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Research Article

Unbiased DNA Pathogen Detection in Tissues: Real-World Experience With Metagenomic Sequencing in Pathology

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ABSTRACT

Pathogen detection in formalin-fixed paraffin-embedded (FFPE) tissue remains challenging. We implemented metagenomic next-generation sequencing (mNGS) in our clinical diagnostic workflow to evaluate its feasibility, diagnostic yield, and pathogen spectrum in routine infectious pathology cases. Between November 2021 and April 2025, we analyzed 623 FFPE tissue samples using a low-depth mNGS workflow on the Thermo Fisher Ion Torrent platform with a CLC Genomics Workbench (Qiagen) bioinformatics pipeline. Our assay was designed to detect DNA pathogens. When possible, results were validated by orthogonal methods, including species-specific PCRs, *16S*/internal transcribed spacer PCR, and immunohistochemistry on tissue sections. Among 623 samples analyzed, at least 1 potentially pathogenic and plausible microorganism was identified in 229 samples (36.8%), whereas 334 (53.6%) were negative and 60 (9.6%) were uninterpretable due to quality control failures or suspected contamination. Of the 229 positive samples, 145 (63.3%) involved bacteria, 37 (16.2%) viruses, 28 (12.2%) fungi, and 9 (3.9%) parasites; mixed infections with >1 pathogen were detected in 10 (4.4%) samples. The most frequently identified bacterial family was Mycobacteriaceae (n = 27), including *Mycobacterium xenopi* (n = 8), which is not routinely covered by syndromic multiplex PCR panels. Notable viral and fungal detections included a novel human circovirus and *Coccidioides posadasii*. Despite variable sample quality and DNA input, mNGS yielded reliable results in a wide range of tissue types. mNGS is a feasible, valuable addition to routine infectious pathology diagnostics, particularly in complex or inconclusive cases. The assay improved the diagnostic yield compared with conventional PCR, expanded the range of detectable pathogens, and proved robust even in

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low-quality FFPE samples. These results support broader adoption of mNGS in tissue-based pathogen diagnostics.

Introduction

Diagnosing infectious diseases in formalin-fixed paraffin-embedded (FFPE) tissue samples remains a significant challenge in routine pathology.[1] In addition to microscopic evaluation of tissues with special stains or immunohistochemistry, routine pathology practice relies on pathogen-specific PCR assays to detect infections in FFPE tissue.[2] Although targeted PCR assays have long been a cornerstone of molecular pathogen detection, their diagnostic value is limited by nucleic acid degradation in FFPE samples, the need for prior assumptions about potential pathogens, and the clinical heterogeneity of chronic and immune-mediated infections. Unusual or rare pathogens are often not covered by targeted PCR and conventional sequencing.[3] For pathogens without commercially available assays, laboratory-developed tests that require extensive validation must be established.[4,5] Furthermore, stepwise testing for multiple pathogens is time consuming, consumes precious material from small biopsies, and delays diagnosis and adequate treatment of patients. The number of tests is constrained by the limited amount of DNA that can be extracted from small tissue biopsies. Despite extensive testing with conventional methods, the majority of pathogens and infectious diseases in pathology remain without a definitive diagnosis, largely because of nucleic acid degradation in FFPE samples, the restricted scope of targeted assays, and limited biopsy material. These limitations are particularly pronounced in cases involving persistent, rare, unculturable, or emerging organisms.[6,7]

Metagenomic next-generation sequencing (mNGS) offers an unbiased alternative, enabling simultaneous detection of bacteria, viruses, fungi, and parasites in a single assay without pathogen-specific primers.[8] Initially developed for research applications, mNGS has increasingly been adopted in diagnostic laboratories due to improvements in cost effectiveness and workflow integration.[9,10] To date, most clinical applications have focused on acute infections using samples with low host background, such as cerebrospinal fluid or plasma cell-free DNA, where the detection of a microbial species is usually sufficient evidence of infection.[9,11-15] However, the application of mNGS to FFPE tissue poses unique challenges, including degraded nucleic acids, high human background, and the contextual complexity of chronic inflammation.

In late 2021, we implemented a cost-efficient, low-depth mNGS assay, optimized for FFPE tissues, into our routine diagnostic workflow for infectious disease pathology (for comparison with other mNGS studies, see Supplementary Table S1).[16] As a national referral center, we receive highly complex cases from external institutions, often with limited material, inconclusive prior results, and challenging histopathology. Over the past 3.5 years, we have applied mNGS to 623 patient samples across diverse tissue types and clinical scenarios, including persistent inflammation, rare or novel pathogens, and diagnostically ambiguous cases.

In this study, we present our real-world experience with mNGS in tissue-based infectious diagnostics. We assess its performance and accuracy, highlight advantages over conventional methods, and discuss its role in detecting nonculturable and novel pathogens. We also address practical considerations, such as input limitations, contamination risks, and the importance of diagnostic stewardship. Our findings demonstrate the feasibility and clinical value of integrating mNGS into routine pathology workflows.

Methods

Patients and Sample Collection

Data for this study were derived from samples submitted for routine clinical diagnostics between November 2021 and April 2025. External providers either explicitly requested mNGS or ordered a panel of PCR tests, indicating a nonspecific suspicion of pathogen presence. In the latter cases, mNGS was performed at our discretion, particularly when sample quantity was limited. The cohort includes 623 samples from 568 patients, most of whom were referred from external health care institutions (Table 1). Typically, a single FFPE tissue block was available per case. Hematoxylin and eosin staining was performed for histopathological assessment, after which the remaining tissue was processed for nucleic acid extraction. Additional immunohistochemical stains were performed when necessary to validate metagenomic findings. Where available, patient metadata, such as immune status, clinical history, symptoms, and histopathological findings (eg, the presence of

Table 1
Patient characteristics

Patient characteristics		No. (% of patients, n=568)
Sex	Female	269 (47.4%)
	Male	299 (52.6%)
Age (y)	<18	21 (3.7%)
	18-64	359 (63.2%)
	65+	188 (33.1%)
Immunosuppression	Any immunosuppression	73 (12.9%)
	HIV	10
	Hematopoietic stem cell transplant	13
	Solid organ transplant	9
	Chemotherapy	8
	Leukemia	16
	Lymphoma	11
	Lupus	2
	Rheumatoid arthritis	4

Demographic and clinical characteristics of patients whose formalin-fixed, paraffin-embedded tissue samples were submitted for metagenomic next-generation sequencing (mNGS) analysis. A total of 568 patients were included in the study, with 623 samples analyzed overall, as some patients underwent >1 mNGS analysis. The table includes sex distribution, age groups, and presence of immunosuppressive conditions, including HIV infection, transplantation status, hematologic malignancies, and autoimmune diseases.

acid-fast rods in cases with suspected mycobacterial infection), were included to contextualize the sequencing results (Table 2).

Sample Preparation

FFPE blocks were sectioned using a microtome under a stringent cleaning protocol, including blade cleaning with DNase treatment, to minimize crosscontamination and microbiome carryover between samples. When sufficient material was available, tissue sections were mounted on glass slides, and areas of interest were selectively microdissected under the guidance of a board-certified pathologist (K.D.M.). For samples with limited material (<5 mm of tissue), paraffin rolls were collected directly from the block and processed without microdissection.

DNA extraction was performed using the EZ1/2 DNA Tissue Kit (Qiagen; catalog number: 953034) on the EZ1 Advanced XL (Qiagen; catalog number: 9001874) and EZ2 Connect (Qiagen; catalog number: 9003210) automated platforms. DNA concentrations were quantified using the Qubit dsDNA HS Assay (Thermo Fisher Scientific; catalog number: Q32854) on Qubit 2.0 (Thermo Fisher Scientific; catalog number: Q32866) and DeNovix QFX fluorometers.

Table 2
Clinical indications for metagenomic next-generation sequencing testing across all analyzed samples

Clinical indications	No. (%) of samples (n = 623)
Lung tumor/lesion	104 (17.0%)
Lymphadenopathy/lymphadenitis	99 (15.9%)
Inflammatory dermatoses	67 (10.7%)
Inflammatory conditions of the gastrointestinal tract (colitis: 7, enteritis: 10, esophagitis: 5, gastritis: 3, proctitis: 6)	31 (5.0%)
Bronchitis/pneumonia	22 (3.5%)
Arthritis/synovialitis/bursitis	20 (3.2%)
Liver tumor/lesion	18 (2.9%)
Soft tissue inflammation	17 (2.8%)
Hepatitis/cholangitis	16 (2.6%)
Mucositis/ulcer of mucous membranes	12 (1.9%)
Spondylodiscitis	12 (1.9%)
Endocarditis/myocarditis	11 (1.8%)
Brain tumor/lesion	10 (1.6%)
Eye inflammation	10 (1.6%)
Interstitial nephritis	9 (1.4%)
Osteomyelitis	9 (1.4%)
Placental pathologies	9 (1.4%)
Abnormal blood count	8 (1.3%)
Encephalitis	8 (1.3%)
Prosthetic joint infection	8 (1.3%)
Peritonitis/pleuritis/pericarditis	7 (1.1%)
Inflammatory conditions of the male reproductive tract	6 (1.0%)
Mastitis	6 (1.0%)
Skin fistula/ulcer	6 (1.0%)
Pyelonephritis	5 (0.8%)
Vascular damage/inflammation	4 (0.6%)
Bone tumor/lesion	3 (0.5%)
Inflammatory conditions of the female reproductive tract	4 (0.6%)
Pancreatitis	3 (0.5%)
Bladder tumor/lesion	2 (0.3%)
Meningitis/meningoencephalitis	2 (0.3%)
Splenitis	2 (0.3%)
Miscellaneous	23 (3.7%)
Insufficient clinical information	50 (8.0%)

Overview of clinical indications for metagenomic next-generation sequencing based on 623 FFPE tissue samples submitted between 2021 and 2025. Samples were grouped according to the primary pathological question or affected organ system, as documented on the pathology request forms or accompanying clinical information. The most frequent indications included lung lesions, lymphadenopathy, and inflammatory dermatoses. Gastrointestinal inflammation is further broken down by anatomical location (eg, colitis and enteritis). In 50 cases, the available clinical information was insufficient for classification. This table illustrates the wide range of diagnostic contexts in which metagenomic next-generation sequencing was applied.

Pathogen Detection Via Metagenomic Sequencing

NGS was performed as previously described.[16] For more recent cases that began in January 2025, we introduced a spike-in positive control consisting of fragmented genomic DNA from *Thermus thermophilus* HB8 (Takara; catalog number: 3071), which was added prior to end repair during library preparation.

Bioinformatic analyses were performed using CLC Genomics Workbench (versions 22-25; Qiagen) with the Microbial Genomics Module. Taxonomic profiling was conducted with the following settings: “Auto-detect paired distances” (disabled), “Minimum seed length” (30), and “Adjust for read length variation” (enabled). To enhance host-read depletion, we implemented an additional mapping step against the CHM13 human reference genome.[17] This optimization significantly improved signal-to-noise ratios, particularly for low-abundance fungal and parasitic reads.

The taxonomic classification database was continuously expanded during the study period by incorporating additional reference genomes from RefSeq,[18] prioritizing clinically relevant species. A complete and updated list of reference organisms used is available in Supplementary Data S1, which includes 6066 bacteria, 9165 viruses, 2255 fungi, and 334 parasite and protozoa genomes. As described in our previous publication, only bacterial, fungal, and parasite/protozoa genomes with a minimum length of 100,000 bp and viral genomes longer than 1000 bp were included.[16]

Criteria for Pathogen Positivity

Our workflow is designed as a low-cost and shallow-depth mNGS assay for detecting clinically relevant pathogens in FFPE tissue samples. It is not intended for microbiome profiling or high-resolution strain typing, which require greater sequencing depth. The resolution and sensitivity of our mNGS assay are inherently limited by its intentionally shallow sequencing depth, which is optimized to keep costs low. The primary objective is to detect pathogens present at sufficient abundance to account for the clinical and histopathological manifestations. In such cases, the causative pathogen is expected to generate enough reads to rank among the top hits in its taxonomic category.

Due to variability in DNA quality and quantity, sequencing depth, and tissue-specific microbial backgrounds, no universal read count threshold was applied. Instead, positivity was determined contextually, integrating read counts, pathogen rank, and case-specific features, such as tissue type and histopathological findings. For example, low-abundance pathogens in sterile samples (eg, brain and cerebrospinal fluid) may be readily detected with just a few dozen reads. In contrast, samples from microbiota-rich environments, such as the colon or skin, may require higher thresholds, as moderately abundant pathogens

can be masked by the background signal of resident microbial species, which often generate thousands of reads. These dynamics are further influenced by the amount of host (human) DNA present in the sample, which can dilute the detectable pathogen signal.

We used *Cutibacterium acnes* as an internal benchmark for assessing relative abundance. This species is consistently detected in nearly all samples in our data set and correlates with input DNA quantity. Hits near or exceeding the abundance of *Cutibacterium acnes* were evaluated for plausibility and reported when considered clinically relevant. Species with fewer than 5 reads were generally disregarded, except for obligate and well-characterized low-load pathogens such as *Mycobacterium tuberculosis*, which were carefully interpreted and systematically validated (eg, by species-specific PCR assays). For pathogenic viruses known to reactivate under immunosuppression, a reporting threshold of 20 reads was applied. For example, Epstein-Barr virus (EBV) was detected in 58 samples, but only 3 showed sufficiently high read counts to be considered clinically significant.

Controls and Quality Assurance

Positive and negative controls were integrated into our routine workflow throughout the study. A paraffin-only FFPE block was included in each processing batch and sequenced at 1 million reads to assess background contamination. This control typically yields only low read counts but allows us to identify persistent environmental or laboratory contaminants.

The *T. thermophilus* HB8 (Takara; 3071) spike-in control, diluted to yield ~500 reads per sample, served as a positive process control, verifying successful library preparation and detecting potential PCR inhibitors or workflow issues.

Validation of Metagenomic Next-Generation Sequencing Findings

To ensure diagnostic accuracy, mNGS results were validated, when possible, using orthogonal methods. These included a targeted amplicon-based PCR approach: a broad-range 16S ribosomal RNA gene PCR for detecting eubacterial DNA and an internal transcribed spacer (ITS)–based PCR for pan-fungal screening. DNA previously extracted from the FFPE samples was used directly for these confirmatory assays without further purification. The primer sets used for both *16S* ribosomal RNA and ITS amplification have been previously published and validated for broad-range microbial detection in clinical specimens.[19,20] Library preparation was performed using the Rapid Barcoding Kit 96 (SQK-RBK114.96), and sequencing was carried out on the GridION platform with SpotON Flow Cells (FLO-MIN106D, R9.4.1) (all: Oxford Nanopore Technologies). Resulting reads were aligned against the National Center for Biotechnology Information's 16S_ribosomal_RNA and ITS_RefSeq_Fungi BLAST databases (version 2024-05-16).[21] For each read, up to 100 top-ranked hits were retained, and lower-ranked matches were discarded. Subsequently, for each sample, we filtered the alignment results for entries matching the expected mNGS species name and counted the number of unique reads assigned to that species.

In-house pathogen-specific PCR assays and immunohistochemistry using validated antibodies were also employed to confirm selected mNGS findings. Details are provided in Supplementary Table S2.

External results were systematically taken into account and assessed alongside mNGS findings and in-house validations. In cases where external microbiological data were available—such as culture, PCR, or serology results from referring institutions—they were carefully reviewed and integrated into the diagnostic interpretation, particularly when they supported or contradicted the sequencing results. These complementary validations provided additional confidence in our mNGS assay, particularly for rare or low-abundance findings, and were used to refine interpretation criteria during the workflow's implementation phase.

Our interpretation process minimizes the risk of false-positive results. Access to the full metagenomic data is restricted to trained personnel who are familiar with common background species and typical contaminants, enabling reliable filtering of irrelevant hits. The candidate pathogens that remain are subsequently evaluated in a clinical context by the pathologist, who determines which findings merit reporting. Moreover, the comprehensive nature of mNGS allows for a clearer distinction between true pathogens and background organisms—an advantage over pan-fungal or pan-bacterial PCRs, which lack this level of taxonomic resolution.

Results

Since its introduction in late 2021, our mNGS assay has been applied to 623 samples (Fig. 1A). Initially reserved for complex cases or inconclusive pan-bacterial/pan-fungal PCRs, mNGS has become widely accessible to both pathologists and clinicians.[16] Over the past 4 years, the number of mNGS assays performed has steadily increased, whereas the use of species-specific PCR assays declined, highlighting a shift toward mNGS as a preferred diagnostic tool in tissue pathology (Supplementary Fig. S1A). Most samples were submitted by external pathologists (83.9%, Fig. 1A) and typically consisted of a single, representative FFPE block (Table 1). Clinical information varied widely, from minimal to detailed histories, including immune status, treatments, prior diagnostics, and travel history. Importantly, all FFPE blocks were processed at the referring institutions.

Most samples were submitted to investigate unresolved clinical questions, including radiologically detected tumorous lung lesions, lymphadenopathies/lymphadenitis, and inflammatory dermatoses, often in the context of unclear histopathological findings or persistent inflammation (Table 2).

Of the 623 samples, at least 1 potentially pathogenic microorganism was identified in 229 cases (36.8%), whereas 334 samples (53.6%) were negative and 60 (9.6%) were uninterpretable due to quality control failures or suspected contamination (Fig. 1A). The mNGS positivity rate exceeded that of most individual PCR assays in our laboratory, which typically remains <20%, with the exception of pan-fungal and *Echinococcus* PCRs, likely due to their easier histopathological detection and targeted preselection. The modestly higher failure rate of mNGS compared with PCR likely reflects its higher DNA input requirement (250 ng), which can be challenging to obtain from small FFPE samples, and its ability to exclude contaminated samples, based on unexpected microbial profiles. In contrast, although individual PCRs are more sensitive and require less input, they are more susceptible to degradation-related artifacts, such as limited template length. Consequently, a significant number of PCRs failed due to insufficient amplicon generation, as indicated by internal control (human β-globin) failure (Supplementary Fig. S1B).

Among the 229 mNGS-positive cases, 145 (63.3%) identified bacteria, 37 (16.2%) viruses, 28 (12.2%) fungi, and 9 (3.9%) parasites. In 10 cases (4.4%), >1 pathogen was detected (Fig. 1A),

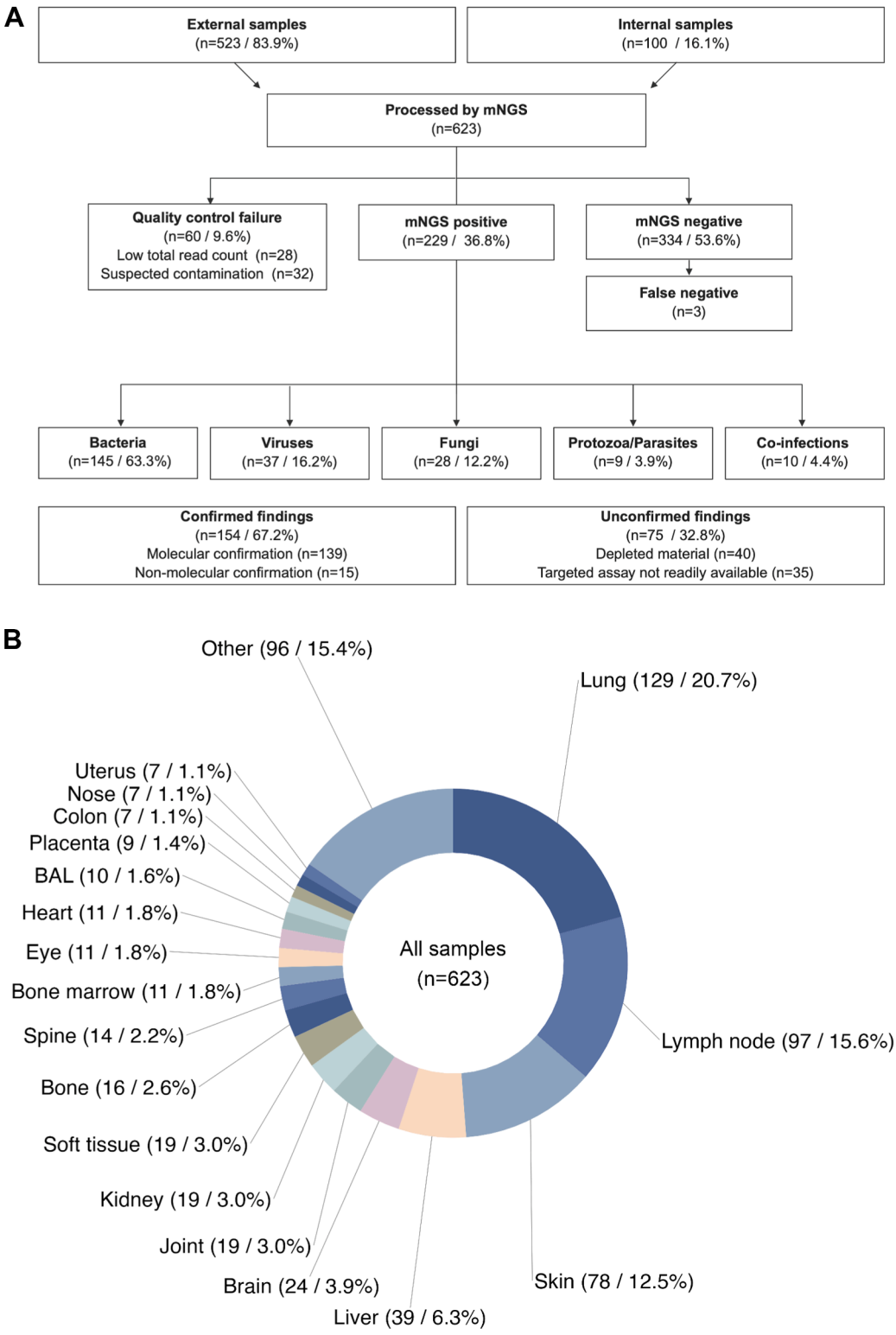

Figure 1.
Overview of sample characteristics and detection rates. (A) Total number of formalin-fixed paraffin-embedded tissue samples submitted for metagenomic next-generation sequencing (mNGS) between November 2021 and April 2025 (n = 623), stratified by diagnostic outcome: mNGS positive (pathogen detected), mNGS negative (no pathogen detected), or uninterpretable due to quality control failure or suspected contamination. Of the 229 positive cases, 154 (67.2%) were confirmed by orthogonal methods, including in-house PCRs, immunohistochemistry, special stains, and/or microbiology results (Fig. 3). For the remaining 75 (32.8%) cases, the positive mNGS result could not be confirmed due to either insufficient residual material for further testing or the absence of a readily available targeted assay. (B) Distribution of tissue types submitted for mNGS analysis. Lung, lymph node, and skin were the most frequently represented tissues, reflecting common clinical indications for infection diagnostics in these organs. BAL, bronchoalveolar lavage.

highlighting the breadth of detectable organisms and the versatility of the mNGS assay in routine infectious pathology. The mNGS assay was applied to a broad spectrum of FFPE samples. Lungs were the most frequent tissue type (129, 20.7%), followed by lymph nodes (97, 15.6%) and skin (78, 12.5%) (Fig. 1B). Relevant pathogens were identified across all tissue types analyzed (Supplementary Fig. S1C).

To assess the added value of mNGS over PCR, we categorized our findings by prevalence and culturability (Fig. 2A, Supplementary Data S2). A substantial proportion represented unexpected or unculturable pathogens, underscoring the advantage of an unbiased sequencing approach. Among bacterial findings (n = 171), *Mycobacteriaceae* was most frequent (n = 27), followed by *Enterobacterales* (n = 14), *Fusobacteriaceae* (n = 12), *Streptococcaceae* (n = 11), and *Staphylococcaceae* (n = 11) (Fig. 2B, Supplementary Fig. S2). Although mycobacteria are frequently suspected and routinely targeted by PCR (eg, *M. tuberculosis*, *Mycobacterium avium*, and *Mycobacterium leprae*), mNGS allowed detection of atypical species like *Mycobacterium xenopi* (n = 8), which are not routinely covered.

Viral (n = 43) and fungal (n = 33) detections were less frequent but included clinically relevant and unexpected pathogens (Fig. 2C, D). Notable examples include a novel human circovirus in an immunocompromised patient and 2 cases of *Coccidioides*, a nonendemic pathogen in Switzerland.[22] EBV was the most frequently detected virus. However, in the vast majority of EBV-positive cases, only very low read counts (up to 20 reads) were observed, indicative of low-level reactivation without clinical significance. Accordingly, these low-level detections were generally not reported. EBV was considered clinically relevant and reported as the likely causative pathogen in only 3 cases, based on the combination of clinical, histopathological, and molecular findings. RNA viruses were not covered by our assay.

Parasitic infections were rare in our cohort (n = 13), likely due to diagnostic reliance on blood/stool samples and histopathology (Fig. 2E). mNGS nonetheless enabled the detection of *Leishmania braziliensis* and *Leishmania panamensis*, not identifiable by our in-house *Leishmania* PCR.

Although the assay was validated on large autopsy lung tissues and culture standards, it performed well on routine FFPE samples, including those with limited tissue quantity or quality (Fig. 3, Supplementary Data S3). Input DNA requirements (250 ng) were met in 67.1% of cases (n = 418; median, 16.0 ng/μL; IQR, 4.12-37.1 ng/μL), though lower inputs (as low as 100 ng) also yielded interpretable results. Lower DNA quantities were common in acellular or fibrotic tissues and liquids. Input quality directly impacted library yield, which reached the target depth of 5 to 10 million reads in 83.5% of samples (n = 520; median, 12,505,916 reads; IQR, 7,220,944-16,347,085 reads).[16] Even suboptimal libraries (read count < 5 M and/or concentration < 6.4 ng/μL DNA) yielded relevant findings, with pathogens identified in 18 of 96 such samples. Bone and bone marrow samples frequently failed due to nucleic acid degradation caused by decalcification. One false-negative mNGS result occurred in such a sample, where *M. tuberculosis* was detected only by conventional PCR/real-time PCR. A second false negative involved a lymph node biopsy with low DNA input, in which *Francisella tularensis* was not detected by mNGS but was identified by serology and subsequently confirmed by PCR. In a third case, mNGS failed to detect *Oxyporus corticola*, a fungus for which no reference genome is currently available. Although a systematic review of all negative mNGS cases was not feasible, parallel species-specific PCRs were performed in 208 (33.4%) cases and did not reveal any additional false-negative results.

Most quality control failures occurred in samples with low input and poor library yield (Fig. 3, bottom left quadrant). However, we also refrained from interpretation in samples with adequate metrics when contamination was suspected or when results appeared implausible (eg, mismatched microbiome profiles or unlikely environmental species such as plant pathogens). Microtome-related contamination was a common concern, particularly in low-microbiome samples (eg, brain, joint and synovial tissue) processed/sectioned after microbiome-rich tissues (eg, gut). Since sectioning is often performed by external institutions, contamination introduced during this step is difficult to identify without sequencing-based quality control. For example, repeated detection of *Burkholderia cenopacia* in DNA extracts from 1 single institution (submitted as preextracted DNA rather than tissue blocks) raised suspicion of environmental contamination. Another common scenario involved low-input samples (< 100 ng) yielding unexpectedly rich microbial profiles, likely due to overamplification of environmental DNA in the absence of sufficient host material.

Of 229 positive cases, 154 (67.2%) were confirmed by in-house PCRs, immunohistochemistry, special stains, and/or microbiology results (Fig. 3; full vs empty circles, Supplementary Fig. S3A). Rare pathogens were validated in collaboration with the Microbiology and Virology Units of our institution. Unconfirmed cases could not be validated due to depleted material or lack of suitable assays, precluding orthogonal confirmation.

mNGS proved particularly useful in replacing multiple targeted PCRs. In cases where clinicians requested several assays (sometimes up to 8), mNGS offered a comprehensive alternative with comparable input requirements. Such requests often reflect diagnostic uncertainty, as pathogens rarely display distinctive histopathological features and are difficult to identify using routine stains, even for experienced pathologists. We reviewed cases in which a specific pathogen was suspected based on clinical information and found that <20% of these suspicions were confirmed by mNGS (Supplementary Fig. S3B).

The morphological assessment of tissue inflammation often fails to provide pathogen-specific clues. In particular, granulomatous inflammation can appear remarkably similar across a wide range of causative agents, including mycobacteria and other bacteria, fungi, and selected viruses (Fig. 4,[23] Supplementary Fig. S4). Despite the diversity of underlying etiologies, the histological patterns overlap substantially, often showing non-caseating or necrotizing granulomas, and in some cases, indistinct inflammatory changes.

Discussion

This study provides a comprehensive overview of mNGS use in infectious disease pathology >3.5 years in routine diagnostic practice, encompassing 623 samples and identifying plausible pathogens in 229 of these samples. Initially reserved for complex or inconclusive cases, mNGS has since become broadly accessible to both pathologists and clinicians and has proven valuable in resolving diagnostically challenging cases.[11] The assay performed reliably across a wide range of real-world FFPE samples, despite variable input DNA quantity and quality.[1]

Unlike most mNGS approaches focusing on critically ill patients and requiring deep, high-cost sequencing for rapid pathogen detection, our approach prioritizes accessibility and cost efficiency in routine pathology.[8] Developed for FFPE samples from nonacute cases, it balances sensitivity and affordability,

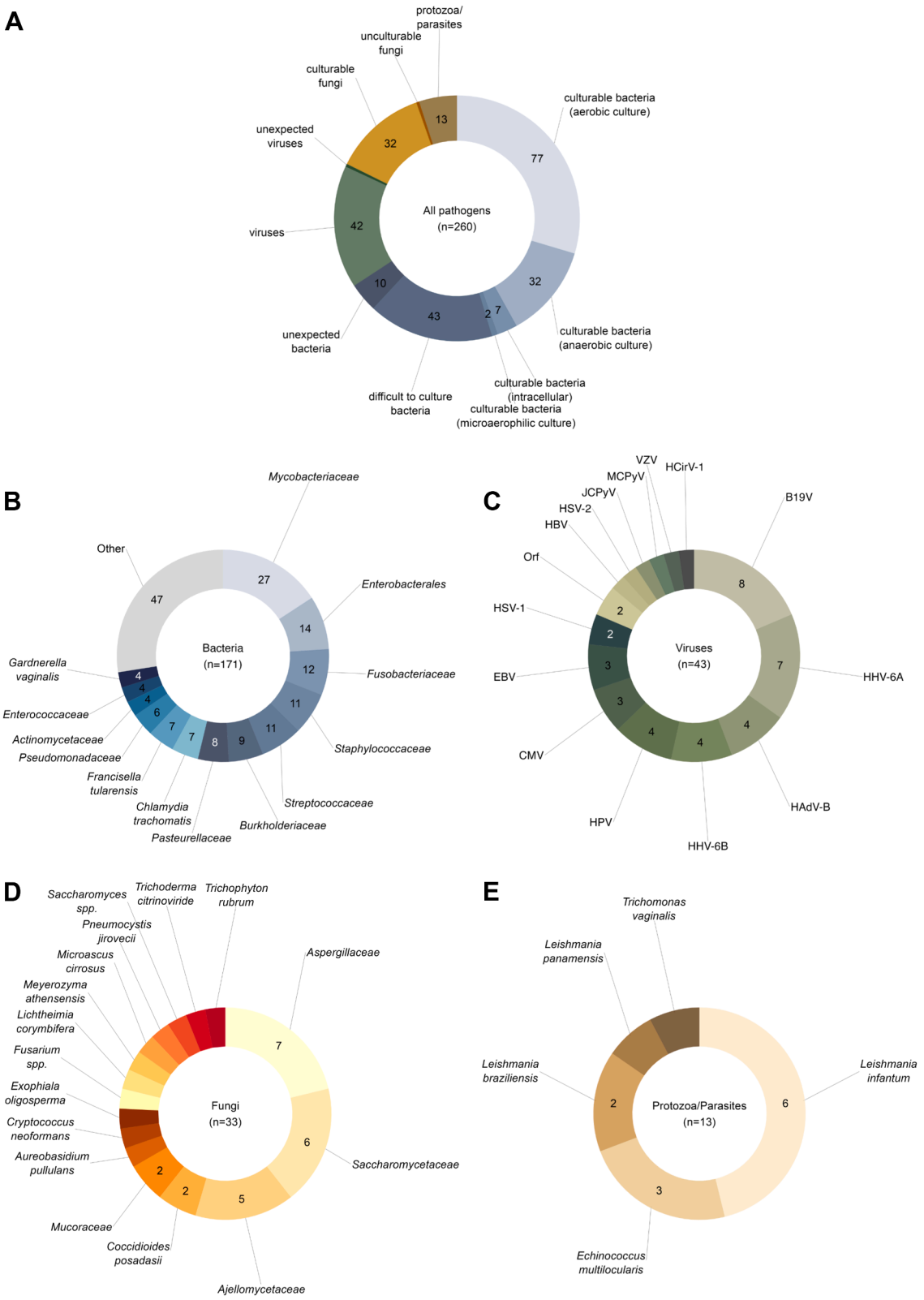

Figure 2.

Diversity and clinical relevance of pathogens identified by metagenomic next-generation sequencing (mNGS). (A) Overview of all pathogens detected (n = 260), grouped by microbial class (bacteria, viruses, fungi, and parasites), culturability (culturable vs unculturable), and prevalence, highlighting the added value of mNGS in identifying unexpected or difficult-to-culture organisms (Supplementary Data S2). (A) Distribution of detected bacterial taxa (n = 171), with Mycobacteriaceae being the most common family. The mNGS assay identified both typical and atypical species, including *Mycobacterium xenopi* (n = 8), which are often missed by routine PCR panels. (B) Detected DNA viral taxa (n = 43), predominantly from the Herpesviridae family. Occasional detection of unusual or novel viruses, such as human circovirus type 1 (HCirV-1), underscores the added value of unbiased sequencing. (C) Fungal pathogens (n = 33) spanned a broad phylogenetic spectrum, including rare or nonendemic species such as *Coccidioides*

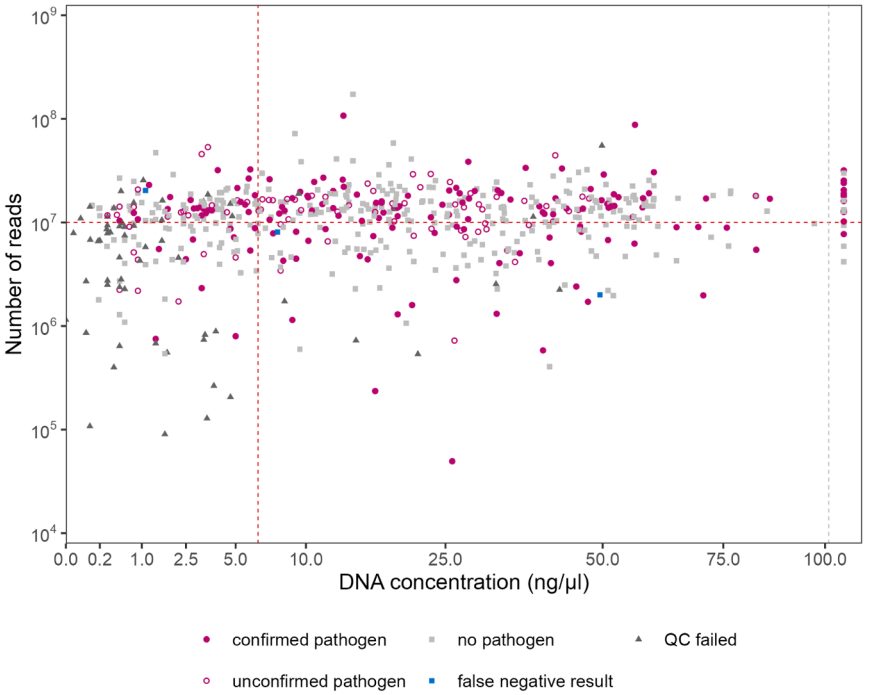

Figure 3.
Metagenomic next-generation sequencing assay performance and interpretation parameters. Input DNA quantity (DNA concentration) plotted against library read count, with points shaded by final interpretation status: positive with orthogonal confirmation by microbiology, PCR, or immunohistochemistry (full pink circles), positive without confirmation (empty pink circles), negative (light gray squares), false negatives detected by PCR (blue squares), and uninterpretable (dark gray triangles). Quality control (QC) and contamination considerations: libraries with low input often failed QC or showed environmental microbiome profiles. Even some well-performing libraries were excluded due to implausible findings (QC failed). DNA concentrations > 100 ng/µL were set arbitrarily to 105 ng/µL. The horizontal dashed line indicates the target read output (10 M), and the vertical line indicates the target input concentration (6.4 ng/µL).

enabling routine detection of persistent, elusive, or unculturable pathogens, or organisms associated with chronic infections.[1,8,24,25] This cost-effective approach expands the benefits of unbiased pathogen detection to a broader patient population in a real-world diagnostic setting.

Despite its lower sequencing depth and lack of pathogen-specific enrichment, our mNGS assay demonstrated a higher positivity rate than individual PCRs, indicating that its sensitivity was sufficient for detecting clinically relevant pathogens. Although up to 100-fold less sensitive than PCR, as shown in our validation, routine diagnostic yield was more often constrained by tissue quality and low pathogen abundance than by methodological sensitivity.[16] These observations illustrate the difficulty of selecting the appropriate targeted assay and emphasize the added value of unbiased mNGS approaches in pathology. Although RNA viruses were not yet covered, parallel PCR testing in a subset of cases revealed no additional false negatives.

A key strength of mNGS was its utility in cases where clinicians ordered multiple PCRs, reflecting diagnostic uncertainty.[12] Unlike pan-bacterial or pan-fungal PCRs, which often yield ambiguous results, mNGS provides species-level resolution, even at low read depth, enhancing differentiation of pathogens from commensals or contaminants. Such scenarios highlight the inherent challenge of choosing the correct targeted assay and underscore the clinical utility of hypothesis-free approaches such as mNGS. The unbiased design of mNGS overcomes the limitations of targeted panels, enabling detection of rare or novel pathogens missed by conventional assays.[22]

In pathology, mNGS uniquely detects rare viruses,[26] identifies bacteria and fungi that grow poorly or not at all, and confirms nonculturable organisms, such as *Leishmania* or *Mycobacterium* species, not covered by standard PCRs.[27] These capabilities are especially valuable when conventional methods, including culture, fail or are not applicable due to sample type or prior treatment.[28] Infectious disease pathology provides a distinct advantage by integrating morphology with molecular diagnostics. This correlation of histological, clinical, and mNGS findings creates a unique diagnostic interface between morphology and microbiology, particularly valuable in the diagnosis of chronic infections and tissue-persistent pathogens. A striking example is granulomatous inflammation, which represents a common, yet morphologically nonspecific, pattern shared across numerous infectious etiologies. Histological features such as necrotizing or nonnecrotizing granulomas often appear indistinguishable regardless of the underlying pathogen. Without molecular testing, such cases remain diagnostically inconclusive and therapeutically ambiguous. Metagenomic NGS not only enables precise pathogen identification but also guides targeted treatment, which is especially critical in infections that would otherwise go unrecognized or be mismanaged due to their nonspecific histological presentation.

posadasii. These findings demonstrate the capability of mNGS to detect fungi outside the scope of geographically or clinically preselected diagnostic algorithms. (D) Parasitic organisms (n = 13), including rare *Leishmania* spp. not targeted by routine PCR, were also identified. In several cases, genus-level assignments by mNGS were further resolved to *Leishmania infantum* using genus-specific PCR.

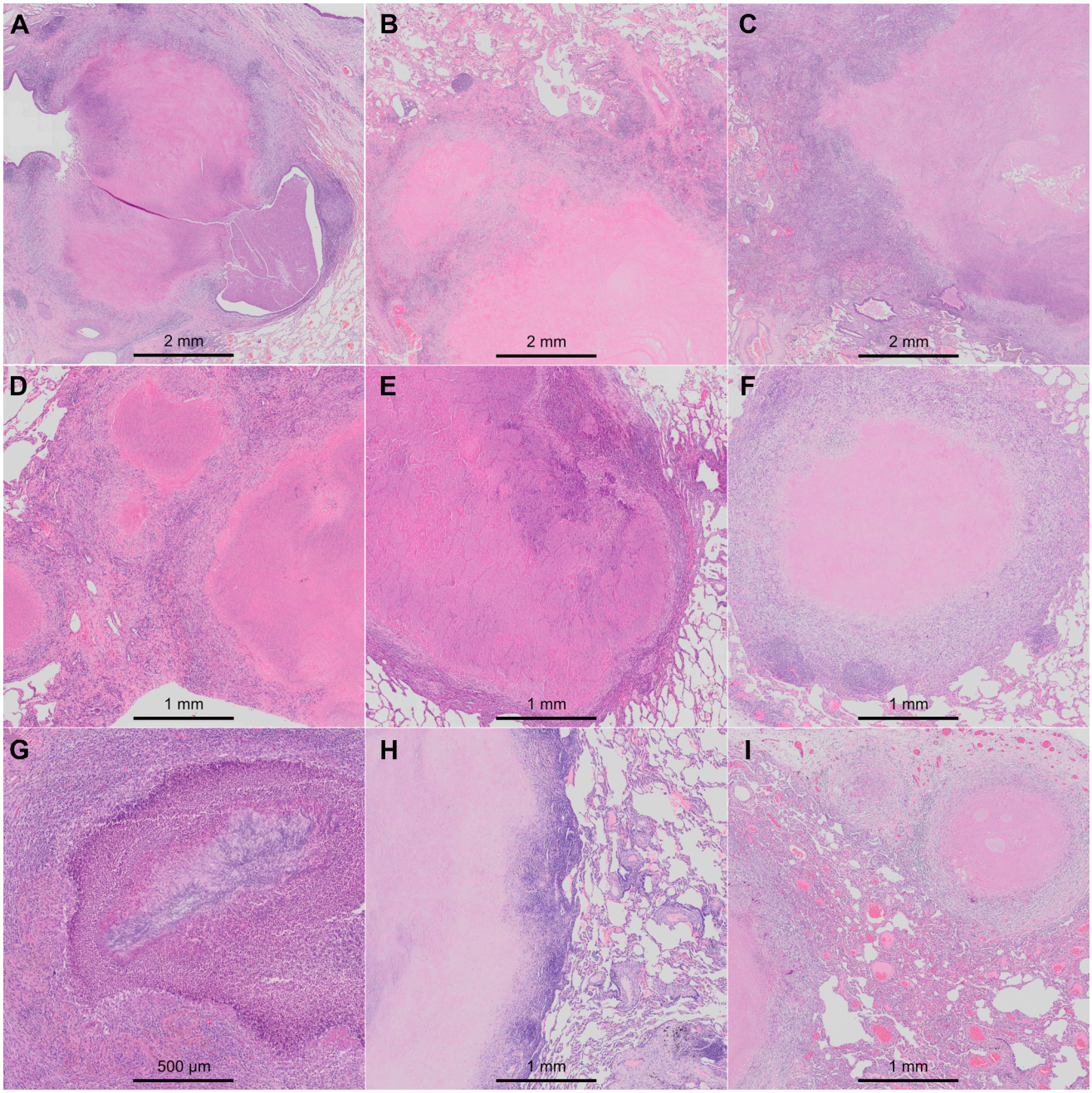

Figure 4.
Representative histological sections from different cases of granulomatous inflammation in formalin-fixed paraffin-embedded lung tissue samples, caused by various confirmed pathogens. Despite varied etiologies, the morphological features overlap significantly, demonstrating the limitations of histology in establishing a specific diagnosis and underscoring the need for molecular pathogen detection. (A) *Mycobacterium tuberculosis*, (B) *Mycobacterium avium*, (C) *Mycobacterium xenopi*, (D) *Francisella tularensis*, (E) *Human mastadenovirus B* (HAdV-B),[23] (F) *Human alphaherpesvirus 3* (VZV), (G) *Aspergillus fumigatus*, (H) *Coccidioides posadasii*, and (I) *Pneumocystis jirovecii*.

A limitation of our study is the lack of detailed clinical information for many cases. Since most samples were submitted from external institutions, we could not systematically assess the impact of clinical variables, such as prior antibiotic treatment or immunosuppression.[27] Moreover, our operational setting does not routinely allow for follow-up once results are reported, making it difficult to evaluate the clinical impact and therapeutic consequences of mNGS beyond the diagnostic phase. This limitation constrains our ability to assess how mNGS findings influence patient management and outcomes. Nevertheless, these aspects have been explored by other groups implementing mNGS in integrated clinical settings that allow for longitudinal feedback on treatment decisions and efficacy.[11,13,29]

Our workflow's scalability and cost effectiveness were enabled by existing sequencing infrastructure, originally established for molecular oncology. The parallels between oncology and infectious disease mNGS—particularly the use of degraded DNA from FFPE material and low nucleic acid inputs—suggest a shared trajectory in diagnostics.[30] As seen in oncology, mNGS

could replace the current patchwork of targeted assays with a single flexible platform.[31]

Cost remains a key consideration in the clinical adoption of mNGS. Although still more expensive than a single targeted PCR, its cost has steadily declined and is now increasingly comparable to—or even lower than—the cumulative cost of multiplex panels typically ordered for broad differential diagnoses. In selected contexts, such as immunocompromised patients at risk for rare infections, mNGS may offer both diagnostic and economic advantages.[32]

Implementing mNGS in routine diagnostics presents distinct challenges. Unlike typical mNGS use in controlled hospital settings, our laboratory processes externally sourced FFPE blocks, often sectioned at different institutions. Contamination introduced during sectioning is difficult to control without sequencing-based quality assessment.[33] To monitor for environmental background and crosscontamination, we include an empty paraffin block processed alongside patient samples as a batch control. Although imperfect, this approach is practical in our setting. Further complexity arises from the lack of suitable external quality assurance schemes for FFPE-based mNGS. Existing round-robin trials do not systematically reflect the high human DNA content typical of FFPE samples, affecting pathogen detection thresholds. In addition, mNGS reagents themselves may introduce microbial background, complicating interpretation. Flexible, yet robust, quality standards are urgently needed to support diverse mNGS applications in clinical settings.[34]

Regulatory and interdisciplinary considerations are also critical. Our assay is embedded in a pathology institute but draws on expertise in microbiology and virology. To ensure diagnostic accuracy, mNGS reports are jointly reviewed by a pathologist (K.D.M.) and clinical microbiologists (C.N., P.M.K.) and virologist (K.L.). We have also established an interdisciplinary infectious disease board, modeled after molecular tumor boards, to support collaborative case interpretation.[8]

Despite initial barriers related to validation, quality control, and regulatory requirements, mNGS offers clear advantages for laboratory consolidation. A single assay capable of detecting virtually all pathogens reduces the need to maintain and validate numerous PCRs and related quality management protocols.[27]

The future success of mNGS in infectious pathology depends not only on technological refinement but also on diagnostic stewardship. Careful case selection—integrating histology, immunohistochemistry, clinical context, and assay characteristics, such as bias, sensitivity, and input requirements—is essential to avoid overuse and misinterpretation. As we move toward broader, hypothesis-free testing, the thoughtful application of mNGS will be key to maximizing diagnostic value while minimizing unnecessary cost.[24]

Author Contributions

B.H. and S.H. designed and performed the experiments and drafted the manuscript. C.N., R.G., and M.T.R. contributed to confirmatory testing and manuscript revisions. J.H., M.S., N.M., R.B., B.K.W., and M.C. carried out experimental measurements. A.N., R.A.K., V.B., M.W.R., S.T., and N.K. provided clinical context and input on the manuscript. A.T. conducted pathology review and provided critical feedback. R.N. contributed expertise in sequencing. C.M.F. and P.S. supported bioinformatics analyses. K.L. and P.M.K. coordinated confirmatory testing and reviewed the manuscript. K.D.M. conceived the study, supervised the project, and edited and finalized the manuscript. All authors read and approved the final paper.

Data Availability

The data presented in this study will be made available upon publication in the EBI online repository (https://www.ebi.ac.uk/ena) under the accession number PRJEB89466. The CLC workflow, taxonomic profiling databases, and report template are available upon request.

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Declaration of Competing Interest

The authors have no competing interests to declare.

Ethical Approval and Consent to Participate

This study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of Northwestern and Central Switzerland (Project-ID 2023-02250). Patient consent was obtained for sample collection and research use through the general consent form of the referring institution.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the author(s) used AI/AI-assisted technologies (Mistral, DeepL) to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Supplementary Material

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