osmium tetroxide and dehydrated in a graded series of ethanol, and embedded in Spurr's resin. Approximately 60 nm of sections were cut with a diamond knife on a LKB Nova ultramicrotome. The cut skin tissue sections were placed in a 200 mesh copper grid and stained with uranyl acetate (saturated in 50% ethanol, acidified) and lead citrate (modified Reynold's). The ultra thin sections were examined in a JEOL FX 2000 transmission electron microscope using 80KV accelerating voltage.

9.2.2 Preparation of tissue culture from freshwater crocodile (*Crocodylus johnstoni*) embryo

Three freshwater crocodiles (*Crocodylus johnstoni*) eggs (15 days old) were used to culture tissues from the embryo. The media used for tissue culture was described in Appendix 6. The whole embryo was separated from the remainder of the egg, placed in a sterile Petri plate and washed twice with Dulbecco's Modified Eagle's Medium (DMEM) to remove some mucus and blood. The cleaned embryo was transferred to another Petri plate, cut into small pieces (<1 mm^2), and 10 mL DMEM added. The embryo suspension was mixed gently, transferred to a 100 mL conical flask and allowed to stand for few minutes to settle down large particles. The supernatant was decanted into a 25 mL tissue culture flask, 1.5 mL of bovine serum was

added, and labelled as primary culture. The remaining large tissue particles or sediments were added to 10 mL DMEM and 1 mL Antibiotic Versene and Trypsin (ATV) solution, and mixed gently. The suspension was decanted into a plastic, sterile centrifuge, and centrifuged at 1,000 × g for 15 minutes. The supernatant was collected into a 25 mL tissue culture flask marked "second collection", and 1.5 mL of foetal calf serum was added. Both the first and second collections were incubated at 28°C. The tissue culture was examined under the microscope every three days, and DMEM was changed after two to three weeks if necessary.

9.2.3 *In vitro* culture of poxvirus

The pox lesions were carefully excised from five skin samples using a sterile scalpel blade, and ground with a mortar and pestle. Prepared DMEM solution (see Appendix 6) was added with 1× concentration of antibiotics – the final concentration of penicillin at 200 units/mL, streptomycin at 200 µg/mL, kanamycin at 80 µg/mL and polymyxin B at 5 µg/mL. When the tissues were completely homogenised, 1 mL of DMEM was added. After mixing, the suspension was transferred to Eppendorf tubes and centrifuged for 5,000× g for two minutes to remove tissue particles. The supernatant was decanted, and the remaining pellets were stored at –80°C. Three tissue culture flasks containing cell cultures of *vero* cells and another three flasks with baby hamster kidney cells (BHK) were examined under the microscope to make sure the cells were healthy. At least 200 µL of the poxvirus suspension was inoculated into each of two tissue culture flasks containing BHK and another two flasks of *vero* cells, and incubated at 28°C. The two non-inoculated remaining flasks of *vero* and BHK tissue cultures were also incubated as controls. After one week, the initial culture was subcultured each into at least two flasks, and subsequent four passages were carried out. The inoculated cell cultures and the controls were examined under the inverted microscope (Olympus CK2) for any changes in the cells every after three days for two weeks.

9.2.4 Inoculation into chicken chorioallantois and crocodile embryo

The protocol for preparing the homogenised poxvirus inoculum was modified by using pH 7.0 PBS containing 5× concentration of antibiotic instead of DMEM to avoid possible bacterial contamination particularly from *Dermatophilus* sp. The suspension was held at room temperature for an hour before inoculation (Tripathy and Hanson, 1980) to prevent possible temperature shock of the embryo. Inoculation with presumed poxvirus infective tissue was performed by injecting 100 µL of homogenised suspension through the chorioallantoic membrane (CAM) in ten chicken eggs and 100 µL of sterile pH 7.0 PBS in two control eggs

following the procedure described by Mitchell-Hoskins (1967). After inoculation, the eggs were incubated at approximately 37°C. Poxvirus inoculation was repeated in five batches of a dozen (11 days old) chicken eggs to have more specimens for histology and electron microscopy examination. Another poxvirus inoculation was performed in two 16-day-old *C porosus* eggs. Approximately 100 µL of poxvirus suspension was injected through the chorioallantoic cavity (Webb, Manolis, Dempsey and Whitehead, 1987), and incubated at 28°C. Both chicken and crocodile eggs were examined six to eight days and one to two weeks, respectively, after inoculation.

9.2.5 Pilot transmission experiment of poxvirus

The Berrimah Farm, Department of Primary Industries and Fisheries, Darwin, Northern Territory supplied 17 *C porosus* hatchlings used in this study. The hatchlings were seven weeks old with an average length of 35 cm from snout to the tip of the tail, and were kept in a concrete pen with a sloping floor (filled with approximately 200 L of water) and a dry area. The transmission experiment on poxvirus was carried out on the same day after the inoculation of *Dermatophilus* sp in the treatment and control studies (Chapter 8).

Prior to animal inoculation, approximately 2 mL of homogenised poxvirus inoculum kept for at least a year in –80°C was transferred immediately into a bucket of ice, and thawed gently to prevent possible temperature shock. At least ten different sites on the skin of the abdomen, chest, neck, jaws, tail and footpads in nine animals were slightly scarified using a sterile scalpel blade. A sterile cotton swab dipped in the homogenised poxvirus inoculum was gently and repeatedly rubbed on the scarified area of the skin. The inoculated animals were kept in plastic crates for an hour before being returned to the pen containing eight non-inoculated in-contact control animals. All inoculated animals included the non-inoculated in-contact control were restrained and examined individually at post-inoculation days 1, 3, 7 and 10.

9.3 Results

9.3.1 Gross and microscopic examination

The gross and microscopic appearance of skin lesions infected with poxvirus was consistent in all 11 hatchlings. The poxvirus lesions were located on the skin of the limbs, abdomen, neck and chest, but mostly on the skin of the tail and footpads. Marked circumscribed grey-white lesions up to 3 mm dia. (Figure 9.1 and Figure 9.2) were present on the pigmented skin particularly on

the lateral sides of the tail, back, lower limbs and footpads. The lesions on the tail were slightly pitted onto the skin and located between scales (Figure 9.3) whereas the lesions on the abdomen, neck and chest were diffused irregular and translucent (Figure 9.4). Many of the skin lesions were ulcerated and accompanied by reddening of adjacent skin.

Microscopic examination on the affected skin revealed the presence of an ulcer, and the epidermal layer extending from a relatively normal skin into the ulcerated part had hypertrophic cells of varying sizes. Affected cells of the epidermis were enlarged and contained either a single large intracytoplasmic inclusion body or multiple small intracytoplasmic inclusion bodies of different sizes. The nucleus was distorted and displaced to the edge of the cell as a result of the accumulation of the inclusion bodies. The intracytoplasmic inclusion bodies were eosinophilic and bigger as the surface cells were approached (Figure 9.5). Degenerating or dead epidermal cells containing the inclusions were intensely eosinophilic and flattened, which filled the concave portion of the affected epidermis. There was not a marked inflammatory reaction present in the dermis beneath the pox lesions except for cases where the pox lesions were adjacent to 'brown spot' lesions.

9.3.2 Transmission electron microscopy

Transmission electron microscope examination of the skin lesions confirmed the presence of poxvirus particles. The inclusion bodies consisted mainly of numerous oval or brick shape virions that were at least 200 nm long (Figure 9.6). Inside the virions there were different forms of nucleocapsid such as rounded dense material, 'sausage' shape but mostly elongated biconcave bodies with rounded ends forming a 'dumb bell' shape (Figure 9.7). An outer thin membrane enclosed some virions present inside the inclusion bodies. In the cytoplasm outside the inclusion bodies there were different structures such as circular bodies and a crescent shape with granular materials adjacent to the concave side (Figure 9.8). Inside the circular bodies varied from homogeneous to localised aggregation of materials sometimes forming a 'sausage' shape inner core. All circular bodies were enclosed with an outer membrane.

Figure 9.1 Pox skin lesions showing grey-white lesions (arrows) on the back of *C porosus* hatchling.

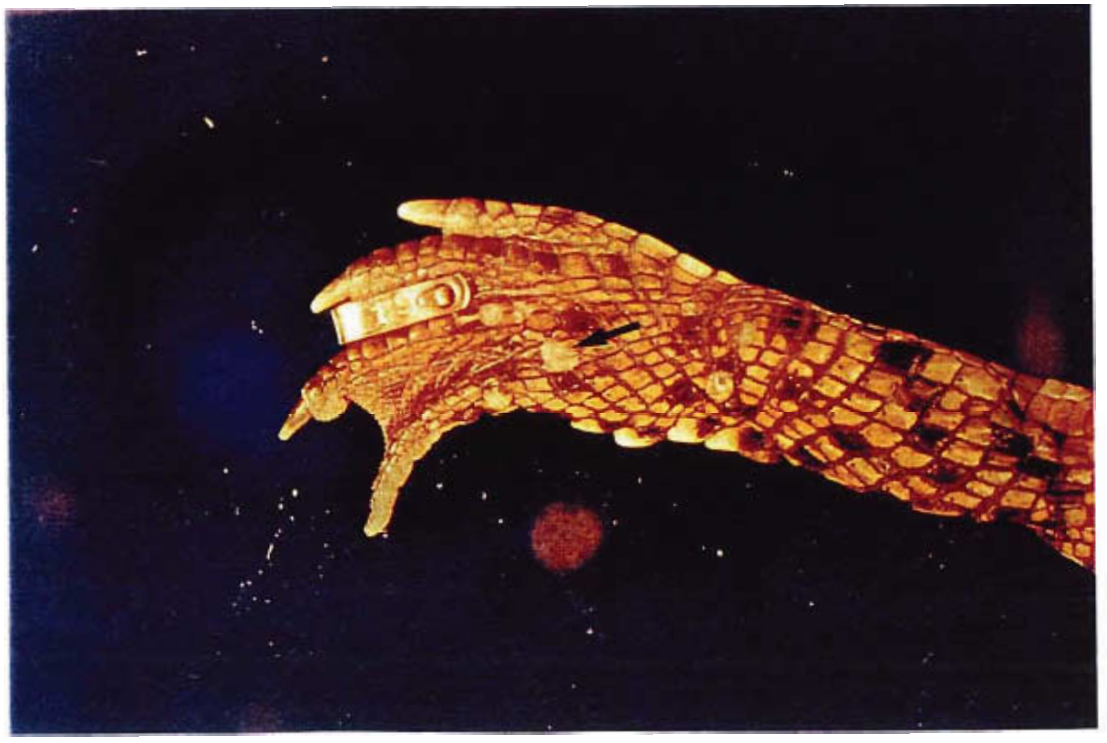

Figure 9.2 Circumscribed grey-white pox lesions on the lower limb and foot pad (arrow).

Figure 9.3 Pox lesions on the tail. Note grey-white lesions (arrow heads) between scales and few discrete lesions on the raised scutes (arrows).

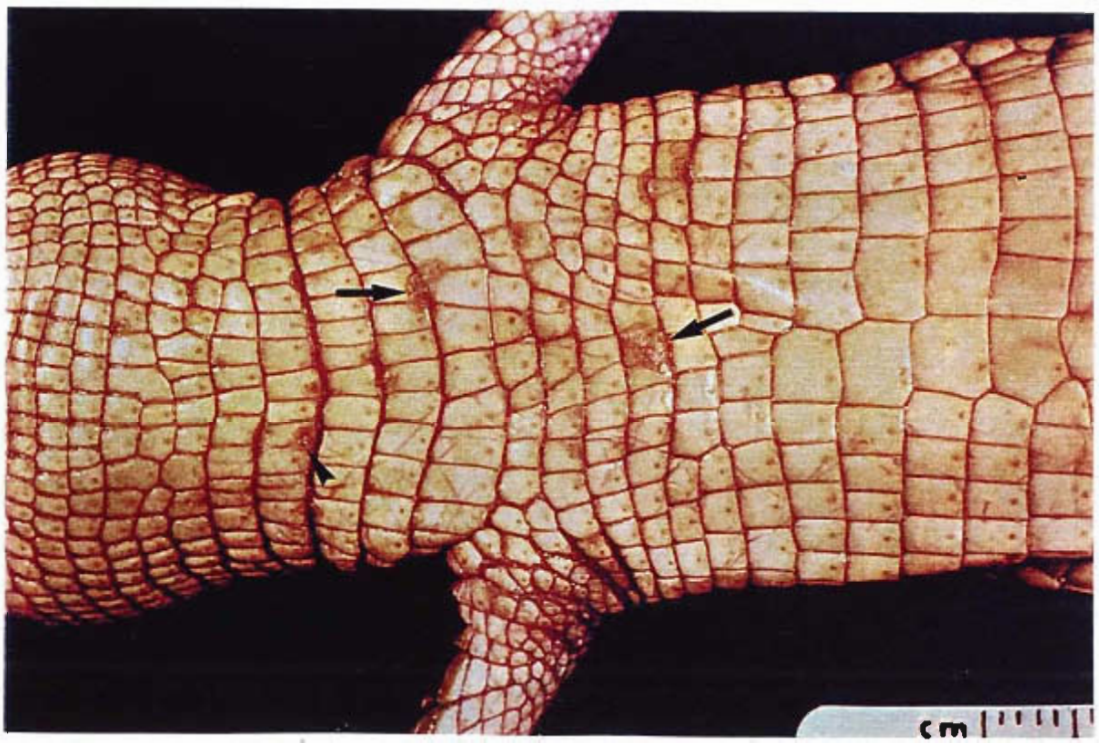

Figure 9.4 Pox lesions on the chest were translucent and irregular to crescent shape (arrows) and small discrete lesions on the neck (arrow head).

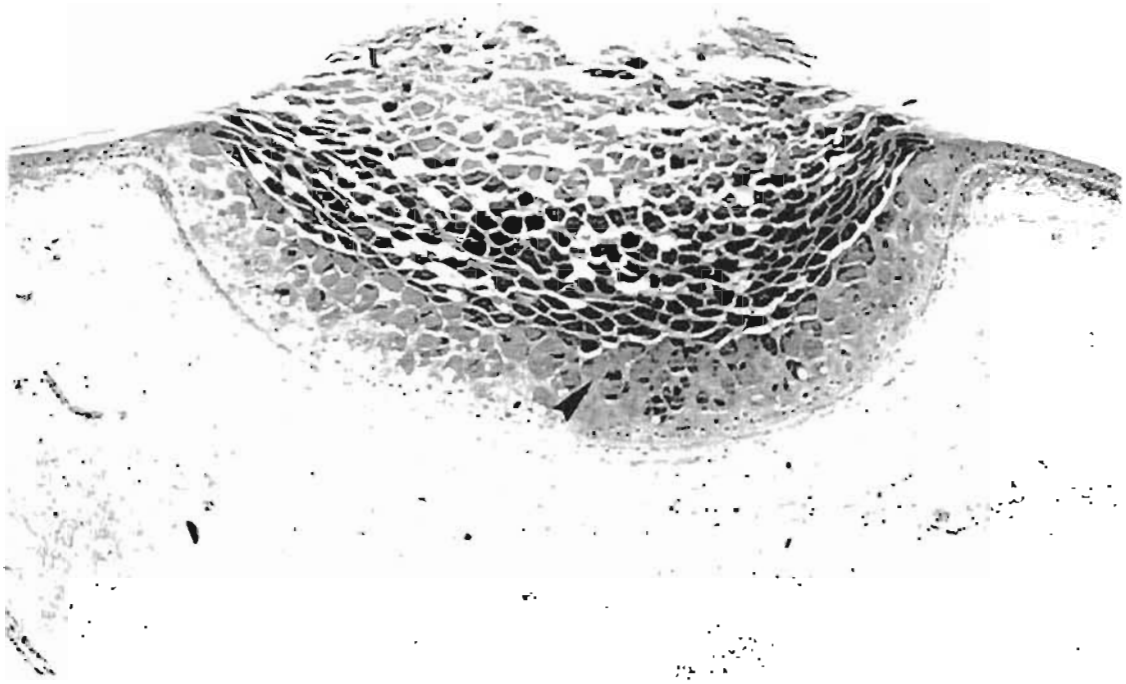

Figure 9.5 Photomicrograph of pox skin lesion showing marked thickening of the epidermis. Note enlarged cells with nucleus displaced to the edge (arrow head). Haematoxylin and eosin ×71.

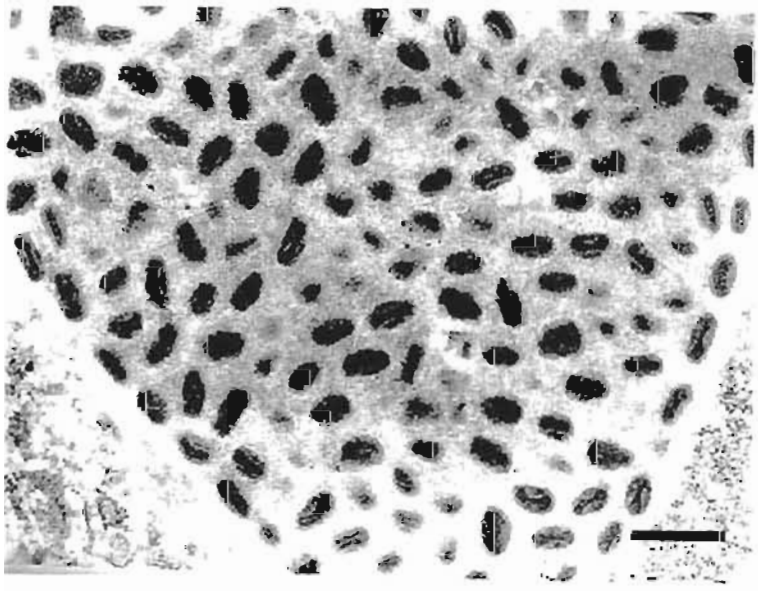

Figure 9.6 Aggregation of oval to brick shape poxvirus particles inside the inclusion body. Bar = 400 nm.

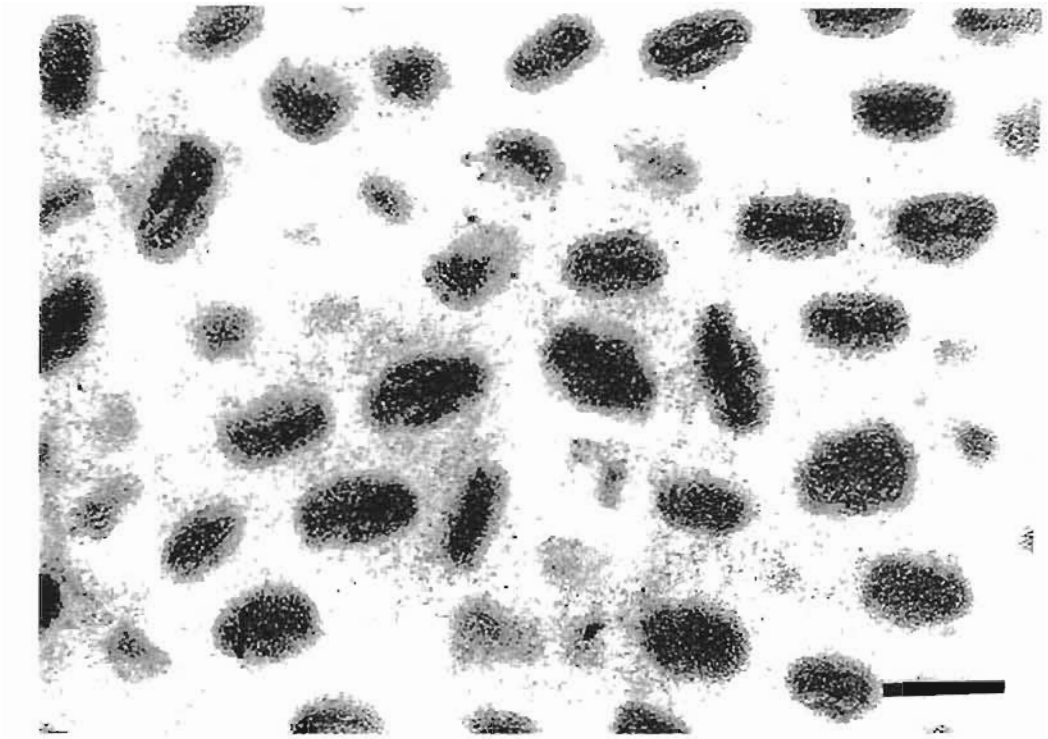

Figure 9.7 Higher magnification than Figure 9.6, showing many 'dumb bell' shaped bodies inside the virions. Bar = 200 nm.

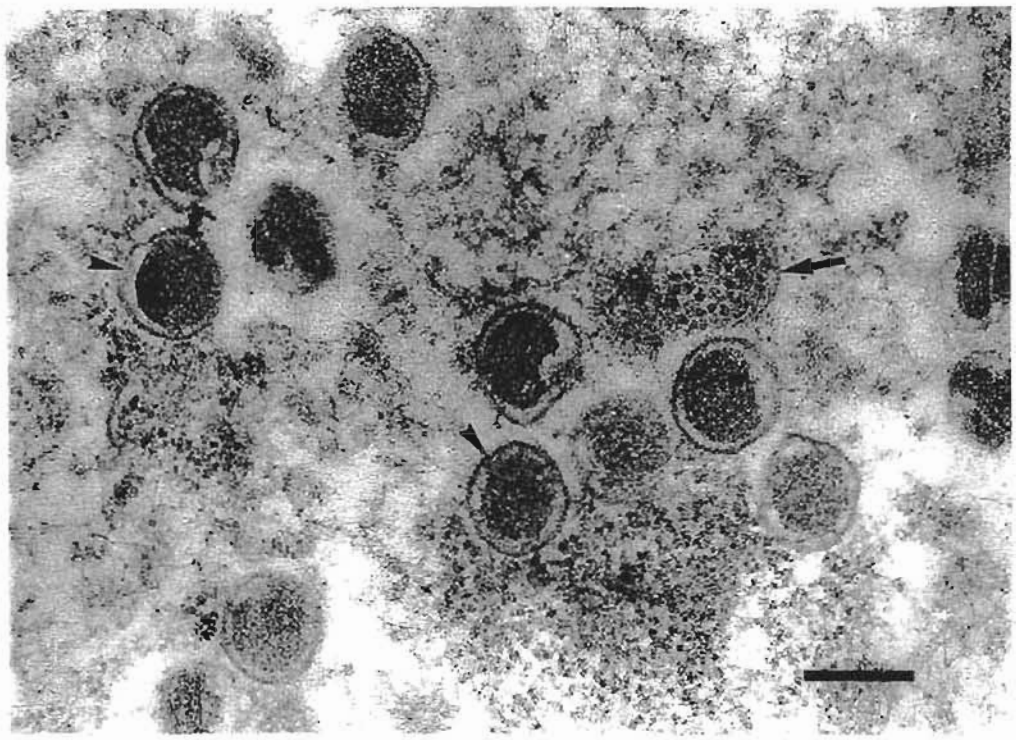

Figure 9.8 Same section as in Figure 9.6. Note many circular bodies containing homogeneous to dense materials inside (arrow heads). Occasional crescent shaped bodies (arrow) had granular materials on the concave side. Bar ≈ 200 nm.

9.3.3 Attempted inoculation of poxvirus in cell culture, chicken chorioallantois, crocodile embryo and *C porosus* hatchlings

Several attempts to grow the poxvirus in *vero* and BHK cell cultures failed to produce any cytopathic effect. Initial growth of fibroblast and epithelial cells from both the primary and secondary cultures of crocodile embryo were satisfactory. However, the cells gradually began to degenerate and died after four passages, hence the inoculation of poxvirus into crocodile cell culture was not carried out.

One to two focal nodular lesions (2 to 5 mm in diameter) were produced on the chicken chorioallantoic membrane of all chicken embryos produced. The lesions were white to opaque colour, and varied from slightly raised to rounded foci. Sometimes two to three small white foci (1 to 2 mm dia.) were present on the chorioallantoic membrane. Histologically, the white to opaque focal lesions contained foci of hyperplastic and hypertrophic cells. A small number of enlarged cells contained empty vacuoles or eosinophilic material that resembled the inclusion body of poxvirus. However, poxvirus particles were not present from several samples of infected chorioallantoic membranes examined with the transmission electron microscope.

In the pilot experiment, no gross lesions characteristic of poxvirus were seen on clinical examination. Instead seven animals developed 'brown spot' lesions on clinical examination at day 10 PI.

9.4 Discussion

The lesions described in this study were different from those skin lesions seen during the survey on skin diseases in crocodiles (Chapter 4). It seems that the gross lesions of skin infected with poxvirus develops in different forms. Poxvirus infected skin complicated with either bacteria or fungi as was earlier reported (Buenviaje *et al.*, 1998a), had brown ulcerative lesions or wart-like growths (Horner, 1988), while uncomplicated poxvirus infections were grey-white circular lesions. The lesions described in this study was considered a mild infection compared with numerous grey-white papular lesions scattered throughout the body of infected caimans (Jacobson *et al.*, 1979). Subtle inflammatory changes present in the dermis beneath the pox lesions in this study were also noted in poxvirus infection in other studies (Penrith *et al.*, 1991).

The appearance of multiple to a single large eosinophilic intracytoplasmic inclusion body containing viral inclusions is similar to reports of poxvirus infection in crocodiles (Buenviaje *et al.*,

1992; Buoro, 1992) and caimans (Jacobson *et al.*, 1979) and are pathognomonic of poxvirus lesions. Many circular and occasional crescent bodies present in the cytoplasm adjacent to the inclusion bodies in this study were immature virions at different stages of development (Jacobson *et al.*, 1979; Moss, 1990). Since the virions contained inside the inclusion bodies have uniform 'dumb bell' shape nucleocapsid it probably indicates that the inclusion bodies in the cytoplasm provide a suitable environment for the maturity of the virus particle.

The source of poxvirus infection in this study could not be determined because the hatchlings were hatched artificially at Queensland Department of Primary Industry Veterinary Laboratories, Townsville from eggs provided by crocodile farms and parks. In addition, the hatchlings were kept in separate enclosed pens and not in contact with other crocodiles. Therefore the disease may have been transmitted by mosquitoes similar to outbreaks of poxvirus infection in poultry (Tripathy and Cunningham, 1984). Although poxvirus infection of crocodiles in Australia did not affect a large number of animals (Buenviaje *et al.*, 1992; 1998a) compared with Zambia (Pandey *et al.*, 1990), the high morbidity rate (68.8%) in this study suggests a possibility of future outbreaks in farmed crocodiles.

The failure to grow poxvirus in tissue culture, chicken chorioallantoic membranes and embryonated crocodile eggs could have been due to inability of crocodile poxvirus to survive over a prolonged period of storage at –80°C. Although there was hypertrophy and hyperplasia associated with some 'inclusion-like' bodies from a small number of cells in the chorioallantoic membranes, the absence of poxvirus particles from such lesions suggest that attempted poxvirus inoculation was unsuccessful. Attempts by other workers to grow crocodile poxvirus in both avian and reptilian cells including chicken chorioallantoic membranes, were unsuccessful (Jacobson *et al.*, 1979; Horner 1988), although in the paper by Huchzermeyer *et al.* (1991) there is a reference to a personal communication from Zimbabwe where poxvirus was cultured in crocodile embryo cells. Attempts to reculture the cells from a crocodile embryo was not undertaken because of time constraints and the unavailability of crocodile eggs.

CHAPTER 10

GENERAL DISCUSSION

In crocodile farming, the major focus is the production of high quality skins to supply the expanding demand for high quality leather. In recent years, there has been an increasing demand for quality crocodile skin currently in short supply, presumably because of the occurrence of diseases affecting the skin. As a consequence, it is critical that any diseases of the skin should be controlled and damage should be kept to the minimum. Although considerable research work on crocodiles has been published, the topics have been mainly limited to the behaviour and population demography and enforcement guidelines for the crocodile industry. There has been a limited published information on the aetiology, pathogenesis, treatment and control of crocodile diseases particularly affecting the skin.

To date, no detailed, systematic scientific studies have been conducted on skin diseases in crocodilians. The lack of information on skin diseases has precluded the development of strategies that will minimise, if not arrest, the spread of infection. This thesis represents an initial attempt to record the diseases affecting the skin of farmed crocodiles in Australia. The diseases were identified through an epidemiological survey in which samples from either dead or sick crocodiles collected mostly from farms in Queensland and the Northern Territory were examined macroscopically and microscopically. In addition, a retrospective study of all microslides of crocodile skin lesions was undertaken and each lesion was classified according to suspected or known aetiological agent.

The results extrapolated from the survey of skin diseases of farmed crocodiles in this study serve as benchmark information, which are important in many ways for improved husbandry management. In 1992, superficial fungal infection was the most prevalent skin disease in farmed crocodiles especially during winter months (May to August) (Buenviaje *et al.*, 1994). Superficial fungal infection in this study was not considered a serious problem under systems of improved husbandry management, especially if the water temperature was maintained at 32°C.

Although four major diseases of skin in farmed crocodiles were identified in the epidemiological survey, dermatophilosis or 'brown spot' disease was the most frequently diagnosed. It was considered the most important disease from an economic point of view, being present on six of nine crocodile farms in Australia. The expeditious spread of 'brown spot' disease to the non-inoculated control group in transmission studies strongly suggests that the disease is highly infectious (see Chapter 6). 'Brown spot' disease can develop rapidly to cause damage even to

healthy animals, probably because the aquatic environment facilitates the spread of infection. Direct contact or mechanical transfer of *Dermatophilus* by insects was confirmed in the experimental transmission study of *D congolensis* using both *Stomoxys calcitrans* and *Musca domestica* in rabbits (Richard and Pier, 1966). However, the transmission studies of dermatophilosis reported here revealed that scarification of the skin was not necessary to effectively produce 'brown spot' lesions. Therefore it was presumed that this highly pathogenic organism required no predisposing factors such as stress or minor scratches on the skin to enable it to establish infection.

Based on the experimental transmission studies, the organism was capable of causing 'brown spot' lesions in a short period (three days after inoculation). The 'brown spot' lesions pitted the scales to various depths especially in the abdomen, in a manner similar to 'brown spot' in alligators (Newton, 1992). It has been reported that *D congolensis* produced significant amounts of keratinase during a 12-day incubation period, and was considered to cause extracellular proteolytic activity responsible for keratinised tissues being the initial target of infection (Hanel, Kalisch, Keil, Marsch and Buslau, 1991). It is presumed that the *Dermatophilus* sp used to inoculate the crocodiles in the transmission studies (Chapter 6) also produced a keratolytic enzyme similar to *D congolensis* (Hermoso *et al.*, 1993). Of particular interest in cases involving dual or multiple skin infections in farmed crocodiles, *Dermatophilus* sp was the most prevalent microorganism which clearly suggests its role as the primary pathogen inducing initial damage to the superficial epidermis. Skin is the first line of defence for the body and once damaged it becomes the portal of entry of other opportunistic pathogens such as fungi, bacteria and viruses, which in turn may invade the underlying tissues causing severe damage.

A Gram-positive filamentous organism isolated from a spontaneous outbreak of 'brown spot' disease in farmed crocodiles was used as the challenge isolate in transmission studies. Both the isolate used for inoculation and the recovered isolates were identical based upon standard microbiological procedures and 16S rDNA sequence analysis, thus confirming its role in causing 'brown spot' disease.

Although the initial microbiological examinations revealed a close resemblance of the crocodile isolate to *Dermatophilus congolensis* type strain (ATCC 14637), there were a number of morphological, cultural and biochemical characteristics that differentiated the crocodile isolate from *D congolensis*. Comparison between *Dermatophilus* sp from *D chelonae* (Masters *et al.*, 1995), the aetiological agent of dermatophilosis in turtles, also revealed substantial differences in morphological, cultural and biochemical characteristics. The transverse and longitudinal

septation considered typical of *D congolensis* in domestic animals (Roberts, 1981) and lizards (Montali *et al*., 1975) was not a feature of the *Dermatophilus* sp isolated from crocodiles. The rate of growth and the colonial morphology of the crocodile isolates differed significantly from *D chelonae* and *D congolensis*. The latter grew faster, and the colonies were more rugose and crateriform and golden yellow colour whereas the former grew more slowly and the colonies were white umbonate with a smooth surface. Besides, the *Dermatophilus* sp isolated from crocodiles was nutritionally more fastidious than *D congolensis* with a requirement for blood to grow on Mueller-Hinton agar.

A 16 S rDNA analysis clearly discriminated the *Dermatophilus* sp crocodile isolate from the *D congolensis* type strain. The differences in the number of nucleotide bases between the *Dermatophilus* sp isolated from crocodiles and the *D congolensis* type strain strongly enforces the conclusion that the crocodile isolates are distinct from *D congolensis* both genotypically and phenotypically. Although there was greater homology of 16S rDNA sequence between *Dermatophilus* sp and *D chelonae*, the phenotypic differences were sufficient to conclude that the crocodile *Dermatophilus* sp is different from *D chelonae*. Overall, both the phenotypic and genotypic differences were sufficient to suggest that the crocodile isolate is a distinct species of *Dermatophilus*, thus a new species of *Dermatophilus* – *Dermatophilus crocodyli* sp nov is proposed.

In the control and treatment of 'brown spot' disease, copper sulphate was found to be the most effective antibacterial agent based upon both *in vitro* and *in vivo* studies. Copper sulphate prevented the further development of 'brown spot' to more severe lesions in crocodiles experimentally infected with *Dermatophilus* sp. It has also been shown that copper sulphate was effective against superficial fungal infection and abscesses. Following copper sulphate treatment, all animals recovered and importantly without evidence of scarring of the affected skin. Although initially there were recurrences of 'brown spot' lesions and the emergence of new cases in non-infected crocodiles under treatment, the protocol for administering the copper sulphate through the water strongly suggests that the animals did not receive sufficient exposure to medication. As such, animals recovering from a sub-therapeutic dose could be the source of recurrent and prolonged infections. Furthermore, a water temperature below 32°C overnight probably contributed to the recurrence of infection (Buenviaje *et al*., 1994) after the immersion heaters failed.

It was concluded that the concentration of 1 ppm copper sulphate can only be maintained if 'dipping bath' pens are provided. In a management system employing flowing water, treatment

was given twice to help maintain therapeutic levels of copper sulphate. There was a response to the medication, only when infected animals were immersed in copper sulphate-treated water for at least 15 minutes twice a day. Based on the four copper sulphate treatment protocols evaluated, the forced immersion of crocodiles in 1 ppm of copper sulphate was the most effective method for the treatment of skin lesions. However, the handling of animals should be minimised to avoid unnecessary stress.

Although it appears that flowing water did restore the normal appetite of infected animals earlier than those kept in static water, there was no effect on the control of 'brown spot' lesions. The practice of providing flowing water in most crocodile farms in hatchling and grower pens probably should be re-evaluated to consider the cost and the effectiveness in preventing 'brown spot' disease. As flowing water did not prevent or reduce the occurrence of 'brown spot' disease it is probably more practical and cost effective to clean and replenish with clean water daily.

Poxvirus infection is another major concern in crocodile farms in Australia. In the epidemiological study on skin diseases of farmed crocodiles, the incidence of poxvirus infection is lower and presently not as important as in South Africa (Pandey *et al.*, 1990; Gitao and Mwendia, 1999). However, the diagnosis of poxvirus infection in 11 of 16 (68.8%) hatchlings and the severity of lesions in a separate transmission study on dermatophilosis is a cause for concern. The possibility of future outbreaks in farmed crocodiles must be considered. The source of infection could not be established because the infected hatchlings were hatched from eggs collected from the wild, and were kept in a separate enclosed pen not in contact with other crocodiles. The role of mosquitoes or other insect vectors implicated in outbreaks of poxvirus infection in poultry (Tripathy and Cunningham, 1984) needs to be investigated.

In conclusion, the work undertaken and presented in this thesis has contributed substantially to our understanding of a wide range of skin diseases in farmed crocodiles in Australia. The objectives of this project as defined at the commencement of the study were achieved. The pathology of the skin diseases particularly 'brown spot' disease was described both macroscopically and microscopically. 'Brown spot' disease was identified as the most prevalent and probably the most important disease in farmed crocodiles in Australia. The aetiological agent of 'brown spot' disease was identified, and a new species of *Dermatophilus* was proposed. Copper sulphate at 1 ppm concentration was proven to be effective against 'brown spot' disease, however it is highly recommended that the crocodile farms should provide 'dipping bath' pens for treatment of sick animals.

The outcome of the research reported in this thesis has provided the basis for a number of future studies on the skin diseases of crocodile. Research areas that require consideration include:

- Further evaluation on the application of copper sulphate in water in different management system as prophylaxis against diseases affecting the skin with particular emphasis on 'brown spot' disease.

- Further DNA studies utilising DNA-DNA hybridization techniques and infectivity studies of crocodile isolate TVS 96-490-9B, which is phenotypically similar to crocodile isolates TVS 96-490-7B and TVS 367-15.

- Poxvirus infection in crocodiles needs to be re-investigated. This includes the isolation and propagation of the virus, and detailed characterisation of the virus, conduct experimental transmission studies and determine the pathogenesis of the disease.

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APPENDIX

TABLES 1.1 — 1.8

APPENDIX TABLE 1

MICROBIOLOGICAL CHARACTERISTICS OF DERMATOPHILUS SPECIES

Table 1.1 Cultural characteristics of crocodile isolates on blood agar and Mueller-Hinton agar.

Media	TVS 96-4907B (FI 1) TVS 96-490-9B (Fi 2) TVS 96-367-15A (Fi 3)	96-366-5A (Fi 4)	96-700-1C (FI 5)	97-124-WS (water sample)
1) Blood agar at 37°C, 5% CO_2				
After 24 hours	No growth	The colonies were 1mm in diameter, grey colour and pitting into the medium.	The colonies were 1mm in diameter, grey and pitting into the medium.	The colonies were 1mm in diameter, grey and pitting into the medium.
After 48 hours	Very tiny and barely visible colonies.	The colonies were 1 to 3 mm, dark grey colour and umbonate which were divided into four lobes.	The colonies were bigger (1 to 2 mm in diameter) with few white aerial hyphae.	The colonies were 1 to 2 mm and yellow colour.
After 72 hours	The colonies were 1 to 3 mm in diameter, haemolytic and pitting into the medium. The colonies were pale yellow colour, slightly rugose and sometimes crateriform.	The colonies were almost covered with aerial hyphae.	The colonies were grey to yellow colour, rugose, crateriform and sometimes haemolytic.	The colonies were rugose.
After five days	The colonies were bigger (2 to 5mm diameter), tough and leathery.	Occasional small yellow colonies were budding off from the original colonies. The size of the colonies were 4 to 6 mm in diameter	All colonies (up to 4 mm in diameter) were yellow colour and more aerial hyphae	All colonies were 2 to 3 mm in diameter, excessively folded or rugose and sometimes haemolytic.
2) Blood agar at 28°C				
After 48 hours	Very tiny grey to pale yellow colonies	The colonies (0.5 to 1 mm in diameter) were light grey colour and pitting into the medium.	The colonies were 0.5 to 1 mm in diameter, light grey colour and pitting into the medium.	Not tested
After five days	The colonies were pale yellow colour, very sticky and caseous. A zone of haemolysis was also present	The colonies were grey to yellow colour and crateriform. White aerial hyphae were present	The colonies were grey to yellow colour and sometimes crateriform.	

Table 1.1 (cont'd)

Media	TVS 96-4907B (Fi 1) TVS 96-490-9B (Fi 2) TVS 96-367-15A (Fi 3)	96-366-5A (Fi 4)	96-700-1C (Fi 5)	97-124-WS (water sample)
3) **Mueller-Hinton agar with 9% sheep blood**	After 48 hours incubation, the colonies were 1 to 2 mm in diameter. The colonies were pale yellow, pitting into the medium, haemolytic and crateriform	Not tested.	Not tested.	Not tested.
4) **Mueller-Hinton agar**	No growth	Not tested.	Not tested.	Not tested.

Table 1.2 Cultural characteristics of *Dermatophilus congolensus* and *Dermatophilus chelonae* on blood agar and Mueller-Hinton agar.

Media	QVLDPI 22204 (DCC 1)	ACM 530 (DCD) ACM 531 (DCS 1) ACM 532 (DCS 2)	*Dermatophilus congolensis* type strain (ATCC 14637)	*Dermatophilus chelonae* (DCH 1 and DCH 2)
1) Blood agar at 37°C, 5% CO_2				
After 24 hours	No growth	The colonies were very small, grey colour and pitting into the medium.	The colonies were 1 mm in diameter, grey and pitting into the medium.	No growth.
After 48 hours	Very tiny colonies (< 0.5 mm in diameter).	The colonies were 1 to 2 mm, grey to yellow colour and sometimes haemolytic.	The colonies were bigger (1 to 2 mm in diameter) and deeply pitted into the medium.	No growth
After 72 hours	The colonies were 1 to 2 mm in diameter, yellow colour and crateriform.	The colonies were 2 to 4 mm, crateriform and sometimes with aerial hyphae.	The colonies were golden yellow colour, more rugose and crateriform and haemolytic.	Very tiny and grey colonies.
After five days	The colonies were 3 mm in diameter, crateriform but less rugose. Sometimes the colonies were haemolytic.	The colonies were pale to golden yellow colour.	All colonies were up to 4 mm in diameter, soft and firable.	Very tiny and grey colonies.
2) Blood agar at 28°C				
After 48 hours	The colonies were 0.5 to 1 mm in diameter, yellow colour and pitting into the medium.	The colonies were 0.5 to 1 mm, light grey colour, pitting into the medium and haemolytic.	No growth after 48 hours incubation.	No visible growth.
After five days	The colonies were 0.5 to 2 mm in diameter, yellow colour, mucoid and haemolytic.	The colonies were golden yellow colour, a few crateriform and occasional aerial hyphae.	The colonies were pale yellow, haemolytic and mucoid.	The colonies were very tiny. After 10 days incubation, the colonies were 1 to 2 mm in diameter and haemolytic. A slightly central depressed area was present in the colonies. The colonies were sticky and strongly attached to the medium.

Table 1.2 (cont'd)

Media	QVLDPI 22204 (DCC 1)	ACM 530 (DCD) ACM 531 (DCS 1) ACM 532 (DCS 2)	*Dermatophilus congolensis* type strain (ATCC 14637)	*Dermatophilus chelonae* (DCH 1 and DCH 2)
3) Mueller-Hinton agar with 9% sheep blood	Not tested.	Not tested.	After 4 8 hours incubation, the colonies were 1 to 2 mm in diameter and pitting into the medium.	After five days incubation, the colonies were up to 1 mm in diameter. The colonies were yellow colour, pitting into the medium and haemolytic.
4) Mueller-Hinton agar	Not tested.	Not tested.	The colonies were 1 to 2 mm in diameter, yellow colour and pitting into the medium.	No growth.

Table 1.3 Growth characteristics of *Dermatophilus* species isolates in broth after four days incubation.

Isolates	10% Bovine serum in tryptose phosphate broth	Brain heat infusion broth
A) Crocodile isolates		
1) TVS 96-490-7B (Fi 1) TVS 96-490-9B (Fi 2) TVS 97-367-15A (Fi 3)	The broth medium was clear. The granular flocculents were present at the bottom of the tubes.	The broth medium was clar. The granular flocculents were present at the bottom of the tubes.
2) TVS 96-366-5A (Fi 4)	The broth medium was clear. The white granular flocculents were present at the bottom of the tubes.	The broth medium was clar. The granular flocculents were present at the bottom of the tubes.
3) TVS 96-700-1C (Fi 5)	The granular flocculents were present at the bottom of the tubes and some attached to the side of the tube near the surface.	The granular flocculents were present at the bottom of the tubes and some attached to the side of the tube near the surface.
4) TVS 97-124-WS (water sample)	The granular flocculents were present at the bottom of the tubes and some attached to the side of the tube near the surface.	The granular flocculents were present at the bottom of the tubes and some attached to the side of the tube near the surface.
B) *Dermatophilus congolensis*		
1) QVLDPI 22204 (DCC 1)	The broth medium was clear. The granular flocculents were present at the bottom of the tube and some attached to the side of the tubes.	The broth medium was clear. The granular flocculents were present at the bottom of the tube and some attached to the side of the tubes.
2) ACM 530 (DCD); ACM 531 (DCS 1); ACM 532 (DCS 2)	The broth medium was clear. The granular flocculents were present at the bottom of the tubes.	The broth medium was clear. The granular flocculents were present at the bottom of the tubes.
3) *D congolensis* type strain (ATCC 14367)	The medium was clear. The granular flocculents were present at the bottom of the tube with some forming a ring near the surface of the tube. A membrane flocculent was also present on the surface.	The medium was clear. The granular flocculents were present at the bottom of the tube and some attached to the side of the tubes.
C) *Dermatophilus chelonae* (DCH 1 and DCH 2)	No growth at 37°C. After four days incubation at 28°C, there were granular flocculents present at the bottom of the tubes.	No growth at 37°C. After four days incubation at 28°C , there were granular flocculents present at the bottom of the tubes.

Table 1.4 Morphological characteristics of *Dermatophilus* species isolates in broth after four days incubation.

		Direst smear after 48 hours of incubation				
Isolates	**Hyphae in infected tissue**	**Zoospores**	**Hyphae**	**Kinyoun modified Ziehl-Neelsen**	**Division of hyphae**	**Number of zoospores after five days incubation**
A) Crocodile isolates						
1) TVS 96-490-7B (Fi 1) TVS 96-490-9B (Fi 2) TVS 97-367-15A (Fi 3)	0.8 to 1 µm in diameter	1 µm in diameter	0.8 to 1.2 µm in diameter	Non-acid fast	Transverse	Few zoospores
2) TVS 96-366-5A (Fi 4)	Not applicable	1 µm in diameter	0.8 to 1.2 µm in diameter	Non-acid fast	Transverse	Few zoospores
3) TVS 96-700-1C (Fi 5)	Not applicable	1 µm in diameter	0.8 to 1.2 µm in diameter	Non-acid fast	Transverse	Few zoospores
4) TVS 97-124-WS (water sample)	Not applicable	1 µm in diameter	0.8 to 1.2 µm in diameter	Non-acid fast	Transverse	Few zoospores
B) *Dermatophilus congolensis*						
1) QVLDPI 22204 (DCC 1)	Not applicable	1 µm in diameter	0.5 to 1.5 µm in diameter	Non-acid fast	Transverse and longitudinal	Many zoospores
2) ACM 530 (DCD); ACM 531 (DCS 1); ACM 532 (DCS 2)	Not applicable	1 µm in diameter	0.5 to 1.5 µm in diameter	Non-acid fast	Transverse and longitudinal	Many zoospores
3) *D congolensis* type strain (ATCC 14367)	Not applicable	1 µm in diameter	0.5 to 1.5 µm in diameter	Non-acid fast	Transverse and longitudinal	Many zoospores
C) *Dermatophilus chelonae* (DCH 1 and DCH 2)	Not applicable	1 µm in diameter	0.5 to 1.5 µm in diameter	Non-acid fast	Transverse and longitudinal	Many zoospores

Table 1.5 Biochemical characteristics of *Dermatophilus* species isolates.

Isolates	Catalase	Oxidase	Indole	Urease	Nitrate reduction	Methyl red	Voges-Proskauer	Oxidative/ fermentative
A) Crocodile isolates								
1) TVS 96-490-78 (Fi 1) TVS 96-490-98 (Fi 2) TVS 97-367-15A (Fi 3)	Positive	Negative	Variable	Positive	Variable	Negative	Negative	Positive
2) TVS 96-366-5A (Fi 4)	Positive	Negative	Negative	Positive	Variable	Negative	Negative	Positive
3) TVS 96-700-1C (Fi 5)	Positive	Negative	Negative	Positive	Variable	Negative	Negative	Positive
4) TVS 97-124-WS (water sample)	Positive	Negauve	Negative	Positive	Variable	Negative	Negative	Variable
B) *Dermatophilus congolensis*								
1) QVLDPI 22204 (DCC 1)	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative
2) ACM 530 (DCD); ACM 531 (DCS 1); ACM 532 (DCS 2)	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative
3) *D congolensis* type strain (ATCC 14367)	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Positive
C) *Dermatophilus chelonae* (DCH 1 and DCH 2)	Positive	Negative	Variable	Negative	Variable	Negative	Negative	Negative

Table 1.6 The results of hydrolysis on *Dermatophilus* species isolates.

Isolates	Casein	Xanthine	Tyrosine	Loeffler's serum
A) **Crocodile isolates**				
1) TVS 96-490-7B (Fi 1) TVS 96-490-9B (Fi 2) TVS 97-367-15A (Fi 3)	Variable	Negative	Negative	Positive
2) TVS 96-366-5A (Fi 4)	Negative	Positive	Negative	Negative
3) TVS 96-700-1C (Fi 5)	Negative	Positive	Negative	Positive
4) TVS 97-124-WS (water sample)	Negative	Positive	Negative	Negative
B) ***Dermatophilus congolensis***				
1) QVLDPI 22204 (DCC 1)	Variable	Negative	Negative	Negative
2) ACM 530 (DCD); ACM 531 (DCS 1); ACM 532 (DCS 2)	Variable	Negative	Variable	Negative
3) *D congolensis* type strain (ATCC 14367)	Positive	Negative	Negative	Positive
C) ***Dermatophilus chelonae* (DCH 1 and DCH 2)**	Positive	Negative	Variable	Negative

Table 1.7 Results of the carbohydrate tests on *Dermatophilus* isolates.

Isolates	Dulcitol	Fructose	Glucose	Lactose	Mannitol	Salicin	Sorbitol	Sucrose	Xylose
A) Crocodile isolates									
1) TVS 96-490-7B (FI 1) TVS 96-490-9B (FI 2) TVS 97-367-15A (FI 3)	Negative	Variable	Positive	Negative	Negative	Negative	Negative	Variable	Negative
2) TVS 96-366-5A (Fi 4)	Negative	Variable	Positive	Negative	Negative	Negative	Negative	Variable	Positive
3) TVS 96-700-1C (Fi 5)	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
4) TVS 97-124-WS (water sample)	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
B) *Dermatophilus congolensis*									
1) QVLDPI 22204 (DCC 1)	Negative	Variable	Negative	Negative	Variable	Negative	Negative	Variable	Negative
2) ACM 530 (DCD); ACM 531 (DCS 1); ACM 532 (DCS 2)	Negative	Variable	Variable	Negative	Negative	Negative	Negative	Variable	Negative
3) *D congolensis* type strain (ATCC 14367)	Negative	Variable	Positive	Negative	Negative	Negative	Negative	Variable	Positive
C) *Dermatophilus chelonae* (DCH 1 and DCH 2)	Negative	Negative	Negative	Variable	Negative	Negative	Negative	Variable	Negative

Table 1.8 The results of the antibiotic sensitivity tests on *Dermatophilus* isolates.

Isolates	Ampicillin (10 µg)	Cefotaxime (30 µg)	Ciprofloxacine (5 µg)	Cloxacillin (5 µg)	Erythromycin (15 µg)	Penicillin (10 units)	Streptomycin (10 µg)	Tetracyline (30 µg)
A) **Crocodile isolates**								
1) TVS 96-490-78 (Fi 1) TVS 96-490-9B (Fi 2) TVS 97-367-15A (Fi 3)	Susceptible (34 to 28 mm)	Susceptible (32 to 28 mm)	Susceptible (28 to 35 mm)	Susceptible (30 to 35 mm)	Susceptible (32 to 38 mm)	Susceptible (32 to 35 mm)	Resistant (No zone of inhibition)	Susceptible (32 to 38 mm)
2) TVS 96-366-5A (Fi 4)	Susceptible (35 to 38 mm)	Resistant (No zone of inhibition)	Susceptible (26 mm)	Resistant (No zone of inhibition)	Intermediate (14 to 18 mm)	Resistant (12 mm)	Susceptible (28 to 30 mm)	Susceptible (35 to 38 mm)
3) TVS 96-700-1C (Fi 5)	Susceptible (14 to 17 mm)	Resistant (No zone of inhibition)	Susceptible (24 to 31 mm)	Resistant (No zone of inhibition)	Intermediate (20 to 21 mm)	Resistant (14 mm)	Resistant (14 mm)	Resistant (10 mm)
4) TVS 97-124-WS (water sample)	Susceptible (16 to 18 mm	Resistant (No zone of inhibition)	Susceptible (21 to 28 mm)	Resistant (No zone of inhibition)	Resistant (10 mm)	Resistant (13 to 18 mm)	Susceptible (35 to 36 mm)	Intermediate (14 to 16 mm)
B) ***Dermatophilus congolensis***								
1) QVLDPI 22204 (DCC 1)	Resistant (6 to 8 mm)	Resistant (No zone of inhibition)	Intermediate (15 to 16 mm)	Resistant (No zone of inhibition)	Resistant (8 mm)	Resistant (No zone of inhibition)	Susceptible (34 to 36 mm)	Susceptible (30 to 36 mm)
2) *D congolensis* type strain (ATCC 14367)	Susceptible (32 to 38 mm)	Susceptible (36 to 38 mm)	Susceptible (18 to 25 mm)	Susceptible (34 to 35 mm)	Susceptible (34 mm)	Susceptible (35 to 36 mm)	Susceptible (16 to 25 mm)	Susceptible (34 m)
C) ***Dermatophilus chelonae*** (DCH 2)	Susceptible (34 to 26 mm)	Susceptible (No zone of inhibition)	Susceptible (32 to 34 mm)	Susceptible (34 mm)	Susceptible (34 to 36 mm)	Susceptible (32 to 35 mm)	Resistant (No zone of inhibition)	Susceptible (32 to 35 mm)

Appendix

Table 2.1 — 2.17

16S rDNA Sequences

Table 2.1 *Dermatophilus* sp (TVS 96-490-9B)

ACGGGTGAGT	AACACGTGAG	TAACCTGCCC	TTCACTCTGG	GATAACCACG
GGAAACCGGG	GCTAATACTG	GATATGACAC	ACTGGCGCAT	GATGGTGTGT
GGAAAGATTT	ATTGGTGGAG	GATGGACTCG	CGGCCTATCA	GCTTGTTGGT
GAGGTAATGG	CTCACCAAGG	CGACGACGGG	TAGCCGGCCT	GAGAGGGTGA
CCGGCCACAC	TGGGACTGAG	ACACGGNCCA	GACTCCTGCG	GGAGGCAGCA
GTGGGGAATA	TTGCACAATG	GGCGAAAGCC	TGATGCAGCG	ACGCCGCGTG
AGGGATGAAG	GCCTTCGGGT	TGTAAACCTC	TTTCNCCAGG	GGAAGAAGCG
AAAGTGACGG	TACCTGGAGA	AGAAGCACCG	GCTAACTACG	TGCCAGCAGT
CGCTGTAATA	CATAGGGTGC	GAGCGTTATC	CGGAATTATT	GGGCGTAAAG
CGCGCGTAGG	CGGTTTKTTA	AGTCTGATGT	GAAAGCCCAC	GGCTCAACCG
TGGAGGGTCA	TTGGAAACTG	GAAAACTTGA	GTGCAGAAGA	GGAAAGTGGA
ATTCCATGTG	TAGCGGTGAA	ATGCGCAGAG	ATATGGAGGA	ACACCAGTGG
CGAAGGCGAC	TTTCCTGGTC	CTGTAACTGA	CGCTGATGTG	CGAAAGCGTG
GGGATCAAAC	AGGATTAGAT	ACCCTGGTAG	TCCACGCCGT	AAACGATGAG
TGCTAAGTGT	TAGGGGGTTT	CCGCCCCTTA	GTGCTGCAGC	TAACGCATTA
AGCACTCCGC	CTGGGGAGTA	CGACCGCAAG	GTTGAAACTC	AAAGGAATTG
ACGGGGACCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GAAGCAACGC
GAAGAACCTT	ACCAAATCTT	GACATCCTCT	GACCCCTCTA	GAGATAGAGT
TTTCCCCTTC	GGGGGACAGA	GTGACAGGTG	GTGCATGGTT	GTCGTCAGCT
CGTGTCGWGA	GATGT			

Table 2.2 *Dermatophilus* sp (TVS 96-367-15A)

GGTGGATTAG	TGGCGAACGG	GTGAGTAACA	CGTGAGTAAC	CTGCCCTTCA
CTCTGGGATA	ACCACGGGAA	ACCGGGGCTA	ATACTGGATA	TGACACACTG
GCGCATGATG	GTGTGTGGAA	AGATTTATTG	GTGGAGGATG	GACTCGCGGC
CTATCAGCTT	GTTGGTGAGG	TAATGGCTCA	CCAAGGCGAC	GACGGGTAGC
CGGCCTGAGA	GGGTGACCGG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT
CCTGCGGGAG	GCAGCAGTGG	GGAATATTGC	ACAATGGGCG	AAAGCCTGAT
GCAGCGACGC	CGCGTGAGGG	ATGAAGGCCT	TCGGGTTGTA	AACCTCTTTC
GCCAGGGAAG	AAGCGAAAGT	GACGGTACCT	GGAGAAGAAG	CACCGGCTAA
CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG	TTGTCCGGAA
TTATTGGGCG	TAAAGAGCTT	GTAGGCGGTT	TGTCGCGTCT	GCCGTGAAAA
TCCAAGGCTT	AACTTTGGAC	GTGCGGTGGG	TACGGGCAGG	CTAGAGTGTG
GTAGGGGAGA	CTGGAATTCC	TGGTGTAGCG	GTGAAATGCG	CAGATATCAG
GAGGAACACC	GATGGCGAAG	GCAGGTCTCT	GGGCCATAAC	TGACGCTGAG
AAGCGAAAGC	ATGGGGAGCG	AACAGGATTA	GATACCCTGG	TAGTCCATGC
CGTAAACGTT	GGGCGCTAGG	TGTGGGGCTC	ATTCCACGAG	CTCTGTGCCG
CAGCTAACGC	ATTAAGCGCC	CCGCCTGGGG	AGTACGGCCG	CAAGGCTAAA
ACTCAAAGGA	ATTGACGGGG	GCCCGCACAA	GCGGCGGAGC	ATGCGGATTA
ATTCGATGCA	ACGCGAAGAA	CCTTACCAAG	GCTTGACATA	CACCGGAAAA
GTGCAGAGAT	GTGCTCCCCG	TAAGGTCGGT	GTACAGGTGG	TGCATGGTTG
TCGTCAGCTC	GTGTC			

Table 2.3 *Dermatophilus* sp (TVS 97-124-WS)

TTCGATCCTC	CATCGCSGTG	GATTAGTGGC	GAACGGGTGA	GTAACACGTG
GGCAATCTGC	CCTTCACTCT	GGGACAAGCC	TTGGAAACGA	GGTCTAATAC
CGGATAACAC	TCTCCTCCTC	CTGGGGGTGG	GTTAAAAGCT	CCGGCGGTGA
AGGATGAGCC	CGCGGCCTAT	CAGCTTGTTG	GTGAGGTAAT	GGCTCACCAA
GGCGACGACG	GGTAGCCGGC	CTGAGAGGGC	GACCGGCCAC	ACTGGGACTG
AGACACGGCC	CAGACTCCTA	CGGGAGGCAG	CAGTGGGGAA	TATTGCACAA
TGGGCGAAAG	CCTGATGCAG	CGACGCCGCG	TGAGGGATGA	CGGCCTTCGG
GTTGTAAACC	TCTTTCAGCA	GGGAAGAAGC	GAAAGTGACG	GTACCTGCAG
AAGAAGCGCC	GGCTAACTAC	GTGCCAGCAG	CCGCGGTAAT	ACGTAGGGCG
CAAGCGTTGT	CCGGAATTAT	TGGGCGTAAA	GAGCTCGTAG	GCGGCTTGTC
ACGTCGATTG	TGAAAGCTCG	GGGCTTAACC	CCGAGTCTGC	AGTCGATACG
GGCTAGCTAG	AGTGTGGTAG	GGGAGATCGG	AATTCCTGGT	GTAGCGGTGA
AATGCGCAGA	TATCAGGAGG	AACACCGGTG	GCGAAGGCGG	ATCTCTGGGC
CATTACTGAC	GCTGAGGAGC	GAAAGCGTGG	GGAGCGAACA	GGATTAGATA
CCCTGGTAGT	CCACGCCGTA	AACGGTGGGC	ACTAGGTGTG	GGCAACATTC
CACGTTGTCC	GCGCCGCAGC	TAACGCATTA	AGTGCCCCGC	CTGGGGAGTA
CGGCCGCAAG	GCTAAAACTC	AAAGGAATTG	ACGGGGGCCC	GCACAAGCAG
CGGAGCATGT	GGCTTAATTC	GACGCAACGC	GAAGAACCTT	ACCAAGGCTT
GACATACACC	GGAAACGTCC	AGAGATAGGC	GCCCCCTTGT	GGTCGGTGTA
CAGGTGGTGC	ATGGC			

Table 2.4 *Dermatophilus* sp (TVS 97-124)

GGTGGATTAG	TGGCGAACGG	GTGAGTAACA	CGTGAGTAAC	CTGCCCTTCA
CTCTGGGATA	ACCACGGGAA	ACCGGGGCTA	ATACTGGATA	TGACACACTG
GCGCATGATG	GTGTGTGGAA	AGATTTATTG	GTGGAGGATG	GACTCGCGGC
CTATCAGCTT	GTTGGTGAGG	TAATGGCTCA	CCAAGGCGAC	GACGGGTAGC
CGGCCTGAGA	GGGTGACCGG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT
CCTGCGGGAG	GCAGCAGTGG	GGAATATTGC	ACAATGGGCG	AAAGCCTGAT
GCAGCGACGC	CGCGTGAGGG	ATGAAGGCCT	TCGGGTTGTA	AACCTCTTTC
GCCAGGGAAG	AAGCGAAAGT	GACGGTACCT	GGAGAAGAAG	CACCGGCTAA
CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG	TTGTCCGGAA
TTATTGGGCG	TAAAGAGCTT	GTAGGCGGTT	TGTCGCGTCT	GCCGTGAAAA
TCCAAGGCTT	AACTTTGGAC	GTGCGGTGGG	TACGGGCAGG	CTAGAGTGTG
GTAGGGGAGA	CTGGAATTCC	TGGTGTAGCG	GTGAAATGCG	CAGATATCAG
GAGGAACACC	GATGGCGAAG	GCAGGTCTCT	GGGCCATAAC	TGACGCTGAG
AAGCGAAAGC	ATGGGGAGCG	AACAGGATTA	GATACCCTGG	TAGTCCATGC
CGTAAACGTT	GGGCGCTAGG	TGTGGGGCTC	ATTCCACGAG	CTCTGTGCCG
CAGCTAACGC	ATTAAGCGCC	CCGCCTGGGG	AGTACGGCCG	CAAGGCTAAA
ACTCAAAGGA	ATTGACGGGG	GCCCGCACAA	GCGGCGGAGC	ATGCGGATTA
ATTCGATGCA	ACGCGAAGAA	CCTTACCAAG	GCTTGACATA	CACCGGAAAA
GTGCAGAGAT	GTGCTCCCCG	TAAGGTCGGT	GTACAGGTGG	TGCATGGTTG
TCGTCAGCTC	GTGTC			

Table 2.5 *Dermatophilus* sp (TVS 97-405)

GGTGGATTAG	TGGCGAACGG	GTGAGTAACA	CGTGAGTAAC	CTGCCCTTCA
CTCTGGGATA	ACCACGGGAA	ACCGGGGCTA	ATACTGGATA	TGACACACTG
GCGCATGATG	GTGTGTGGAA	AGATTTATTG	GTGGAGGATG	GACTCGCGGC
CTATCAGCTT	GTTGGTGAGG	TAATGGCTCA	CCAAGGCGAC	GACGGGTAGC
CGGCCTGAGA	GGGTGACCGG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT
CCTGCGGGAG	GCAGCAGTGG	GGAATATTGC	ACAATGGGCG	AAAGCCTGAT
GCAGCGACGC	CGCGTGAGGG	ATGAAGGCCT	TCGGGTTGTA	AACCTCTTTC
GCCAGGGAAG	AAGCGAAAGT	GACGGTACCT	GGAGAAGAAG	CACCGGCTAA
CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG	TTGTCCGGAA
TTATTGGGCG	TAAAGAGCTT	GTAGGCGGTT	TGTCGCGTCT	GCCGTGAAAA
TCCAAGGCTT	AACTTTGGAC	GTGCGGTGGG	TACGGGCAGG	CTAGAGTGTG
GTAGGGGAGA	CTGGAATTCC	TGGTGTAGCG	GTGAAATGCG	CAGATATCAG
GAGGAACACC	GATGGCGAAG	GCAGGTCTCT	GGGCCATAAC	TGACGCTGAG
AAGCGAAAGC	ATGGGGAGCG	AACAGGATTA	GATACCCTGG	TAGTCCATGC
CGTAAACGTT	GGGCGCTAGG	TGTGGGGCTC	ATTCCACGAG	CTCTGTGCCG
CAGCTAACGC	ATTAAGCGCC	CCGCCTGGGG	AGTACGGCCG	CAAGGCTAAA
ACTCAAAGGA	ATTGACGGGG	GCCCGCACAA	GCGGCGGAGC	ATGCGGATTA
ATTCGATGCA	ACGCGAAGAA	CCTTACCAAG	GCTTGACATA	CACCGGAAAA
GTGCAGAGAT	GTGCTCCCCG	TAAGGTCGGT	GTACAGGTGG	TGCATGGTTG
TCGTCAGCTC	GTGTC			

Table 2.6 *Dermatophilus* sp (TVS 97-412)

GGTGGATTAG	TGGCGAACGG	GTGAGTAACA	CGTGAGTAAC	CTGCCCTTCA
CTCTGGGATA	ACCACGGGAA	ACCGGGGCTA	ATACTGGATA	TGACACACTG
GCGCATGATG	GTGTGTGGAA	AGATTTATTG	GTGGAGGATG	GACTCGCGGC
CTATCAGCTT	GTTGGTGAGG	TAATGGCTCA	CCAAGGCGAC	GACGGGTAGC
CGGCCTGAGA	GGGTGACCGG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT
CCTGCGGGAG	GCAGCAGTGG	GGAATATTGC	ACAATGGGCG	AAAGCCTGAT
GCAGCGACGC	CGCGTGAGGG	ATGAAGGCCT	TCGGGTTGTA	AACCTCTTTC
GCCAGGGAAG	AAGCGAAAGT	GACGGTACCT	GGAGAAGAAG	CACCGGCTAA
CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG	TTGTCCGGAA
TTATTGGGCG	TAAAGAGCTT	GTAGGCGGTT	TGTCGCGTCT	GCCGTGAAAA
TCCAAGGCTT	AACTTTGGAC	GTGCGGTGGG	TACGGGCAGG	CTAGAGTGTG
GTAGGGGAGA	CTGGAATTCC	TGGTGTAGCG	GTGAAATGCG	CAGATATCAG
GAGGAACACC	GATGGCGAAG	GCAGGTCTCT	GGGCCATAAC	TGACGCTGAG
AAGCGAAAGC	ATGGGGAGCG	AACAGGATTA	GATACCCTGG	TAGTCCATGC
CGTAAACGTT	GGGCGCTAGG	TGTGGGGCTC	ATTCCACGAG	CTCTGTGCCG
CAGCTAACGC	ATTAAGCGCC	CCGCCTGGGG	AGTACGGCCG	CAAGGCTAAA
ACTCAAAGGA	ATTGACGGGG	GCCCGCACAA	GCGGCGGAGC	ATGCGGATTA
ATTCGATGCA	ACGCGAAGAA	CCTTACCAAG	GCTTGACATA	CACCGGAAAA
GTGCAGAGAT	GTGCTCCCCG	TAAGGTCGGT	GTACAGGTGG	TGCATGGTTG
TCGTCAGCTC	GTGTC			

Table 2.7 *Dermatophilus* sp (TVS 97-427-1)

GGTGGATTAG TGGCGAACGG GTGAGTAACA CGTGAGTAAC CTGCCCTTCA
CTCTGGGATA ACCACGGGAA ACCGGGGCTA ATACTGGATA TGACACACTG
GCGCATGATG GTGTGTGGAA AGATTTATTG GTGGAGGATG GACTCGCGGC
CTATCAGCTT GTTGGTGAGG TAATGGCTCA CCAAGGCGAC GACGGGTAGC
CGGCCTGAGA GGGTGACCGG CCACACTGGG ACTGAGACAC GGCCCAGACT

CCTGCGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGCG AAAGCCTGAT
GCAGCGACGC CGCGTGAGGG ATGAAGGCCT TCGGGTTGTA AACCTCTTTC
GCCAGGGAAG AAGCGAAAGT GACGGTACCT GGAGAAGAAG CACCGGCTAA
CTACGTGCCA GCAGCCGCGG TAATACGTAG GGTGCGAGCG TTGTCCGGAA
TTATTGGGCG TAAAGAGCTT GTAGGCGGTT TGTCGCGTCT GCCGTGAAAA

TCCAAGGCTT AACTTTGGAC GTGCGGTGGG TACGGGCAGG CTAGAGTGTG
GTAGGGGAGA CTGGAATTCC TGGTGTAGCG GTGAAATGCG CAGATATCAG
GAGGAACACC GATGGCGAAG GCAGGTCTCT GGGCCATAAC TGACGCTGAG
AAGCGAAAGC ATGGGGAGCG AACAGGATTA GATACCCTGG TAGTCCATGC
CGTAAACGTT GGGCGCTAGG TGTGGGGCTC ATTCCACGAG CTCTGTGCCG

CAGCTAACGC ATTAAGCGCC CCGCCTGGGG AGTACGGCCG CAAGGCTAAA
ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGCGGAGC ATGCGGATTA
ATTCGATGCA ACGCGAAGAA CCTTACCAAG GCTTGACATA CACCGGAAAA
GTGCAGAGAT GTGCTCCCCG TAAGGTCGGT GTACAGGTGG TGCATGGTTG
TCGTCAGCTC GTGTC

Table 2.8 *Dermatophilus* sp (TVS 97-472)

GGTGGATTAG	TGGCGAACGG	GTGAGTAACA	CGTGAGTAAC	CTGCCCTTCA
CTCTGGGATA	ACCACGGGAA	ACCGGGGCTA	ATACTGGATA	TGACACACTG
GCGCATGATG	GTGTGTGGAA	AGATTTATTG	GTGGAGGATG	GACTCGCGGC
CTATCAGCTT	GTTGGTGAGG	TAATGGCTCA	CCAAGGCGAC	GACGGGTAGC
CGGCCTGAGA	GGGTGACCGG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT
CCTGCGGGAG	GCAGCAGTGG	GGAATATTGC	ACAATGGGCG	AAAGCCTGAT
GCAGCGACGC	CGCGTGAGGG	ATGAAGGCCT	TCGGGTTGTA	AACCTCTTTC
GCCAGGGAAG	AAGCGAAAGT	GACGGTACCT	GGAGAAGAAG	CACCGGCTAA
CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG	TTGTCCGGAA
TTATTGGGCG	TAAAGAGCTT	GTAGGCGGTT	TGTCGCGTCT	GCCGTGAAAA
TCCAAGGCTT	AACTTTGGAC	GTGCGGTGGG	TACGGGCAGG	CTAGAGTGTG
GTAGGGGAGA	CTGGAATTCC	TGGTGTAGCG	GTGAAATGCG	CAGATATCAG
GAGGAACACC	GATGGCGAAG	GCAGGTCTCT	GGGCCATAAC	TGACGCTGAG
AAGCGAAAGC	ATGGGGAGCG	AACAGGATTA	GATACCCTGG	TAGTCCATGC
CGTAAACGTT	GGGCGCTAGG	TGTGGGGCTC	ATTCCACGAG	CTCTGTGCCG
CAGCTAACGC	ATTAAGCGCC	CCGCCTGGGG	AGTACGGCCG	CAAGGCTAAA
ACTCAAAGGA	ATTGACGGGG	GCCCGCACAA	GCGGCGGAGC	ATGCGGATTA
ATTCGATGCA	ACGCGAAGAA	CCTTACCAAG	GCTTGACATA	CACCGGAAAA
GTGCAGAGAT	GTGCTCCCCG	TAAGGTCGGT	GTACAGGTGG	TGCATGGTTG
TCGTCAGCTC	GTGTC			

Table 2.9 *Dermatophilus* sp (TVS 97-474)

GGTGGATTAG TGGCGAACGG GTGAGTAACA CGTGAGTAAC CTGCCCTTCA
CTCTGGGATA ACCACGGGAA ACCGGGGCTA ATACTGGATA TGACACACTG
GCGCATGATG GTGTGTGGAA AGATTTATTG GTGGAGGATG GACTCGCGGC
CTATCAGCTT GTTGGTGAGG TAATGGCTCA CCAAGGCGAC GACGGGTAGC
CGGCCTGAGA GGGTGACCGG CCACACTGGG ACTGAGACAC GGCCCAGACT

CCTGCGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGCG AAAGCCTGAT
GCAGCGACGC CGCGTGAGGG ATGAAGGCCT TCGGGTTGTA AACCTCTTTC
GCCAGGGAAG AAGCGAAAGT GACGGTACCT GGAGAAGAAG CACCGGCTAA
CTACGTGCCA GCAGCCGCGG TAATACGTAG GGTGCGAGCG TTGTCCGGAA
TTATTGGGCG TAAAGAGCTT GTAGGCGGTT TGTCGCGTCT GCCGTGAAAA

TCCAAGGCTT AACTTTGGAC GTGCGGTGGG TACGGGCAGG CTAGAGTGTG
GTAGGGGAGA CTGGAATTCC TGGTGTAGCG GTGAAATGCG CAGATATCAG
GAGGAACACC GATGGCGAAG GCAGGTCTCT GGGCCATAAC TGACGCTGAG
AAGCGAAAGC ATGGGGAGCG AACAGGATTA GATACCCTGG TAGTCCATGC
CGTAAACGTT GGGCGCTAGG TGTGGGGCTC ATTCCACGAG CTCTGTGCCG

CAGCTAACGC ATTAAGCGCC CCGCCTGGGG AGTACGGCCG CAAGGCTAAA
ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGCGGAGC ATGCGGATTA
ATTCGATGCA ACGCGAAGAA CCTTACCAAG GCTTGACATA CACCGGAAAA
GTGCAGAGAT GTGCTCCCCG TAAGGTCGGT GTACAGGTGG TGCATGGTTG
TCGTCAGCTC GTGTC

Table 2.10 *Dermatophilus congolensis* (ACM Number 530)

CACATGCAAG	TCGAACGATG	AAGCCCAGCT	TGCTGGGTGG	ATTAGTGGCG
AACGGGTGAG	TAACACGTGA	GTAATCTACC	CCTCACTTTG	GGATAAGCCC
CGGAAACGGG	GTCTAATACT	GAATATGACC	TTTCCTCGCA	TGAGGTTTGG
TGGAAAGTTT	TTTCGGTGGG	GGATGTGCTC	GCGGCCTATC	AGCTTGTTGG
TGAGGTAACG	GCTCACCAAG	GCGACGACGG	GTAGCCGGCC	TGAGAGGGTG
AACGGCCACA	CTGGGACTGA	GACACGGCCC	AGACTCCTAC	GGGAGGCAGC
AGTGGGGAAT	ATTGCACAAT	GGGCGAAAGC	CTGATGCAGC	GACGCCGCGT
GAGGGATGAA	GGCCTTCGGG	TTGTAAACCT	CTTTCAGCAG	GGGAGAAGCG
AAAGTGACGG	TACCTGCAGA	AGAAGCACCG	GCTAACTACG	TGCCAGCAGC
CGCGGTAATA	CGTAGGGTGC	GAGCGTTGTC	CGGAATTATT	GGGCGTAAAG
AGCTTGTANG	CGGTTTGTCG	CGTCTGCTGT	GAAAACCCAG	GGCTTAACCC
TGGACGTGCA	GTGGGTACGG	GCAGGCTAGA	GTGTGGTAGG	GGAGACTGGA
ATTCCTGGTG	TAGCGGTGAA	ATGCGCAGAT	ATCAGGAGGA	ACACCGATGG
CGAAGGCAGG	TCTCTGGGCC	ATTACTGACG	CTGAGAAGCG	AAAGCATGGG
TAGCGAACAG	GATTAGATAC	CCTGGTAGTC	CATGCCGTAA	ACGTTGGGCG
CTGGGTGTGG	GGTCCATTCC	ACGGATTCTG	CGCCGTAGCT	AACGCATTAA
GCGCCCCGCC	TGGGGAGTAC	GGCCGCAAGG	CTAAAACTCA	AAGGAATTGA
CGGGGGCCCG	CACAAGCGGC	GGAGCATGCG	GATTAATTCR	ATGCAACGCG
AAGAACCWTA	CCAAGGCTTG	ACATACACCG	GAAAAGTGCA	GAGATGTACT
CCCCTTTTTG	GTCGG			

Table 2.11 *Dermatophilus congolensis* (ACM Number 531)

GGGTGGATTC	CTGGCGAACG	GGTGAGTAAC	ACGTGAGTAA	TCTGCCCCTC
ACTTTGGGAT	AAGCCCCGGA	AACGGGGTCT	AATACTGAAT	ATGACCTTTC
CTCGCATGAG	GTTTGGTGGA	AAGTTTTTTC	GGTGGGGGAT	GTGCTCGCGG
CCTATCAGCT	TGTTGGTGAG	GTAACGGCTC	ACCAAGGCGA	CACGGGTAGC
CAGGGCCTGA	GAGGGTGAAC	CGGCCACACT	GGGACTGAGA	CACGGCCCAG
ACTCCTACGG	GAGGCAGCAG	TGGGGAATAT	TGCACAATGG	GCAGAAAGCC
TGATGCAGCG	ACGCCGCGTG	AGGGATGAAG	GCTTTCGGGT	TGTAAACCTC
TTTCAGCAGG	GGAGAAGCGA	AAGTGACGGT	ACCTGCAGAA	GAAGCACCGG
CTAACTACGT	GCCAGCAGCC	GCGGTAATAC	GTAGGGTGCG	AGCGTTGTCC
GGAATTATTG	GGCGTAAAGA	GCTAGTAGGC	GGTTTGTCGC	GTCTGCTGTG
AAAATCAGGG	CTTAACCCTG	GACGTGCAGT	GGGTACGGCA	GGCTAGAGTG
TGTAGGGAGA	CTGAATTCCT	GGTGTACGGT	GAAATGCGCA	GATATCAGAG
ACACCGATGC	AAGCAGGTCT	CTGGCATACT	GACGCTAGAA	GCGAAAGCAT
GGGTAGCGAA	CAGGATTAGA	TACCCTGGTA	GTCCATGCCG	TAACGTTGGG
CGCTGGGTGT	GGGGTCCATT	CCACGGATTC	TGCGCCGAGC	TAACGCATTA
AGCGCCCCGC	CTGGGGAGTA	CGCCGCAAGG	CTAAAACTCA	AAGGAATTGA
CGGGGGCCCG	CACAAGCGGC	GGAGCATGCG	GATTAATTCG	ATGCAACGCA
AGAACCTTAC	CAAGGCTTGA	CATACACCGG	AAAAGTGCAG	AGATGTACTC
CCCTTTTTGG	TCGGTGTACA	GGTGGTGCAT	GGTTGTCGTC	AGCTCGTGTC
GTGAGATGTT	GGGTT			

Table 2.12 *Dermatophilus congolensis* (OVLDPI Number 22204)

GCGGCGTGCT TTAATACATG CAAGTCGAGC GAACAGACGA GGAGCTTGCT
CCTCTGACGT TAGCGGCGGA CGGGTGAGTA ACACGTGGAT AACCTACCTA
TAAGACTGGG ATAACTTCGG GAAACCGGAG CTAATACCGG ATAATATATT
GAACCGCATG GTTCAATAGT GAAAGACGGT TTTGCTGTCA CTTATAGATG
GATCCGCGCC GCATTAGCTA GTTGGTAAGG TAACGGCTTA CCAAGGCAAC

GATGCGTAGC CGACCTGAGA GGGTGATCGG CCACACTGGA ACTGAGACAC
GGTCCAGACT CCTACGGGAG GCAGCAGTAG GGAATCTTCC GCAATGGGCG
AAAGCCTGAC GGAGCAACGC CGCGTGAGTG ATGAAGGTCT TCGGATCGTA
AAACTCTGTT ATTAGGGAAG AACAAATGTG TAAGTAACTA TGCACGTCTT
GACGGTACCT AATCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG

TAATACGTAG GTGGCAAGCG TTATCCGGAA TTATTGGGCG TAAAGCGCGC
GTAGGCGGTT TTTTAAGTCT GATGTGAAAG CCCACGGCTC AACCGTGGAG
GGTCATTGGA AACTGGAAAA CTTGAGTGCA GAAGAGGAAA GTGGAATTCC
ATGTGTAGCG GTGAAATGCG CAGAGATATG GAGGAACACC AGTGGCGAAG
GCGACTTTCT GGTCTGTAAC TGACGCTTGA TGTGCGAAAG CGTGGGGATC

AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA
GTGTTAGGGG GTTTCCGCCC CTTAGTGCTG CAGCTAACAG CATTAAGCAC
TCCGCCTGGG GAGTACGACC GCAAGGTTGA AACTCAAAGG AATTGACGGG
GACCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGAAGA
ACCTTACCAA ATCTT

Table 2.13 *Dermatophilus congolensis* type strain (ATCC Number 14637)

TTGATCCTGG CTCAGGACGA ACGCTGGCGG CGTGCTTAAC ACATGCAAGT
CGAACGATGA AGCCCAGCTT GCTGGTGGAT TAGTGGCGAA CGGGTGAGTA
ACACGTGAGT AATCTACCCC TCACTTTGGG ATAAGCCCCG GAAACGGGTC
TAATACTGAA TATGACCTTT CCTCGCATGA GGTTTGGTGG AAAGTTTTTT
CGGTGGGGGA TGTGCTCGCG GCCTATCAGC TTGTTGGTGA GGTAACGGCT

CACCAAGGCG ACGACGGGTA GCCGGCCTGA GAGGGTGAAC GGCCACACTG
GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT
GCACAATGGG CGAAAGCCTG ATGCAGCGAC GCCGCGTGAG GGATGAAGGC
CTTCGGGTTG TAAACCTCTT TCAGCAGGGG AGAAGCGAAA GTGACGGTAC
CTGCAGAAGA AGCACCGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT

AGGGTGCGAG CGTTGTCCGG AATTATTGGG CGTAAAGAGC TTGTAGGCGG
TTTGTCGCGT CTGCTGTGAA AATCCAGGGC TTAACCCTGG ACGTGCAGTG
GGTACGGGCA GGCTAGAGTG TGGTAGGGGA GACTGGAATT CCTGGTGTAG
CGGTGAAATG CGCAGATATC AGGAGGAACA CCGATGGCGA AGGCAGGTCT
CTGGGCCATT ACTGACGCTG AGAAGCGAAA CGATGGGTAG CGAACAGGAT

TAGATACCCT GGTAGTCCAT GCCGTAAACG TTGGGCGCTG GGTGTGGGGT
CCATTCCACG GATTCTGCGC CGTAGCTAAC GCATTAAGCG CCCCGCCTGG
GGAGTACGGC CGCAACGGTA AAACTCAAAG GAATTGACGG GGGCCCGCAC
AAGCGGCGGA GCATGCGGAT TAATTCGATG CAACGCGAAG AACCTTACCA
AGGCTTGACA TACAC

Table 2.14 *Dermatophilus chelonae* (WA 1305)

GGTGGATTAG	TGGCGAACGG	GTGAGTAACA	CGTGAGTAAC	CTGCCCTTCA
CTCTGGGATA	ACCACGGGAA	ACCGGGGCTA	ATACTGGATA	TGACACACTG
GCGCATGATG	GTGTGTGGAA	AGATTTATTG	GTGGAGGATG	GACTCGCGGC
CTATCAGCTT	GTTGGTGAGG	TAATGGCTCA	CCAAGGCGAC	GACGGGTAGC
CGGCCTGAGA	GGGTGACCGG	CCACACTGGG	ACTGAGACAC	GGCCCAGACT
CCTGCGGGAG	GCAGCAGTGG	GGAATATTGC	ACAATGGGCG	AAAGCCTGAT
GCAGCGACGC	CGCGTGAGGG	ATGAAGGCCT	TCGGGTTGTA	AACCTCTTTC
GCCAGGGAAG	AAGCGAAAGT	GACGGTACCT	GGAGAAGAAG	CACCGGCTAA
CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG	TTGTCCGGAA
TTATTGGGCG	TAAAGAGCTT	GTAGGCGGTT	TGTCGCGTCT	GCCGTGAAAA
TCCNGGGCTT	AACCCCGGAC	GTGCGGTGGG	TACGGGCAGG	CTAGAGTGTG
GTAGGGGAGA	CTGGAATTCC	TGGTGTAGCG	GTGAAATGCG	CAGATATCAG
GAGGAACACC	GATGGCGAAG	GCAGGTCTCT	GGGCCATAAC	TGACGCTGAG
AAGCGAAAGC	ATGGGGAGCG	AACAGGATTA	GATACCCTGG	TAGTCCATGC
CGTAAACGTT	GGGCGCTAGG	TGTGGGACTC	ATTCCACGAG	TTCTGCGCCG
CAGCTAACGC	ATTAAGCGCC	CCGCCTGGGG	AGTACGGCCG	CAAGGCTAAA
ACTCAAAGGA	ATTGACGGGG	GCCCGCACAA	GCGGCGGAGC	ATGCGGATTA
ATTCGATGCA	ACGCGAAGAA	CCTTACCAAG	GCTTGACATA	CACCGGAAAC
ACGCAGAGAT	GTGTGCCCCG	TAAGGTCGGT	GTACAGGTGG	TGCATGGTTG
TCGTCAGCTC	GTGTC			

Table 2.15 *Geodermatophilus obscurus* (Genbank Accession Number X92359)

GGGGGGATGA GTGGCGAACG GGTGAGTAAC ACGTGGGCAA CCTGCCCCCG
GCTCTGGGAT AACTCCAAGA AATTGGGGCT AATACCGGAT GTTCACCGGC
TCCCGCATGG TGGTGGGTGG AAAGGGTTTC CGGCTGGGGA TGGGCCCGCG
GCCTATCAGC TTGTTGGTGG GGTAGTGGCC TACCAAGTCG ACGACGGGTA
GCCGGCCTGA GAGGGTGACC GGCCACACTG GGACTGAGAC ACGGCCCAGA

CTCCTACGGG AGGCAGCAGT GGGGAATATT GCGCAATGGG CGGAAGCCTG
ACGCAGCGAC GCCGCGTGGG GGATGACGGC CTTCGGGTTG TAAACCTCTT
TCAGCAGGGA CGAAGCGGAA GTGACGGTAC CTGCAGAAGA AGCACCGGCC
AACTACGTGC CAGCAGCCGC GGTAATACGT AGGGTGCAAG CGTTGTCCGG
AATTATTGGG CGTAAAGAGC TCGTAGGCGG TTCGTCGCGT CGGCTGTGAA

AACCCGGAGC TCAACTCCGG GCCTGCAGTC GATACGGGCG GACTTGAGTT
CGGCAGGGGA GACTGGAATT CCTGGTGTAG CGGTGAAATG CGCAGATATC
AGGAGGAACA CCGGTGGCGA AGGCGGGTCT CTGGGCCGAT ACTGACGCTG
AGGAGCGAAA GCGTGGGGAG CGAACAGGAT TAGATACCCT GGTAGTCCAC
GCCGTAAACG TTGGGCGCTA GGTGTGGGGG CCATTCCACG GTCTCCGTGC

CGCAGCTAAC GCATTAAGCG CCCCGCCTGG GGAGTACGGC CGCAAGGATA
AAACTCAAAG GAATTGACGG AGGCCCGCAC AAGCGGCGGA GCATGTTGCT
TAATTCGATG CAACGCGAAG AACCTTACCT AGGCTTGACA TGCACGGAAA
TCTCGCAGAG ATGCGGGGTG CCTTTGGCGT CGTGCACAGG TGGTGCATGG
TTGTCGTCAG CTCGT

Table 2.16 *Frankia* sp (Number AF034776)

GACGAACGCT GGCGGCGTGC TTAACACATG CAAGTCGAGC GGGGGACTTC
GGTCTTCAGC GGCGAACGGG TGAGTAACAC GTGGGCAACC TGCCCCGAGC
TCTGGGATAA CTTCGGGAAA CCGGGGCTAA TACCGGATAT GACATTGCCG
GGCATCTGGT GGTGTGGAAA GATTTATCGG CTCGGGATGG GCCCGCGGCC
TATCAGCTTG TTGGTGGGGT GATGGCCTAC CAAGGCGACG ACGGGTAGCC

GGCCTGAGAG GGCGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC
CTACGGGAGG CAGCAGTGGG GAATATTGCG CAATGGGCGG AAGCCTGACG
CAGCGACGCC GCGTGGGGGA TGACGGCCTT CGGGTTGTAA ACCTCTTTCA
GCAGGGACGA AGCGCAAGTG ACGGTACCTG CAGAAGAAGC ACCGGCCAAC
TACGTGCCAG CAGCCGCGGT AATACGTAGG GTGCAAGCGT TGTCCGGAAT

TATTGGGCGT AAAGAGCTCG TAGGCGGCTT GTCGCGTCGG CTGTGAAATC
CCGGGGCTCA ACTCCGGGCG TGCAGTCGAT ACGGGCAGGC TAGAGTCCGG
CAGGGGAGAC TGGAATTCCT GGTGTAGCGG TGAAATGCGC AGATATCAGG
AAGAACACCG GTGGCGAAGG CGGGTCTCTG GGCCGGTACT GACGCTTAGG
AGCGAAAGCG TGGGGAGCGA ACAGGATTAG ATACCCTGGT AGTCCACGCC

GTAAACGTTG GGCGCTAGGT GTGGGGGACT TTCCACGGCC TCCGTGCCGC
AGCTAACGCA TTAAGCGCCC CGCCTGGGGA GTACGGCCGC AAGGCTAAAA
GTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGCGGAGCA TGTGGCTTAA
TTCGATGCAA CGCGAAGAAC CTTACCAAGG CTTGACATGC AGGGAAATCT
CGTAGAGATA CGGGG

Table 2.17 *Streptomyces* sp (Number AF012741)

GCGGCGTGCT TAACACATGC AAGTCGAACG ATGAACCACT TCGGTGGGGA
TTAGTGGCGA ACGGGTGAGT AACACGTGGG CAATCTGCCC TTCACTCTGG
GACAAGCCCT GGAAACGGGG TCTAATACCG GATACTGACC CTTGCAGGCA
TCTGCGAGGT TCGAAAGCTC CGGCGGTGAA GGATGAGCCC GCGGCCTATC
AGCTTGTTGG TGAGGTAATG GCTCACCAAG GCGACGACGG GTAGCCGGCC

TGAGAGGGCG ACCGGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC
GGGAGGCAGC AGTGGGGAAT ATTGCACAAT GGGCGAAAGC CTGATGCAGC
GACGCCGCGT GAGGGATGAC GGCCTTCGGG TTGTAAACCT CTTTCAGCAG
GGAAGAAGCG AAAGTGACGG TACCTGCAGA AGAAGCGCCG GCTAACTACG
TGCCAGCAGC CGCGGTAATA CGTAGGGCGC AAGCGTTGTC CGGAATTATT

GGGCGTAAAG AACTCGTAGG CGGCTTGTCA CGTCGGTTGT GAAAGCCCGG
GGCTTAACCC CGGGTCTGCA GTCGATACGG GCAGGCTAGA ATTCGGTAGG
GGAGATCGGA ATTCCTGGTG TTTCGGTGAA ATGCGCAGAT ATCAGGAGGA
ACACCGGTGG CGAAGGCGGA TCTCTGGGCC GATACTGACG CTGAGGAGCG
AAAGCGTGGG GAGCGAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA

ACGGTGGGCA CTAAGTGTGG GCAACATTCC ACGTTGTCCG TGCCGCAGCT
AACGCATTAA GTGCCCCGCC TGGGGAGTAC GGCCGCAAGG CTAAAACTCA
AAGGAATTGA CGGGGGCCCG CACAAGCGGC GGAGCATGTG GCTTAATTCG
ACGCAACGCG AAGAACCTTA CCAAGGCTTG ACATACACCG GAAAGCATCA
GAGATGGTGC CCCCC

Appendix 3

Bacterial Culture Media and Reagents

3.1 General

The water used in the preparation of media, reagents and stains was initially distilled (distillation apparatus model 6T triple distillation system Labglass Pty. Ltd. Brisbane, Australia) followed by a series of deionisation (Megahom-CM, Sybron/Barnstead, Boston, Massachusetts, USA). The deionised water was autoclaved at 110 kPa (121°C) for 15 minutes.

3.2 Blood agar

Blood agar base (Oxoid, Unipath Ltd, Basingstoke, Hampshire, England)	40 g
Distilled water	1000 mL

The blood agar base was dissolved in distilled water in a boiling waterbath at least 20 minutes, then autoclaved at 121°C for 15 minutes. When the media cooled up to a tolerable temperature by holding it with your hands, 50 mL of whole sheep blood was added. The blood agar was mixed gently and poured in Petri plates.

Note: Polymyxin B (Sigma Chemicals Co., Steinheim, Germany) at the rate 1000 units/mL of media was added. This special medium was intended for the initial screening of *Dermatophilus* sp from skin samples.

3.3 Mueller-Hinton agar

Mueller-Hinton agar (Oxoid)	38 g
Distilled water	1000 mL

The Mueller-Hinton agar (MHA) was dissolved in distilled water in a boiling water bath and sterilised by autoclaving at 121°C for 15 minutes. When MHA was cooled down, it was poured in to Petri plates.

Note: The Mueller-Hinton agar was supplemented with 9% whole sheep blood for the used in antibiotic sensitivity testing. The sheep blood was added to the agar when the temperature was cooled down.

3.4 Tryptose phosphate broth

Tryptose (Oxoid)	10 g
D-glucose (Unilab Ajax Chemicals, Auburn, NSW, Australia)	1.0 g
Sodium chloride (Sigma)	2.5 g
Disodium hydrogen orthophosphate (Sigma)	1.25 g
Distilled water	500 mL

All ingredients were dissolved in distilled water in a boiling water bath. The pH was adjusted to 7.3±02 after the broth was cooled down, then autoclave at 121°C for 15 minutes. The broth was allowed to cool down before a sterile 10% bovine serum filtered through a 0.45 µm membrane filters (Sartorius Autralia Pty. Ltd., East Oakleigh, Victoria), was added. Aliqouts of 10 mL were prepared in Bijoux bottles.

3.5 Brain heart infusion broth

Brain heart infusion broth (Acumedia Manufacturer Inc., Baltimore, Maryland)	37 g
Distilled water	1000 mL

The Brain heart infusion broth (37 g) was dissolved in 1000 mL of distilled water in a boiling water bath. Aliquots of 10 mL quantity in Bijoux bottles were autoclaved at 121°C for 15 minutes.

3.6 Gram staining (DIFCO Laboratories, Detroit, Michigan)

A kit purchased from DIFCO laboratories was used in Gram-staining. A small amount of the organisms to be stained was emulsified in a drop of distilled water on the slide, air dried and heat fixed. The slides were flooded with Gram-crystal violet for one minute, and immediately rinsed with tap water. Excess water was removed, and the slides were flooded with Grams-iodine for one minute. Again, the slides were rinsed with tap water, then decolorised with Gram-decoloriser for not longer than five seconds. The slides were rinsed in flowing water and

counterstained with Gram-safranine for one minute. Again, the slides were rinsed, air dried and examined under the microscope.

3.7 Kinyoun stain for partial acid fast

Solution 1 Carbolfuschsin

Basic fuschin (Searle Diagnostic, Bucks, England)	4 g
Phenol (CSR Ltd Distilleries Group, Yarraville, Victoria)	8 mL
Ethanol (95%) (CSR)	20 mL
Distilled water	100 mL

Basic fuschin was dissolved in ethanol and while shaking, distilled water was added slowly. The phenol was melted at 56°C in water bath, and phenol was added to the stain using a pipette provided with a rubber bulb.

Solution 2 Decoloriser

Ethanol (95%) (CRC)	97 mL
Concentrated HCL (APS Finechem, NSW, Australia)	3 mL

The concentrated hydrochloric acid (HCL) was added slowly to ethanol, working under a chemical fume hood.

Solution 3 Counterstain

Methylene blue (Fluka Chemika, Switzerland)	0.3 g
Distilled water	100 mL

Procedure for staining

A very small amount of organisms to be stained was emulsified in a drop of distilled water on the slide. A known positive control such as *Nocardia* species and a negative control (*E coli*) were stained along with the unknown strain. The slides were air dried, heat fixed and were flooded with the Kinyoun's carbolfuchsin stain. The stain was allowed to remain on the slide for three minutes. The slides were rinsed with tap water, removed the excess water, and decolorised briefly no longer than 3 to 5 seconds with 3% acid ethanol. Counterstained with Kinyoun's methylene blue (above) for 30 seconds, and rinsed again with tap water. The slides were air

dried and examine under the microscope. Partially acid-fast organisms showed reddish to purple filaments, compared to non-acid fast that are blue only.

3.8 Nigrosin-methylene blue capsule staining

Solution 1 Nigrosin

Nigrosin (Sigma)	10 g
Formalin, as preservative (APS, Ajax chemicals)	0.5 mL
Distilled water	100 mL

Nigrosin was dissolved in distilled water in a water bath at 56°C. Formalin was added after the Nigrosin was completely dissolved. The solution was filtered, labelled and stored at room temperature.

Solution 2 Loeffler's alkaline methylene blue

Methylene blue (Fluka)	0.45 g
Ethanol (95%) (CSR)	30 mL
Potassium Hydroxide, 1% aqueous solution (Unilab, Ajax chemicals)	1 mL
Distilled water	99 mL

The methylene blue was dissolved in ethanol. The potassium hydroxide 1% aqueous solution was added to the distilled water. The methylene blue solution and the potassium hydroxide solution were mixed thoroughly and filtered before used.

Procedure for staining

A small amount of culture was placed on the glass slide, and one loopful of solution 1 was added. After mixing, the slide was air dried and fixed with gentle heat. The solution 2 was applied for 30 seconds, ringed rapidly in water, blot carefully and dry with gentle heat. *Klebsiella pneumoniae* and *E coli* as were used as positive and negative controls, respectively. Bacterial cells: blue, capsule unstained against a dark grey background of nigrosin.

3.9 Hanging drop motility test

A small amount of organisms from at least 72 hours culture in blood agar plates was mixed up with a drop of distilled water on the coverslip. On the slide a ring of Petrolatum 2 cm in diameter was made and gently attached the slide to the coverslip without touching the inoculum. The slide and coverslip should be completely sealed otherwise 'draughts' can cause pseudo-motility. With a quick movement, the slide was inverted so that the coverslip was uppermost. The specimen was examined under the microscope focusing first onto the edge of the 'drop' at low power magnification, then to a 40 magnification.

3.10 Methyl red and Voges-Proskauer (MR-VP) test

Solution 1 Methyl red solution

Methyl Red (BDH Chemicals Ltd, Poole, England)	0.1 g
Ethyl alcohol 95% (CSR)	300 mL
Distilled water	200 mL

The methylene red was dissolved in the alcohol before adding the distilled water. The solution was placed in a brown bottle to protect against exposure to light, and stored in the refrigerator.

Solution 2 Voges-Proskauer reagent A

a-Naphthol (Sigma)	5 g
Absolute ethyl alcohol (CSR)	100 mL

The a-naphthol was dissolved in a small amount of ethyl alcohol first, then gradually bring the volume to 100 mL in a volumetric flask or cylinder. The alcohol should be almost colourless. The solution was transferred to a brown bottle or a bottle covered with alfoil, and stored in the refrigerator.

Solution 3 VP reagent B

Potassium hydroxide (Unilab, Ajax Chemicals)	40 g
Distilled water	100mL

The potassium hydroxide was quickly weighed out, as it is hygroscopic and will become caustic when moist. Cold distilled water (100 mL) was added to the flask containing the potassium hydroxide to prevent overheating, and stored in a refrigerator.

Solution 4 MR-VP broth base (Glucose-phosphate medium)

Peptone (Oxoid)	0.5 g
K_2HPO_4 (Sigma)	0.5 g
Distilled water	100 mL
Glucose (Unilab, Ajax Chemicals)	0.5 g

Peptone and K_2HPO_4 were dissolved in water bath at 56°C, filtered and the pH was adjusted to 7.5. Glucose was added and mixed. The solutions were distributed in 5 mL volumes in Bijoux bottles and sterilised at 115°C for 10 minutes.

Procedure for MR-VP test

MR/VP broth base was inoculated with a loopful of test isolates and incubated for 48 hours. The broth was equally divided, each tube containing 2.5 mL of bacterial suspension. To the first tube, 0.6 mL or six drops of VP reagent A (a-naphthol) was added, then 0.2 mL or two drops of VP reagent B. The tubes were mixed gently and allowed to set for 15 minutes. The formation of a pink to red colour, indicating the presence of acetoin (acetyl methyl carbinol) was noted. A pink colour in the medium is indicative of a positive result. A negative result will appear colourless or yellow.

While the VP reaction was developing, the remaining 2.5 mL of substrate was tested for acidity by adding 0.5 mL or five drops of methyl red (solution 1), and a changed of colour was observed. If the indicator remains red, the methyl red test result is positive. A change to yellow indicates the pH of the medium is greater than 6.0, a negative MR test. If the reagent remains orange, the test must be repeated after a longer incubation period.

3.11 Indole test

Solution 1 Kovacs' reagent

p-Dimethylaminobenzaldehyde (Sigma)	10 g
Absolute Isoamyl or isobutyl alcohol (Sigma)	150 mL
Concentrated hydrochloric acid (APS Finechem)	50 mL

The p-dimethylaminobenzaldehyde was dissolved in alcohol, and slowly added with HCL acid while constantly stirring the mixture. The reagent should be pale coloured and should be stored in a brown bottle, refrigerated, and if it turns brown or if the quality control organisms failed to give correct reaction, discard the reagent and make a fresh lot. The aldehyde may require gentle heating in order to go into solution.

Solution 2 Tryptone water

Tryptone (Oxoid)	10 g
NaCl (Sigma)	2.5 g
Distilled water	500 mL

Tryptone and salt were dissolved in distilled water, and the pH was adjusted to 7.5. Aliquots of 5 mL of trytone water in Bijoux bottles were autoclaved at 121°C for 15 minutes.

Procedure

A loopful of organisms was inoculated into Tryptone water and incubated for at least 24 hours. Most commercial peptone, pancreatic enzymatic casein hydrolysate, or tryptone (a pancreatic digest of casein) broths will contain enough tryptophan for use in this test. Note that acid hydrolysate of casein broths that do not contain tryptone are not suitable for the indole test. To perform the test, 2 mL of the broth suspension was transferred to a second tube and 0.5 mL or five drops of Kovacs' reagent was added. The tube was mixed gently, and the presence of a pink colour in a ring around the interface between the broth and the alcoholic reagent, which rises to the surface, was recorded. If the test is negative, the remaining broth may be re-incubated for an additional 24 hours and the test may be repeated.

3.12 Tyrosine or Xanthine agar

Nutrient agar (Oxoid)	23 g
Tyrosine or Xantine (Sigma)	5 g
Distilled water	1000 mL

All ingredients were dissolved in distilled water in a boiling water bath. The pH was adjusted to 7.0 and autoclaved at 121°C for 15 minutes. The agar was dispensed, ensuring the tyrosine or xanthine crystals were evenly distributed in Petri plates. Tyrosine and xanthine agars are recommended for differentiation of species of aerobic actinomycetes. Its use is similar to that of casein agar for hydrolysis.

3.13 Casein Medium

Solution A

Skimmed milk (Oxoid)	10 g
Distilled water	100 mL

The skimmed milk was dissolved in distilled water and sterilised at 121°C for 15 minutes.

Solution B

Distilled water	100 mL
Agar	2 g

The agar was dissolved in distilled water and autoclave at 121°C for 20 minutes. Both solutions A and B were cooled down to approximately 45°C, mixed and poured into sterile Petri plates.

3.14 Conventional Nitrate reduction test

Nitrate broth

Tryptone (Oxoid)	5 g
Neopeptone (Oxoid)	5 g
Distilled water	1000 mL
Potassium nitrate (Sigma)	1 g
Glucose (Unilab, Ajax Chaemicala)	0.1 g

Before the potassium nitrate and glucose were added, tryptone and neopeptone were boiled in a water bath. The pH was adjusted to 7.3 or 7.4. Aliquots of 5 mL were dispensed in Bijoux bottles and were sterilised at 121°C for 15 minutes.

Procedure

The test isolates were inoculated in 5 mL nitrate broth for 24 hours to 48 hours or one to five days (for *Dermatophilus* species only) for poorly growing organisms. A small Durnham tube was placed in an inverted position into the test tube containing the broth to trap bubbles of nitrogen gas that may be formed by nitrate-reducing organisms.

Reagent A

Sulfanilic acid (BDH Chemicals, Australia Pty., Kelsyth, Victoria)	4 g
Acetic acid (5M) (BDH)	500 mL

Reagent B

N,N-dimethyl-1-naphthylamine	3 mL
Acetic acid (5M) (BDH)	500 mL

Procedure

The bacterial suspension in broth was added with three drops, each of reagent A and reagent B. The reaction was observed for 30 minutes for the presence of a red colour, indicating a positive result, which means the nitrate was reduced to nitrite. The presence of unreduced nitrate can be detected by adding a pinch of commercially available zinc powder to the broth if the red colour did not develop after the initial reagents were added.

3.15 Spot oxidase test

1% tetramethyl-p-phenylenediamine dihydrochloride (Sigma)	1 g
Sterile distilled water	99 mL

The tetramethyl-p-phenylenediamine dihydrochloride was dissolved in a sterile distilled water. It should not be, and if it has become deep blue the autoxidation of the reagent maybe retarded by adding 0.1% ascorbic acid.

Procedure

The filter paper was placed into a sterile Petri plate, and several drops of the fresh reagent were placed near the middle of the paper. A small portion of the colony to be tested preferably not more than 24 hours old culture (3 days for *Dermatophilus* species) were pick off with a platinum wire or wooden stick, and rub onto the moisten filter paper. The colour change was observed for the presence of blue or purple within 10 seconds (timing was critical), which indicated a positive result.

3.16 Loeffler's serum medium

Nutrient broth (sterile) pH 7.6 (Oxoid)	250 mL
Glucose (sterile 25% solution) (Unilab, Ajax, Chemicals)	10 mL
Serum (bovine)	750 mL

The glucose solution and serum were sterilised using membrane filters before they were added to the nutrient broth. Mixed well and dispensed aseptically the 3.5 mL volumes into sterile Bijoux bottles. Sterilised bottles containing the broth by inspissation in the slant position, three hours at 75°C on first day, ½ hour at 75°C on second day, ½ hour at 75°C on third day.

3.17 Urease solid medium for Urease test (Kauffmann's method)

Agar (Oxoid)	15 g
Peptone (Oxoid)	1 g
NaCl (Sigma)	5 g
KH_2PO_4 (Sigma)	2 g
Glucose (Unilab, Ajax Chemicals)	1 g
Phenol red (0.6%) (Sigma)	2 mL
Distilled water	1000 mL

All solid ingredients were dissolved in distilled water in a boiling water bath. The solution was adjusted to pH 6.8. Phenol red was added and sterilised at 121°C for 15 minutes. Before used 10 mL of sterile 20% urea solution/100 mL of urease medium was added. Urea solution was filtered through a D 9 filter or 0.45 µm membrane filters, and dispensed 2.5 mL in ¼ oz McCartney bottles.

3.18 Oxidative-Fermentative (O/F) Test for Carbohydrate utilisation OF base medium (glucose)

Peptone or tryptone (Oxoid)	0.4 g
Sodium chloride (Sigma)	1 g
Dipotassium phosphate (Sigma)	60 mg
Bromthymol blue (Sigma)	6.0 mg
Distilled water	200 mL

All ingredients were dissolved in distilled water and the pH was adjusted to 7.1 and sterilised at 121°C for 15 minutes. Glucose (10% aqueous solutions) was sterilised using a 0.2 µm membrane filters. When O-F base has cooled to 55°C after autoclaving, aseptically add 20 mL of the sterile 10% carbohydrate solution to the 200 mL flask of O-F base to achieved a final concentration of 1% carbohydrate. The correctly prepared medium is green, but it appears somewhat bluish while it is still hot. When cooled down, 5 mL glucose O-F media was dispensed into 16 × 125 mm screw cap test tubes. The tubes were allowed to solidify upright (forming a semisoft butt), tightened the caps, and stored in a refrigerator. The medium should be used for several months. If desired, the base can be dispensed without carbohydrates, which can then be added to the melted tubes of O-F base medium as needed (0.5 mL 10% carbohydrate to 5 mL base). For testing the nature of glucose utilization, two tubes of O-F glucose were inoculated using a straight wire. The O-F medium was stabbed at least four times into each tube, and the stabs should extend approximately 5 mm deep into the surface layer of the butt. The medium in one of each of the two identical tubes was overlayed with a sterile melted petrolatum or melted paraffin combined with an equal volume of petroleum jelly approximately 1 cm deep to prevent oxygen from reaching the inoculum. Sterile mineral oil was not recommended for overlaying the medium, because some lots are acidic and can contribute to false positive results. The tubes were incubated at 37°C for as long as four days and examined daily for the production of acid, as indicated by a change in the bromthymol blue indicator from green to yellow.

3.19 Carbohydrate media for fermentation tests

Purple Broth Base

Peptone (Oxoid)	10 g
Beef extract/Lab-lemco powder (Oxoid)	1 g
Sodium chloride (Sigma)	5 g
Bromcresol purple (Sigma)	15 mg
Distilled water	1000 mL

All ingredients were dissolved in distilled water, and the pH adjusted to 6.8. The broth was dispensed in nine flasks, each containing 90 mL and autoclaved at 121°C for 15 minutes. Nine sugars, all purchased from Sigma company included lactose, fructose glucose, sucrose, sorbitol, xylose, dulcitol, mannitol and salicin were prepared in 10% aqueous solutions and sterilised using 0.02 µm membrane filters. To the 90 mL purple broth base was added 10 mL of sugar solutions (1% final concentration). Before dispensing 5 mL carbohydrate broth in screw cap tubes, Durnham tubes was inserted into the tube to detect gas formation.

3.20 Catalase test

A small amount of bacterial colony was placed on a clean glass slide, and a drop of 3% H_2O_2 was added. A positive result showed the presence of bubbles of oxygen.

3.21 Sterile phosphate buffered saline

Sodium chloride (NaCl) (Sigma)	8 g
Potassium chloride (KCl) (BDH)	0.2 g
Potasium dihydrogen phosphate (KH_2PO_4) (Sigma)	0.2 g
Sodium hydrogen phosphate (Na_2HPO_4) [or ($Na_2HPO_4.2H_2O$) (1.44 g)] (Unilab, Ajax, Chemicals)	1.15 g
Distilled water	1000 mL

All ingredients were dissolved in distilled water in a boiling water bath and autoclaved at 121°C for 15 minutes.

3.22 Disk diffusion susceptibility method (Isenberge, 1992)

Mueller-Hinton agar (MHA) supplemented with 9% sheep (see section 1.3) blood (15 mL in a 90 mm Petri plate) and the bacterial inoculum adjusted to a McFarland 0.5 turbidity standard were prepared. A sterile cotton swab dipped into the inoculum was swab three times to the entire surface of the agar plates, rotating the plate approximately 600 between streaking to ensure even distribution. Avoid hitting the sides of Petri plate and creating aerosols. The plates were allowed to stand at room temperature for three minutes but no longer than 15 minutes before applying the disks using the disk dispenser (Oxoid). All antibiotics were purchased from Oxoid, ciprofloxacin (5µg), cefotaxim (30 µg), cloxacillin (5 µg), penicillin (10 units), ampicillin (10 µg), streptomycin (10 µg), erythromycin (15 µg) and tetracycline (30 µg).

Appendix 4

Reagents Used in DNA Extraction

4.1 TE buffer at pH 8

Distilled water	500 mL
100mM Tris, MW 121.1 (Sigma)	5 g
10 mM EDTA, MW 372.2 (Sigma)	1.861 g

Note. To compute for one molar, weigh the chemicals in grams based on the molecular weight of the chemical compound and dissolved in 1 L of distilled water.

4.2 Lysis buffer

Tris at pH 9.5 (Sigma)	10 mM
EDTA (Sigma)	100 mM
Lysozymes (Sigma)	10 mg/mL

Note. Store the solution in a freezer.

4.3 100 mM of EDTA solution

EDTA (MW 372.2) (Sigma)	35.22 g
Distilled water	1000 mL

4.4 20% Sodium dodecyl sulfate (SDS)

SDS (Sigma)	20 g
Distilled water	100 mL

4.5 2 mL Proteinase K (10 mg/mL)

Proteinase K (Sigma)	20 mg
Distilled water	2 mL

Note: Aliquot in two Eppendorf tubes were stored in a freezer.

4.6 100 mL 6 M sodium chloride

Sodium chloride (MW 58.44) (Sigma)	35.064 g
Distilled water	100 mL

4.7 2 mL RNAse A (30mg/mL)

RNAse (Sigma)	60 mg
Sterile distilled water	2 mL

Note: Aliquot in two Eppendorf tubes were stored in a freezer.

4.8 10 mM Tris at pH 9.5

Tris (Sigma)	0.121 g
Distilled water	100 mL

4.9 10X TBE

Tris (Sigma)	108 g
Boric acid (borate) (Sigma)	55 g
0.5 M EDTA (pH 8)	40 mL
Distilled water	1000 mL

4.10 CTAB/NaCl solution

NaCl	4.1 g
Distilled water	80 mL
Hexadecyltrimethyl amonium bromide (CTAB) (Sigma)	10 g

Procedure

Dissolve 4.1 g NaCl in 80 mL of sterile distilled water, and 10 g of CTAB was slowly added while heating and stirring. If necessary, heat to 65°C to dissolve. The final volume was adjusted to 100 mL.

4.11 3 M Sodium acetate

Sodium acetate.$3H_2O$ (Sigma)	408.1 g
Distilled water	800 mL

Procedure

The sodium acetate was dissolved in distilled water and the pH was adjusted to 7.0 with dilute acetic acid. The volume was adjusted to 1000 mL with distilled water and sterilised by autoclaving.

Appendix 5

Reagents for Ribotyping

5.1 200 mM EDTA

EDTA (Sigma)	74.44 g
Sterile distilled water	1000 mL

5.2 6X Gel-loading buffers

Bbromophenol blue (Sigma)	0.25%
EDTA (Sigma)	10 mM
Glycerol in water (APS)	30%

Note: The solution was stored at 4°C.

5.3 1X TBE

Tris (Sigma)	10.8 g
Boric acid (Sigma)	5.5 g
0.5 M EDTA (pH 8.0) (Sigma)	4 mL

5.4 20X SSC

NaCL (Sigma)	175.3 g
Sodium citrate (Sigma)	88.2 g

Procedure

The NaCl and sodium citrate were dissolved in 800 mL of sterile distilled water. The pH was adjusted to 7.0, and the solution was added with sterile distilled water up to 1000 mL volume.

5.5 Ethidium bromide (10 mg/mL)

Ethidium bromide (Boehringer, Mannheim)	1 g
Distilled water	100mL

Procedure

The ethidium bromide was dissolved in distilled water using a magnetic stirrer for several hours to ensure the dye has dissolved. The solution was transferred to a clean bottle and wrapped in aluminum foil and stored at room temperature.

5.6 5X cDNA buffer

Tris at pH 8.5 (Sigma)	250 mM
Magnesium chloride (Sigma)	40 mM
Dithiothreitol (Sigma)	150 mM

5.7 Maleic acid buffer/washing buffer 1

Maleic acid (Sigma)	3.1 M
NaCl (Sigma)	0.02 M

5.8 Washing buffer 2

Maleic acid/washing buffer 1 (Sigma)	1000 mL
Tween 20 (Ajax, chemicals)	0.3%

5.9 2X wash

2X SSC (100mL of 20X SSC + 900mL distilled water)	
SDS (5 mL of 20% SDS or 1 g SDS)	3.2%

5.10 Prehybridisation buffer

5X SSC (250 mL of 20X SSC + 750 mL distilled water)	
N-laurylsarcosine or 1 gram N—laurylysarcosine (Sigma)	3.3%
SDS (1 mL of 20% SDS or 0.2 g SDS)	0.02%
Blocking stock	10%

5.11 0.1X wash

20X SSC	5 mL
Distilled water	995 mL

5.12 Blocking agents stock solution

Blocking reagent (supplied in a kit by Boehringer, Mannheim)	20 g
Maleic acid buffer/buffer 1	200 mL

Procedure

The blocking reagent was dissolved in maleic acid buffer/ buffer 1 in a hot stirring plate at 60°C approximately one hour until in solution, and sterilised by autoclaving.

5.13 Buffer 3/Detection buffer

Tris (pH 9.5) (Sigma)	100 mM (or 12.11 g)
NaCl (Sigma)	100 mM (or 5.844 g)
Distilled water	1000 mL

5.14 Denaturation buffer

NaCl (Sigma)	1.5 M (or 87.67 g)
NaOH (Sigma)	0.5 M (or 20 g)
Distilled water	1000 mL

5.15 Neutralisation buffer

Tris (Sigma)	0.5 M (60.55 g)
NaCl (Sigma)	1.5 M (87.67 g)
EDTA (Sigma)	2.5 mM (0.93 g)
Distilled water	1000 mL

Appendix 6

Tissue Culture Media

6.1 X 2.5 stock DMEM

Ingredients

DMEM, powdered Dulbecco's Modified Eagle's medium for 10 litres with L-Glutamine, without sodium bicarbonate (Multicel, Trace, Cat. No. 50-013-PB)	133.7 g
Penicillin, Na benzyl	1.2 g
Streptomycin sulphate	2.0 g
Polymyxin B	0.05 g
Kanamycin (or Antibiotic solution 100 mL)	0.8 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
1N HCl	5 mL

Method

All the solid ingredients were dissolved in about 3.5 litres of fresh triple distilled water and 1N HCl was added. Four litres of fresh triple distilled water was added and stirred well for about five minutes. The solution was filtered using ONE MediaKap-10 hollow fibre media filter (0.2 µm for 10 litres). The filtered solutions were collected in 600 mL aliquots into suitable sterile bottles with well sealing caps and stored at 4°C.

Normal strength DMEM was prepared by adding 6 mL of fungizone solution for each 600 mL of stock medium. Mix well and measure out 200 mL of stock media into 300 mL of sterile triple distilled water.

6.2 X10 ATV solution

Ingredients

Trypsin (1:250, Sigma, Cat. No. T-0646)	5.0 g
Versene (Na EDTA)	2.0 g
Sodium chloride, NaCl	80.0 g
Potassium chloride, KCl	4.0 g
Sodium bicarbonate, $NaHCO_3$	2.5 g
Glucose	10.0 g
Phenol red, Na salt (Sigma, Cat. No. P-4758)	0.2 g

Method

All ingredients were dissolved in approximately 900 mL of sterile triple distilled water, and stirred for 30 minutes at room temperature. The solution was incubated in a water bath at 45° to 50°C for 30 minutes. The sterile triple distilled water was added to make up 1000 mL solution, and sterilised by filtering through a 0.2 µm Medicap-10 filter. The sterilised solution was dispensed into 100 mL aliquots and stored at –20°C in the freezer.

Buenviaje, G.N., Hirst, R.G., Ladds, P.W. and Millan, J.M. (1997) Isolation of *Dermatophilus* sp from skin lesions in farmed saltwater crocodiles (Crocodylus porosus). Australian Veterinary Journal. 75: 365-367.

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