

Title page

Metagenomic sequencing as a pathogen-agnostic clinical diagnostic tool for infectious diseases: a systematic review and meta-analysis of diagnostic test accuracy studies

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Abstract (249 words)

Background: Metagenomic sequencing is frequently claimed to have the potential to revolutionise microbiology through rapid species identification and antimicrobial resistance (AMR) prediction. We assess progress towards this.

Methods: We perform a systematic review and meta-analysis of all published literature on culture-independent metagenomic sequencing for pathogen-agnostic infectious disease diagnostics to August 12, 2020. Methodologic bias and applicability were assessed using QUADAS-2. (PROSPERO CRD42020163777)

Results: A total of 2023 clinical samples from 13/21 eligible diagnostic test accuracy studies were included in the meta-analysis. Reference standards were culture, molecular testing, clinical decision or a composite measure. Sensitivity and specificity in the most widely investigated sample types were 90%(78-96%) and 86%(45-98%) for blood, 75%(95%CI, 54-89%) and 96%(72-100%) for CSF, and 84%(79-88%) and 67%(38-87%) for orthopaedic samples respectively. We identified limited use of controls, especially negative controls which were used in only 62%(13/21) studies. AMR prediction and comparison to phenotypic results was undertaken in four studies: categorical agreement was 88%(80%-97%), very major and major error rates were 24%(8-40%) and 5%(0-12%) respectively. Better human DNA depletion methods are required: a median 91%(IQR 82-98%)[range 76-98%] of sequences were classified as human. The median(IQR)[range] time from sample to result was 29(24-94)[4-144] hours. The reported consumables cost per sample ranged from \$130-\$685.

Conclusions: There is scope for improving the quality of reporting in clinical metagenomic studies. Although our results are limited by the heterogeneity displayed, our results reflect a promising outlook for clinical metagenomics. Methodological improvements, and convergence around protocols and best practises may improve performance in future.

43 **Keywords:** infection, diagnosis, metagenomics, whole-genome sequencing, meta-analysis

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Introduction

The term metagenomics first appeared in 1998 which referenced the idea that a collection of genes could be sequenced in a single sample without the need for isolation or lab cultivation of a specific species (1). In infectious diseases, metagenomics has the potential to be a disruptive technology that revolutionises diagnostics, in place of traditional culture-based microbiology which has remained fundamentally unchanged over decades. The complete metagenomic pipeline from sample collection to report is shown in Figure 1. Traditionally most diagnostic microbiology depends on culture to identify the causative organism in an infection and to define antimicrobial susceptibilities. However, this process can take several days, or in the case of *Mycobacteria* and other slow growing organisms several weeks. While waiting for results patients are treated empirically, which may result in unnecessarily broad-spectrum treatment in some, and ineffective treatment in others. Additionally, culture is imperfectly sensitive, e.g. many patients with serious infection have negative blood cultures, and culture may be impaired by prior antimicrobial exposure.

Molecular diagnostics based on detection of antigens, antibodies or nucleic acid are increasingly used to complement culture-based diagnosis. For example, multiplex-PCR syndromic panels are able to identify common pathogens and antimicrobial resistance determinants within a few hours. However, these panels can only identify a restricted number of pathogens and resistance determinants and are therefore typically bespoke for a given clinical syndrome.

In contrast, emerging real-time sequencing technologies potentially offer a pathogen and clinical syndrome agnostic platform to identify any pathogens and known resistance determinants present within hours. Here we evaluate progress towards this ambition. Studies

have demonstrated that metagenomic pipelines can provide faster diagnoses than conventional culture-based methods which may hold crucial for critically unwell patients (2). Additionally the detection of pathogens from culture-negative samples may have clinical management implications in several other settings including prosthetic joint infections, chronic wound infections and endocarditis (3–5). Where sufficient knowledge bases exist prediction of antimicrobial resistance is potentially possible and detection of mixed infections is also possible, where a subpopulation of resistant strains can be detected (6).

In this review, our objectives were to assess all current literature published on culture-independent metagenomic sequencing for pathogen-agnostic infectious disease diagnostics in participants with potential infection and to describe the specific challenges and progress in the field.

Methods

Search strategy and selection criteria

We conducted a systematic review on all available literature up to 13th August 2020 in accordance with the Preferred Reporting System for Systematic Reviews and Meta-Analyses for Diagnostic Test Accuracy (PRISMA-DTA) guidelines (7). We searched PubMed and Google Scholar using a combination of relevant MeSH terms (Table S1). Abstracts of studies identified were screened using the inclusion and exclusion criteria specified in Table S2. We focused on pathogen-agnostic sequencing studies and excluded pilot studies, those with <10 samples, and those focused on diagnosis of a specific pathogen or group of pathogens. The reference lists of included manuscripts were also manually searched for relevant studies not identified during the literature search. We assessed methodologic quality and risk of bias using the QUADAS-2 tool for diagnostic accuracy studies and excluded any study with high overall risk of bias (8). Study inclusion, extraction of data and quality evaluation was assessed by two

independent reviewers and any differences were resolved through discussion or by a third reviewer. Peer-reviewed articles that utilised metagenomic sequencing as a tool for clinical diagnostics in infectious diseases were appraised and relevant data was extracted where possible. Principle diagnostic accuracy measures were sensitivity and specificity per clinical sample result. Antimicrobial resistance prediction was assessed using the resistance prediction outcome over each combination of antibiotic and sample tested. This study has been registered with the PROSPERO prospective register of systematic reviews (reference CRD42020163777).

Meta-analysis

The index test was defined as the metagenomic sequencing test method, while the reference standard defining true positives and true negatives were based on a pragmatic approach of each study's best measure of the presence or absence of infection with a specific species using either culture, molecular testing, clinical decision or a combination of these factors. Additional species identified as plausible by studies including polymicrobial infections were considered additional true pathogens and contributed to the numerator and denominator in the sensitivity analysis. Specificity was determined by considering the proportion of samples deemed negative by the reference standard that were identified as negative by metagenomic sequencing. Although it is plausible to consider that any sample can contain a false positive result, including culture-positive samples, studies differed considerably in reporting this. Therefore, we only included reference standard negative samples in specificity calculations. Studies where only sensitivity could be determined and not specificity were included in the meta-analysis, however, a further analyses excluding these studies were also performed.

We used the R meta package for the statistical analysis (9). Outcomes are reported for each study with 95% confidence intervals (CIs), and pooled estimates provided using fixed or random-effects models, depending on the absence or presence of heterogeneity using the I² test respectively. An I² value >50% was considered heterogenous.

Results

A total of 551 manuscripts were screened, 530 were excluded (Supplementary Figure 1), leaving 21 eligible for inclusion. Specificity could not be determined in eight studies, leaving 13 studies to be included in the meta-analysis (Table 1).

Clinical samples and range of pathogens

The range of clinical samples included blood (10–14), positive blood cultures (2), urine (15), respiratory samples (16–19), orthopaedic device sonication fluid and other bone and joint samples (3, 20–22), cerebrospinal fluid (23–26), intraocular fluid (27) and heart valve tissue (5). The range of pathogens found are detailed in Table 1. The median number of samples sequenced per study was 67 (IQR 44-166) [Range 12-348].

Methodology: nucleic acid extraction and sequencing library preparation

Methods to deplete human cells prior to DNA extraction were used in 8/21 (38%) studies (Table S3), including differential centrifugation (2, 15, 22) or filtration with a 5µm membrane (2, 11, 20) to remove human cells, based on their larger mass or size respectively, and

the MolYsis Basic5 kit (Molzylm, Bremen, Germany) (3, 21) which is reported to differentially lyse human cells.

Nucleic acid extraction kits and techniques were heterogeneous across studies (Table S3). Most studies utilised commercial kits, primarily based on membrane/column methods, while non-commercial methods relied mostly on ethanol precipitation or lysis followed by bead-cleaning methods. 3/21 (14%) studies performed a post-extraction enrichment step to facilitate enrichment of microbial DNA from clinical samples and 7/21 (33%) studies used a DNA amplification method prior to library preparation to achieve required input DNA concentrations (Table S3). DNA clean-up with magnetic beads was used in 9/21 (43%) studies to purify extracted DNA prior to sequencing.

Methodology: controls

Studies were assessed for three types of controls, (i) an internal or spiked control, (ii) a negative control and (iii) a positive control. Only 4/21 (19%) studies reported internal control use; this included bacteriophages and synthetic DNA (12, 14, 24, 25). 13/21 (62%) studies reported use of a negative control. The median (IQR) [range] of negative control samples per every 10 samples processed was 1.5 (1.0-1.5) [0.5-1.5]. Only 6/21 (29%) studies reported use of a positive control, including using a mixture of 7 representative organisms and a single *Corynebacterium glutamicum* ATCC 13032 positive control (3, 13, 14, 17, 24, 25).

Sequencing technologies

Illumina technology (CA, USA) was used exclusively in 16/21 (76%) studies (Table S4). One study exclusively used Oxford Nanopore Technologies MinION (ONT, UK) (16) and two studies exclusively used Ion Proton (Life Technologies, USA) (10, 15). One studies compared the use of Illumina sequencing versus ONT MinION (2). The BGISEQ-500/50 (BGI, Tianjin, China) platform was used in one study (23).

Bioinformatic approaches

Supplementary Figure 2 outlines the bioinformatic workflows implemented. The most commonly used classification software packages for species identification were alignment based (n=11) followed by k-mer based (n=8) and marker-gene based (n=1) (see Table S4 for details). The most commonly used species databases originate from the NCBI and included the NCBI RefSeq database (n=9), unspecified NCBI databases (n=7), MetaPhlAn2 database (n=3), NCBI Genbank (n=1) and Karius proprietary database (n=1).

Assessing true species presence versus contamination

Various strategies were employed to identify true presence of an organism verses contamination. The most commonly used methods include an empirical approach setting absolute thresholds based on sequencing known negative and positive samples (n=4) (2, 3, 24, 25) while other studies (n=3) used a parametric approach based on the observed distribution of contaminating reads in control and other samples(14, 17, 21). Other strategies are described in Supplementary Figure 3. By use of negative controls and other approaches, 16/21 (76%)

studies report contamination of some degree while 5/21 (24%) had no details of contamination available. No study reported no evidence of contamination.

Performance: species identification

Methodological quality was assessed using the QUADAS-2 tool (Supplementary Figure 4) (8). Risk of bias was most commonly seen in the patient domain with 2/21 (10%) studies having high risk and 3/21 (14%) having some concern, all relating to selection of specific samples for study, rather than use of a random or consecutive sample. This was followed by the index test domain where 4/21 (19%) studies had some concern relating to the reporting of bioinformatic thresholds and methods to assign a result as positive or negative (Supplementary Figure 6).

A meta-analysis of species identification performance was performed by sample type on 13/21 studies where both test sensitivity and specificity could be determined. Sensitivity in the most widely investigated sample types blood (n=288), CSF (n=133), and orthopaedic (n=297) was 90% (78-96%), 75% (54-89%), and 84% (79-88%) respectively (Figure 2). Varying performance may reflect the number of organisms present in each sample type and the relative abundance of human cells and any other bacteria present. Among CSF studies, Wilson, M et al. (2019) report lower sensitivity, possibly reflecting a greater effort to obtain a diagnosis in the reference standard used for comparison including culture, multiplex molecular diagnostics and obtaining a variety of samples including tissue biopsy and abscess fluid (25). Furthermore, conservative preestablished bioinformatic thresholds were used which resulted in negative results even though species-specific reads might have been used to identify the appropriate organism at lower thresholds. Amongst studies on blood, those with low sample sizes generally performed worse, possibly reflected the most limited experience developed in these studies (10).

Specificity in blood (n=533), CSF (n=314), and orthopaedic samples (n=224) was 86% (45-98%), 96% (72-100%), and 67% (38-87%) respectively (Figure 3). CSF studies performed better compared to blood and orthopaedic studies in part as preestablished conservative thresholds were used, trading off sensitivity for higher specificity (24, 25). Specificity performance varied substantially across orthopaedic studies due to non-standardised thresholds for determining a negative result in the presence of high contamination and low DNA extraction yields, resulting in many organisms of unknown clinical significance (Figure 3). 8/21 (38%) studies did not report specificity and were excluded from the meta-analysis. 20/21 (95%) studies found a median of 7 (4-15) [1-62] additional pathogens with subsequent clinical interpretation varying throughout. In this analysis, positive and negative predictive values grouped by sample type range between 72-98% and 75-90% respectively, and diagnostic accuracy between 77-95% (Table S7).

Using reported per-study means, a median 91% (IQR 82-98%) [Range 76-98%] of sequence data was classified as human DNA. Blood samples had the highest median reported of 91% (98-91) [84-98], and sonication and bone samples had a median of 87% (82-93) [76-98].

Performance: antimicrobial resistance prediction

9 studies attempted genotypic drug susceptibility prediction and compared results to either drug susceptibility phenotype (n=4) or had no comparison (n=5). Four studies that compared results to drug susceptibility phenotypes were included in a meta-analysis (Table S5). Performance depends on the prevalence of resistance and the specific pathogen-antimicrobial combinations tested. In the 4 eligible studies, the median (IQR) [range] number of clinical samples per study was 29 (22-35) [17-39]. A median of 196 (45-350) [35-369] antibiotic predictions were

performed per study. Beta-lactams, quinolones and tetracyclines were some of the most commonly tested classes of antibiotics and 19% (153/795) of pathogen-antimicrobial combinations were phenotypically resistant (Table S5). All studies evaluated prediction of the correct antimicrobial susceptibility as a categorical value, i.e. sensitive or resistant. Categorical agreement rates were 88% (CI, 80% to 97%) across studies using a random effects model. The pooled estimate of very major error rates, defined as the number of samples with phenotypic resistance not predicted by genotype over all samples with phenotypic resistance was 24% (CI, 8% to 40%). The pooled major error rate, defined as predicted resistance by genotype but sensitive phenotype over all phenotypically sensitive samples, was 5% (CI, 0% to 12%) (Figure 4). There was marked heterogeneity in all three metrics (I^2 0.86-0.92; all $p < 0.01$).

Time of complete process

9/21 (43%) studies reported sample processing times (Supplementary Figure 7). Based on the mean time provided from each study, sample extraction/preparation took a median (IQR) [range] 3 (3-3) [3-3] hours and library preparation a median 4 (4-4) [4-4] hours. Reported sequencing using Illumina took 16 (16-16) [16-16] hours. Where sequence analysis was undertaken in real-time using the Nanopore platform, first species prediction took 1 (1-1) [1-1] hour. The total time from sample extraction to speciation and AMR prediction was 29 (24-94) [4-144] hours.

Cost

Only one study reported an average cost estimation per sample which varied from \$130 when samples are multiplexed up to \$685 for processing a single sample (16).

Discussion

In this review we describe all studies found to date assessing the performance of metagenomic sequencing as a tool for pathogen-agnostic diagnosis of clinical infection on a variety of clinical samples including from blood, urine, respiratory samples, cerebrospinal fluid, orthopaedic sonication and synovial fluid, intraocular fluid and cardiac valvular tissue. Sensitivity in the most widely investigated sample types blood (n=288), CSF (n=133), and orthopaedic (n=297) was 90% (78-96%), 75% (54-89%), and 84% (79-88%) respectively (Figure 2). Specificity in blood (n=533), CSF (n=314), and orthopaedic samples (n=224) was 86% (45-98%), 96% (72-100%), and 67% (38-87%) respectively (Figure 3). Analysis by sample type sub-groups demonstrate development of workflows for CSF, blood and orthopaedic fluid with reproducible performance (Figure 2 and 3).

While these results reflect impressive progress in the field, the level of accuracy required is still being broadly assessed as metagenomics is unlike any other patient diagnostic with specific advantages and disadvantages reviewed elsewhere (28, 29). However, it should be remembered that the current gold standard of culture-based microbiology is imperfect, for example two standard blood cultures will result in missing at least 10-18% of episodes with potentially culturable organisms (30). Additionally, as metagenomics may detect potential pathogens not found by culture, reported specificity may be reduced because of the use of an imperfect reference standard. For example, a median of 7 (4-15) [1-62] additional pathogens were found per study. Besides culture, metagenomics can be benchmarked against multiplex syndromic panels however, ultimately expert clinical review is required to evaluate the plausibility of additional potential pathogens detected.

While species identification may change therapy in some settings, e.g. as a result of intrinsic resistance or local epidemiology, rapid and precise antimicrobial resistance prediction linked to specific pathogens would greatly improve the time to administration of targeted antimicrobials. Although the categorical agreement rate with phenotyping was 88% (CI, 80% to 97%), major and very major error rates were 5% (CI, 0% to 12%) and 24% (CI, 8% to 40%) respectively, well above the current regulatory thresholds; the FDA requires major errors <3% and the 95% CI on the very major error rate to be $\leq 7.5\%$ at the upper limit and $\leq 1.5\%$ at the lower limit (31). It is clear that improvements are needed for AMR prediction to reach acceptable performance, including better understanding and cataloguing of the mechanisms underlying antimicrobial resistance and optimised algorithms to detect these within metagenomic data and link them to specific pathogens. This is particularly challenging for plasmid-mediated resistance which may be difficult to assign to a specific species from metagenomic data. Increased yield of pathogen DNA will also likely improve performance. There is a need to establish optimal extraction methods; only one study was found that compared different methods (2). Furthermore, a median of 91% (82-98%) [76-98%] of sequence reads were classified as human even after applying known human depletion laboratory techniques, necessitating the need for improved human DNA depletion methods. Detailed information on library preparation methods can be found in a review by S. Head *et al.* (32).

Quality control is imperative for reliable metagenomic results and will be essential for regulatory approval for clinical applications. Only 19% and 29% of studies used internal and positive controls respectively. Similarly, only 62% used negative controls. We recommend all metagenomic studies consider carefully using all three forms of controls to detect and reduce errors.

300

301 The ability of metagenomics to detect microbes agnostically and at very low concentrations is
302 a major advantage however this also comes with the ability to detect contaminants much more
303 easily which poses a major challenge, especially after DNA amplification. Meticulous
304 laboratory practice is required to minimise contamination risks. However, despite this some
305 residual contamination is likely; 16/21 (76%) studies reported contamination to some degree.
306 Studies used various approaches to classify contamination described in Supplementary Figure
307 3, however there is no convergence on how to exclude contamination and confirm the presence
308 of infection bioinformatically.

309

310 It is yet unclear which sequencing platform will emerge as the ideal device for metagenomic
311 sequencing. Successful platforms will need to be scalable, flexible for different batch sizes and
312 offer stable and predictable performance. For detailed information on sequencing platforms, a
313 review is available by Goodwin S *et al.* (33). Current real-time sequencing devices such the
314 Oxford Nanopore technology yield rapid data, which may be a significant development for
315 infection diagnostics, but are limited by high per read error rates which can be problematic
316 especially if only low coverage depth is achieved of the pathogen genome.

317

318 As the quantity of microbial genetic data increases, bioinformatic approaches need to be
319 optimised to handle large datasets of complex metagenomic data while minimising computing
320 power and memory usage. However, accredited workflows that can be reliably used by non-
321 expert users will be needed for metagenomics to be adopted widely. This will also involve
322 adapting and develop laboratory information management systems to handle different
323 workflows and classes of data.

324

325 In severe infection, it has been demonstrated that a time interval of >1 day before initiation of
326 appropriate antimicrobial therapy is associated with a 2-fold increase in the risk of mortality
327 (34). Clinical metagenomics besides identifying fastidious organisms not identified by culture,
328 may provide a species and antimicrobial resistance results within 4-7 hours in some cases
329 (Supplementary Figure 7), instead of the conventional 24-72 hour with culture. However,
330 practically this may be limited by the number of times that a lab is able to perform sequencing
331 runs within given time intervals.

332 Koch's postulates have played an important role in establishing causal relationships in
333 microbiology, yet have major limitations including the fact that we now know of many
334 pathogens that cannot be conventionally cultured. A reconsideration of these postulates
335 which is relevant and aligns to metagenomics is proposed by Fredericks and Relman (35). If
336 metagenomics can be shown to be diagnostically accurate, it will then become important to
337 understand the clinical correlation and implication of positive results when all other assays
338 are negative. This will necessitate large-scale interventional and longitudinal studies to
339 establish the implications for patient outcomes. Additionally, economic evaluations will be
340 needed. The average consumable cost per sample for clinical metagenomics is reported to
341 range from \$130 when samples are multiplexed up to \$685 for processing a single sample at
342 once. In comparison, the average running cost for blood culture is estimated at <\$50 per
343 sample (36). However, costs associated with changes in patient therapy and management
344 from metagenomics and any impact on outcomes will also need to be considered.

345

346 Clinical laboratories are highly regulated with requirements that vary by jurisdiction, for
347 example in the US FDA clearance is required while in the EU a CE mark is required for

diagnostic platforms. The FDA intends to regulate infectious disease metagenomic high-throughput sequencing devices as systems, including all the components necessary to generate a result. Detailed information including quality control, validation and continuous monitoring may be found in the draft guidance by the FDA (37).

Our study had several limitations. Firstly, the meta-analysis provided does not overcome problems inherent in the design and execution of the primary studies. Furthermore, it does not correct bias as a result of selective publication. 8/21 (38%) of studies did not include negative test results limiting the ability to ascertain false-positive results or contamination within these studies and therefore were excluded from the meta-analysis. Using the QUADAS-2 tool, the main risk of bias arose from selection of specific samples for study, rather than consecutive samples or a random subset which are recommended. Similarly, to avoid index test bias we recommend studies provide further clarity on bioinformatic procedures when determining a positive or negative result. Although studies were grouped by sample type, any observed difference may be confounded by other factors such as differing clinical application, laboratory and bioinformatic methods or the fact that reference standards differed among culture, molecular testing, clinical decision or a composite measure (Table S3-5). Due to the heterogeneity between studies, we are not able to provide specific recommendations on the ideal metagenomics workflow but anticipate by bringing studies to date together this review will provide a mechanism for improving standards and designing better studies in future. We suggest future research conform to requirements set out by the STROBE-metagenomics guideline, STARD, or other equivalent guidelines to improve the quality of study design and reporting (38, 39).

Conclusion

With further advances in extraction methods, sequencing technology and bioinformatic processes, the proficiency of clinical metagenomics is likely to improve. Culture-based diagnostics have remained the backbone of the microbiology lab for over a century (40); however clinical metagenomics has the potential to be the next frontier of clinical microbiology, not only enhancing our understanding of infectious diseases but also detecting polymicrobial infections and pathogens that are conventionally unculturable. This may lead to more effective and narrow-spectrum treatment options reducing antimicrobial resistance and leaving the microbiome unchanged. It is likely that clinical metagenomics will be a part of the clinician's armamentarium in the future to identify and guide therapy of complex infectious diseases. However, convergence around protocols and further metagenomic studies of improved quality and with higher sample sizes are required for the field to progress further.

Potential conflicts of interest

One article included in this meta-analysis was published by the authors (20). DWE has received lecture fees and expenses from Gilead. No other author has a conflict of interest to declare.

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Figures

Figure 1. Metagenomic flow diagram.

Figure 2. Pooled sensitivity of studies grouped by sample type.

Figure 3. Pooled specificity of studies grouped by sample type.

Figure 4. Pooled antimicrobial resistance prediction performance.

Tables

Table 1. Characteristics of studies included in this systematic review

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