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Metagenomic next generation sequencing of bronchoalveolar lavage samples for the diagnosis of lower respiratory tract infections: A systematic review and meta-analysis

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ABSTRACT

Background: Lower respiratory tract infections (LRTI)are known to be diagnosed late or inaccurately. This has fueled the unscrupulous use of antibiotics, as they are often used empirically and clinically, leading to antibiotic abuse and multidrug resistance in patients. Metagenomic next-generation sequencing (mNGS), now widely used in clinical studies, could be a potential intervention to revolutionize microbiology by rapidly identifying unknown species.

Methods: This review and meta-analysis were conducted on eligible studies with respect to metagenomic sequencing on clinical LRTI diagnostics up to May 01, 2022. QUADAS-2 was employed to assess the methodological bias as well as applicability. After that, a meta-analysis was conducted to analyze the accuracy of mNGS, compared with the composite reference standard (CRS), among the enrolled studies.

Results: This work collected 1248 samples in 13/21 qualified articles to factor in the accuracy of the diagnostic test. Typically, methods like molecular testing, culture, composite measures, and clinical decision-making were adopted as the reference criteria. With regard to Bronchoalveolar Lavage Samples, their sensitivity was 89% (82–93%) while their specificity was 90% (66–98%), with obvious heterogeneities in these two factors as demonstrated by different studies. The summary receiver operating characteristic (SROC) curve was plotted for mNGS as a function of LRTI, and the area under the curve (AUC) was 0.94. A Funnel plot with a p-value greater than 0.05 indicated the absence of publication bias. Positive and negative likelihood ratios (PLR and NLR) were >10 and > 0.1, respectively. In this pre-test probability-post-probability-likelihood ratio relationship graph, the values were Prior prob (%) = 20, Post-prob-Pos (%) = 77 and Post-prob-Neg (%) = 4.

Conclusion: The AUC value of SROC suggested a high accuracy of mNGS in diagnosis, with no publication bias and high reliability. The application of mNGS exhibits notable diagnostic efficacy in discerning pathogens present in bronchoalveolar lavage fluid (BALF) among patients afflicted with LRTI. However, mNGS is more meaningful for the definitive diagnosis of the disease rather than the exclusion of the disease. This post-test probability is significantly higher than the pre-test probability.

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1. Introduction

According to the 2019 World Health Organization Global Health Estimates, deaths due to respiratory infections have fallen significantly to 2.6 million deaths in 2019, which comes 460,000 fewer than the count in 2000 [1]. Respiratory infection, especially the case of the lower respiratory tract (LRTI), remains a globally pervasive disorder with the highest mortality and ranks fourth on the list of causes of death. LRTI comes second when low-income places are considered [1]. Lack of early diagnosis and the corresponding target therapy is a major factor in causing LRTI. Therefore, the accuracy and sensitivity of laboratory diagnosis are important parameters for managing and further treating patients with respiratory infections.

At present, the laboratory diagnosis of respiratory infection-related pathogens includes conventional culture, bacterial staining, pathogenic antibodies, and antigens. PCR has been used to devise a multiplex (6/8/13/22) combined real-time PCR for nucleic acid detection of respiratory pathogens [2], which covers several pathogenic microorganisms. However, not all pathogenic microorganisms causing human infections have been identified; hence, some pathogenic microorganisms may be absent in such panels.

The development of a new generation of metagenomic sequencing (mNGS) technology has provided technical support for the detection of unknown infections [3], which can provide unbiased and comprehensive detection of total DNA and RNA levels in the identified pathogens using the genomic DNA sequence information of microorganisms [4].

Various specimens can be obtained for lower respiratory tract infections, such as sputum and blood, but the ideal specimen would be bronchoalveolar lavage fluid (BALF) [5]. There are still many controversies about the use of mNGS on BALF to detect pathogenic bacteria. Owing to constraints of sequencing technology, for example, the detection of pathogenic bacteria can be influenced by human genomes in lavage fluid samples [6]. This article mainly evaluates the sensitivity and specificity of BALF samples using mNGS to diagnose LRTI.

2. Methods

2.1. Registration

This work was registered on the International Platform of Registered Systematic Review and Meta-Analysis Protocols (PROSPERO) (registration number: CRD42022350402) and carried out the following statements as per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses paradigm. Ethical approval was not mandated for this work.

2.2. Search strategy and selection criteria

Articles assessing the accuracy of mNGS in diagnosing LRTI were searched using PubMed, Embase, and the Web of Science Database from inception up to May 1, 2022, by adopting the related MeSH terms (Table S1).

2.3. Inclusion and exclusion criteria

1. We included different types of studies on humans, such as case-control, retrospective, and prospective studies.
2. We included full-text, original research articles that evaluated the effectiveness of mNGS in diagnosing LRTI.
3. We included full-text, original research studies wherein culture, molecular tests, clinical judgment, or a combination of these factors were utilized as reference standards.
4. The values of true positive (TP), false positive (FP), false negative (FN), and true negative (TN) for the assay can be either directly derived or computed from the studies in question.
5. Nevertheless, exclusions were made for case reports, articles not written in English, studies encompassing less than ten specimens, conference reports, preprint articles, pilot studies, and abstracts not accompanied by full articles.

2.4. Study selection

This work imported primary search records in ENDNOTE X9.2, a literature management software, in line with our preset criteria. Thereafter, two researchers were responsible for inclusion studies, extracting data, and evaluating quality, while the disagreements between them were settled by mutual negotiation with the third researcher. Sensitivity and specificity were selected as the primary outcome measures.

2.5. Meta-analysis

The bivariate meta-analysis framework was utilized to calculate the combined sensitivity, specificity, positive/negative likelihood ratio (PLR/NLR), diagnostic odds ratio (DOR), as well as the relevant 95% CI for diagnosing LRTI. In addition, the combined diagnostic significance of mNGS was analyzed by summary receiver operating characteristic (SROC) curve analysis and using the area under SROC curve (AUC) values. After that, the inter-study heterogeneities were analyzed by the chi-square test and I2 statistic. Additionally, the possible heterogeneity source was analyzed by subgroup and sensitivity analysis with Stata 15.0 (Stata Corporation, College Station, TX, USA) and RevMan 5.4(The Nordic Cochrane Centre, The Cochrane Collaboration, London, UK, 2020). $P < 0.05$ was

considered as denoting statistical significance.

2.6. Study quality

By adopting the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS)-2 [7], two reviewers were responsible for evaluating enrolled study quality. (QUADAS)-2 includes four domains regarding patient screening, index test, flow, timing, and the reference standard. Bias risk and clinical utility were evaluated using signaling questions as low, high, or unclear.

3. Results

3.1. Characteristics of the studies

Fig. 1 presents the selection process, as delineated in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart. This work enrolled 551 studies in total, but after the elimination of 530 of them, 21 studies were left initially.

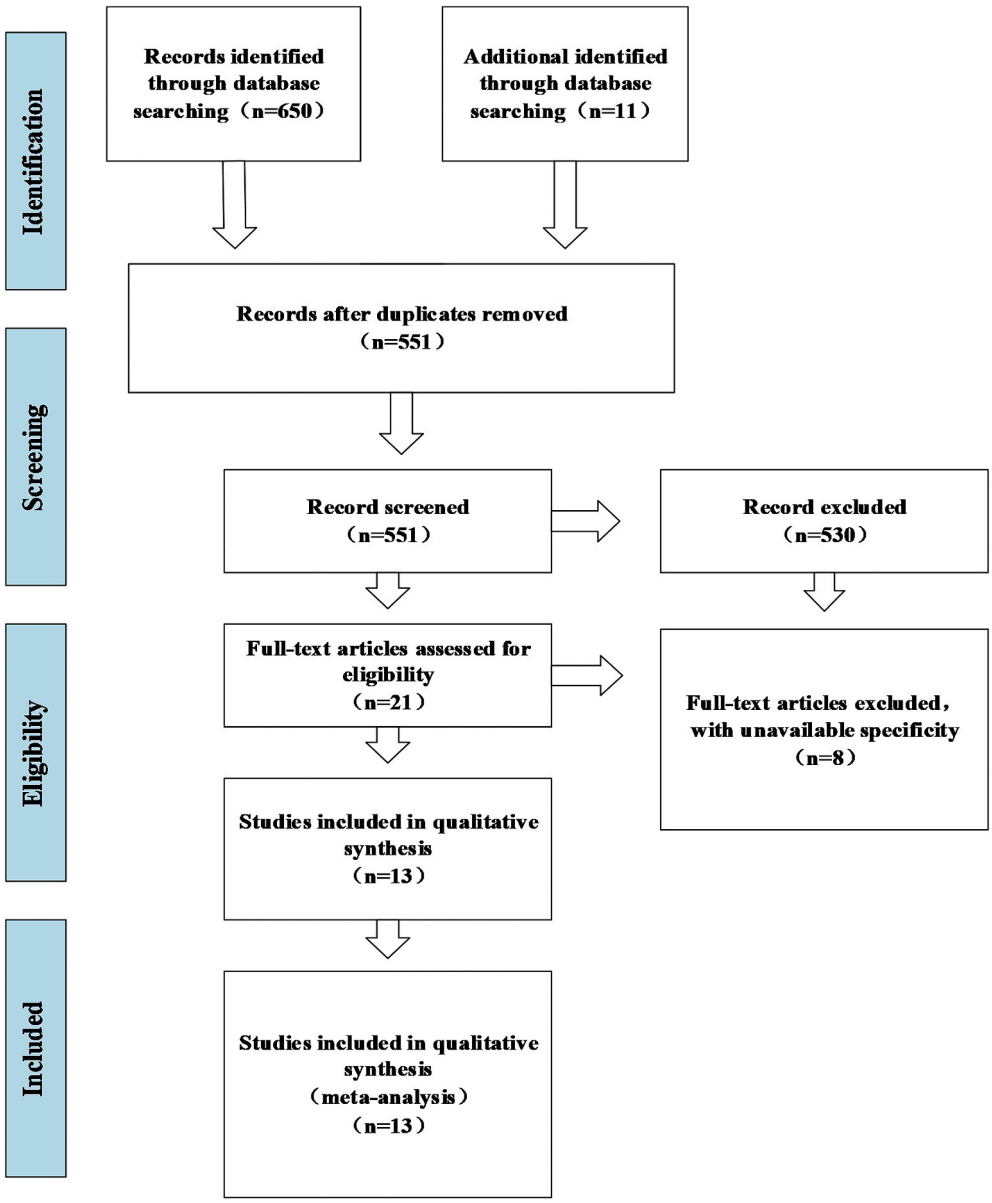

Fig. 1. Flowchart depicting the process of literature search. A total of 551 articles were identified from the databases of Embase, PubMed, and Web of Science.

Later there were eight articles with unavailable specificity, which were subsequently eliminated, leaving 13 articles [8–20] for the final analysis. Each of the articles was published in English, with their publication dates falling between 2020 and 2021. All research specimens were bronchoalveolar lavage fluid. Specimen sizes ranged from 20 to 329 (median, 99), adding up to 1248 specimens. Table 1 displays the enrolled study features.

Fig. 2 below provides a detailed summary of the methodological quality assessment. It is clear that the majority of the reviewed literature demonstrates a low risk of bias. Furthermore, a significant proportion of the articles offer thorough descriptions regarding patient selection, index testing, and reference standards. Consequently, the aggregate quality of the studies incorporated in this analysis is notably high.

3.2. Sequencing technologies

7/13 (53.8%) of the studies used the BGISEQ-500/50 (BGI, Tianjin, China) platform, while 4/13 (30.7%) of the studies employed Illumina technology (CA, USA).

3.3. mNGS diagnostic specificity and sensitivity

mNGS achieved a diagnostic sensitivity of 89% (95%CI: 82–93) with an I2 value of 93.85% (95%CI: 91.30–95.99). The specificity was 90% (95% CI: 66–98) with an I2 value of 94.85% (95% CI: 92.77–98.52) (Fig. 3). The Diagnostic Odds Ratio was around 80 (95% CI: 7856). There were obvious heterogeneities in sensitivity and specificity among the studies. The AUC value of SROC regarding mNGS in diagnosing LRTI was 0.94 (95% CI: 0.91–0.95) (Fig. 4). A Funnel plot with a p-value greater than 0.05 (Fig. 5) indicated no publication bias. A PLR (Positive likelihood ratio) of 13.2 [1.6106.6]> 10 and an NLR (Negative likelihood ratio) of 0.17 [0.09,0.31] > 0.1 indicated that the mNGS is more meaningful for the definitive diagnosis of the disease rather than its exclusion (Fig. 6). In the pre-test probability-post-probability-likelihood ratio relationship graph, the values were: Prior prob (%) = 20, Post-prob-Pos (%) = 77, and the Post-prob-Neg (%) = 4 (Fig. 7). A univariable meta-regression analysis was undertaken, considering the characteristics of the 13 studies, to investigate the potential sources of heterogeneity. The analysis revealed that the factor ‘samemth’ had an impact on sensitivity, whereas ‘predesign’, ‘samemth’, and ‘reftest’ influenced specificity (Fig. 8).

4. Discussion

Respiratory infections may result from microorganisms such as bacteria, fungi, viruses and parasites, which clinically show a high prevalence rate. Since respiratory infections are usually not diagnosed in time, their etiology remains unclear, and a precise clinical treatment hence becomes a far cry. This affects the prognosis and may induce the spread of unknown pathogens in the susceptible population. The first clinical application of NGS was to diagnose neuro *leptospirosis* in a critically ill 14-year-old boy presenting with meningoencephalitis [21]. Owing to the advancement in molecular biology, several studies have recognized the significance of mNGS, which can be particularly used to detect atypical and slow-growing microorganisms. Though mNGS has shown encouraging values in diagnosing different infectious disorders, particularly tuberculous meningitis [22], mNGS still has uncertain diagnostic significance and inaccuracies in lower respiratory tract infections. Therefore, it is warranted to conduct such an investigation.

In this study, mNGS was highly accurate in diagnosing LRTI, and its combined sensitivity, specificity, and AUC were 0.89, 0.90, and 0.94, respectively. The Funnel plot, with p-values greater than 0.05, indicated no publication bias. PLR = 13.2 [1.6106.6]>10 and NLR = 0.17 [0.09,0.31] >0.1 indicated that mNGS was more meaningful for diagnosing diseases rather than for excluding them (Fig. 4). The pooled PLR was 13.2, indicating a 13.2-fold increase in the probability of an accurate diagnosis of LRTI with a positive mNGS test. In addition, the NLR was 0.17, which implied that a negative mNGS result reduced 83% of LRTI.

In the included literature, we found that mNGS has certain advantages compared to traditional microbial diagnostic techniques. First of all, in terms of the positive rate, the sensitivity of [15]the sequencing technology significantly improved compared with a

Table 1
Characteristics of the studies that were included.

First authors	Year	Platform	Patients	TP	FP	TN	FN	Study design	DNA or RNA	Reference
Nana Liu	2020	BGISEQ-50	81	55	2	7	17	Retrospective	DNA + RNA	CRS
Xu Chen	2020	BGISEQ-50	39	13	3	10	3	Retrospective	DNA	CRS
Heping Wang	2020	BGISEQ-100	34	32	1	0	1	Retrospective	DNA	CRS
Cui-Lin Shi	2020	llumina NextSeq	119	74	0	39	6	Prospective	DNA	CRS
Li Liu	2021	BGISEQ	47	31	1	9	6	Retrospective	DNA + RNA	CRS
Hongbin Chen	2021	llumina NextSeq	162	62	17	52	31	Prospective	DNA + RNA	CRS
Xiaowei Fang	2020	BGISEQ-500	72	30	24	17	1	Retrospective	DNA	CRS
Xiaodong Wu	2020	Unknown	329	297	0	25	7	Prospective	DNA + RNA	CRS
Fei Li	2021	llumina MiSeq	48	34	0	9	5	Retrospective	DNA	Multi-PCR
Jingmin Peng	2021	BGISEQ-50	60	30	19	7	4	Retrospective	DNA	CRS
Jinlian Chen	2021	BGISEQ-100	20	17	0	2	1	Retrospective	DNA	CRS
Hongxia Duan	2021	BGISEQ-50	105	60	5	11	29	Retrospective	DNA	CRS
Yangqing Zhan	2021	llumina NextSeq	132	88	0	35	9	Retrospective	DNA	CRS

CRS, composite reference standard; TP, true-positive; FP, false-positive; FN, false-negative; TN, true-negative.

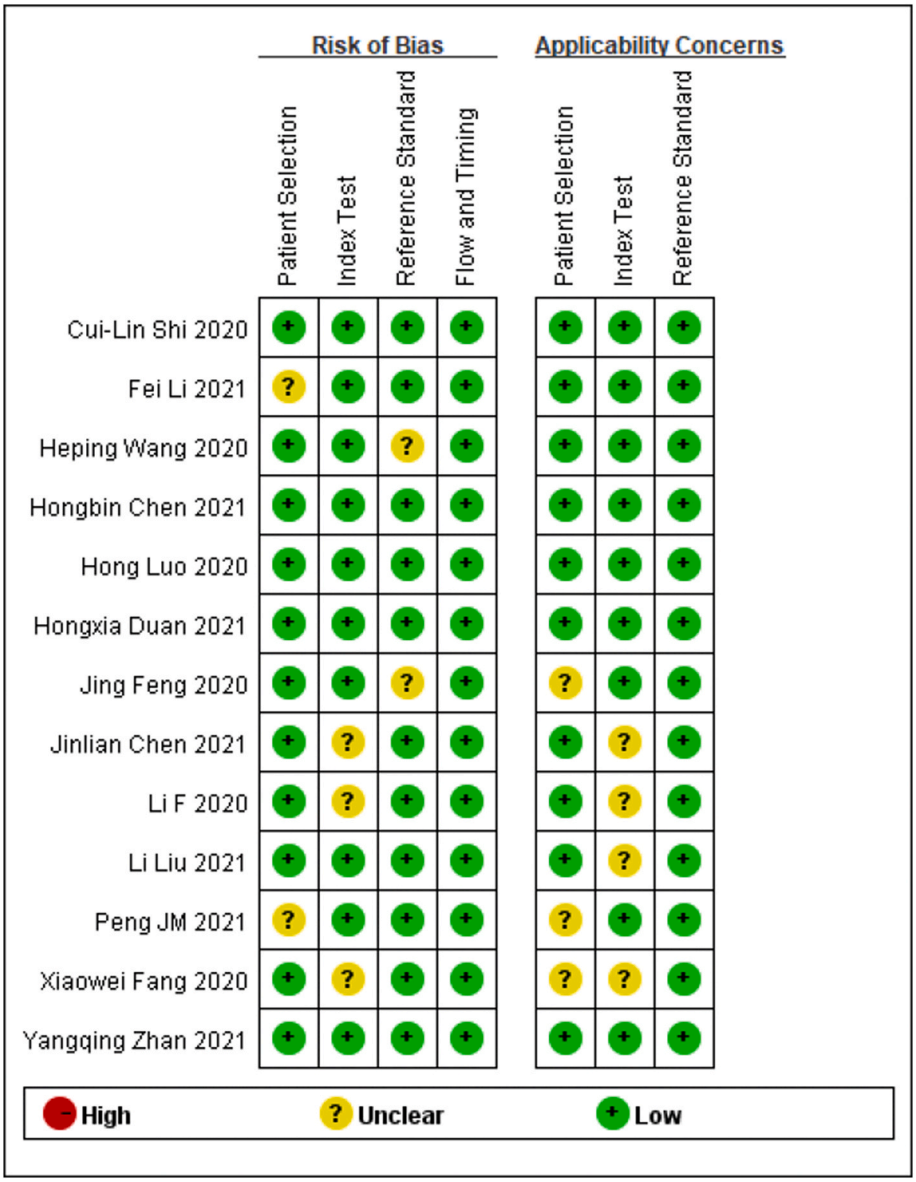

Fig. 2. The methodological quality of the included studies was assessed using a composite reference standard, and the results were presented as percentages in the form of methodological quality graphs, which depict the risk of bias and applicability concerns across the studies.

traditional culture, which is 67.4% and 23.6%, respectively. Among 329 adults with SCAP, a microbial etiology was identified in 304 (92.4%). The overall microbial yield of mNGS was 90.3%, compared to 39.5% in the case of other methods. Moreover, this technology shortens the diagnosis time. For example, routine culture of *Mycobacterium tuberculosis* may take 2–3 weeks. For this slow-growing bacterium, sequencing technology was used to shorten the diagnosis time. Still, the sensitivity was not very far from that of X-pert and traditional culture (which was around 47.92%). Among the articles included in our study, a total of 4 out of 13 (30.7%) conducted both RNA and DNA mNGS, while 8 articles only utilized DNA mNGS. We conducted a subgroup analysis, and the results revealed no significant differences between them. We hypothesize that this observation may be attributed to the specific characteristics of pathogens causing lower respiratory tract infections and the composition of the samples included in the reviewed literature. These factors could potentially account for the absence of significant distinctions in the current findings."

In patients with lower respiratory tract infections, using BALF specimens, mNGS can provide more strain-specific information that could aid in identifying new pathogens and potentially in tracking and controlling outbreaks. In a certain study [15], mNGS alone detected *Tsutsugamushi* and *Coccidiosis*, which cause severe pneumonia. Ever since the outbreak of the pandemic, diagnostic technology of pathogens has become more critical, and one of the discussed studies detected [15] two new *coronavirus* cases simultaneously through sequencing. Regarding co-infection, reports [14]show clear advantages in detecting viruses and identifying co-infections. An

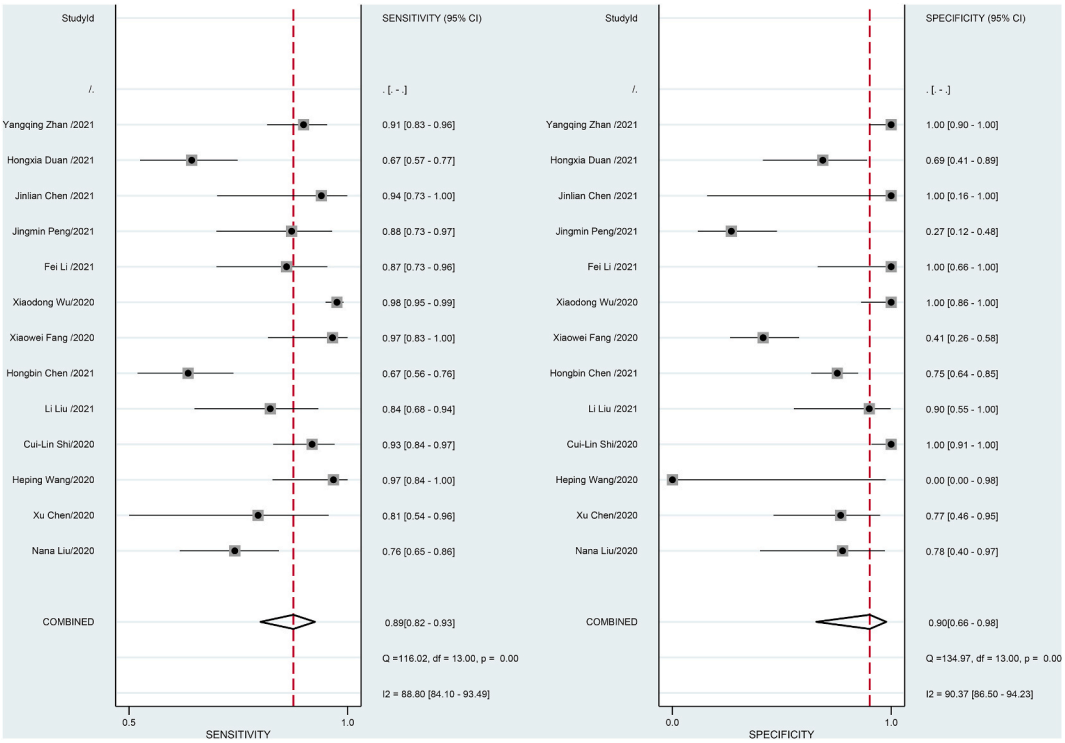

Fig. 3. Forest plot illustrating the sensitivity and specificity of mNGS for diagnosing the condition of interest.

article also pointed out that the diagnosis rate of mixed infection by mNGS technology was 55.6%. Three of the articles we included were prospective studies [11,13,15]. One of these prospective studies [13] used mNGS technology to analyze host transcriptome data to identify differential genes that showed promise in LRTI diagnosis.

In our study, the results of the meta-analysis pointed towards a specificity of 90%, albeit with notable variability. Specificity is calculated based on how many people do not have the disease. It can be calculated using the equation: Specificity = Number of true negatives/(Number of true negatives + Number of false positives). As prior studies have proposed [23], mNGS tends to yield a high false-positive rate. However, a significant majority of the studies we incorporated in this analysis demonstrated low false-positive rates. In some of the articles [15,16], there were zero cases of false positives. This observation has piqued our interest, prompting an analysis of the potential reasons underlying these low false-positive rates. From our perspective, apart from discrepancies stemming from inter-laboratory differences, the determination of true positive results is connected with two screening criteria:

Firstly, threshold criteria are established through dry-lab procedures. On this aspect, the screening standards among all the studies are essentially aligned. Microorganisms were identified as suspected pathogens if their SDSMRN value exceeded 50, and this value was at least threefold higher than that observed in the negative control group. Particularly noteworthy thresholds include Parasite: SDSMRN >100, *Mycobacterium tuberculosis* complex (MTC): SDSMRNG >1. However, it is worth mentioning that there are isolated instances, such as in one article [15], where the threshold for positive selection through dry-lab was not explicitly mentioned.

Secondly, the interpretation of mNGS results requires a reassessment, taking into consideration the patient's clinical symptoms, the inherent characteristics of microorganisms, and other clinical examinations. However, there is currently no unified standard in this regard. This article [24] points out that the interpretation of true positive results first necessitates the classification of the pathogenic probability of microorganisms, followed by clinical decision-making based on clinical features and other relevant tests. Through our analysis of the included literature, it was found that out of the articles considered, 3 articles [13,14,17] showed a relatively higher rate of false positives compared to other articles. These three articles had stringent and clear criteria for determining positive results. For instance, in the article [13], the authors explicitly proposed that pathogens detected by mNGS were classified into 4 categories: definite, probable, possible and unlikely. The article [14] indicated that microorganisms could not be definitively classified as infection, colonization, or pollution based solely on CT and mNGS results. In order to identify the pathogens, two physicians considered clinical features and collaborated to reach a consensus. In some of these articles [15,16], the number of false positive cases is reported as 0. Although the results are directly presented in the articles, there is no detailed explanation of how false positives and negatives were determined. It is briefly mentioned that clinical judgment was used, without a classification of pathogenic probability for microorganisms. This also highlights the challenges in the current clinical application of mNGS technology. Further standardization is required for determining thresholds and interpreting reports.

In addition to needing improvement in report interpretation，mNGS use for diagnosing lower respiratory tract infections is still in its infancy, and many other challenges still remain, as described below.

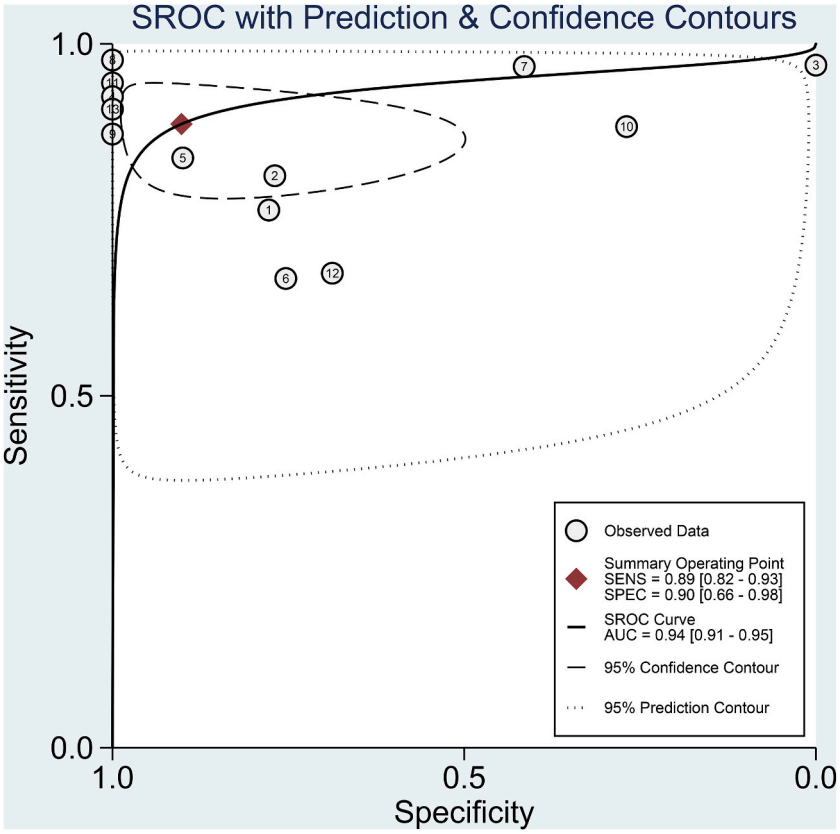

Fig. 4. SROC curve AUC = 0.94.

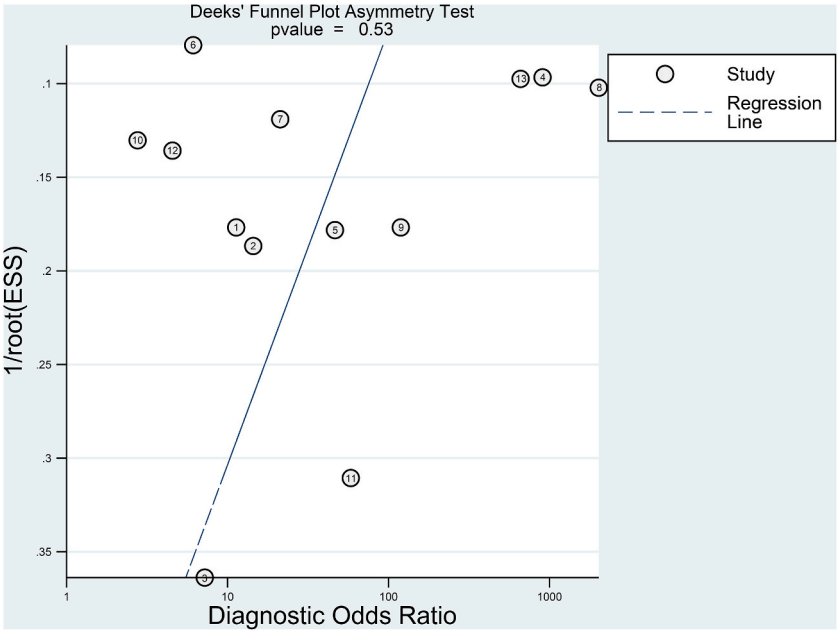

Fig. 5. Funnel plot for publication bias.

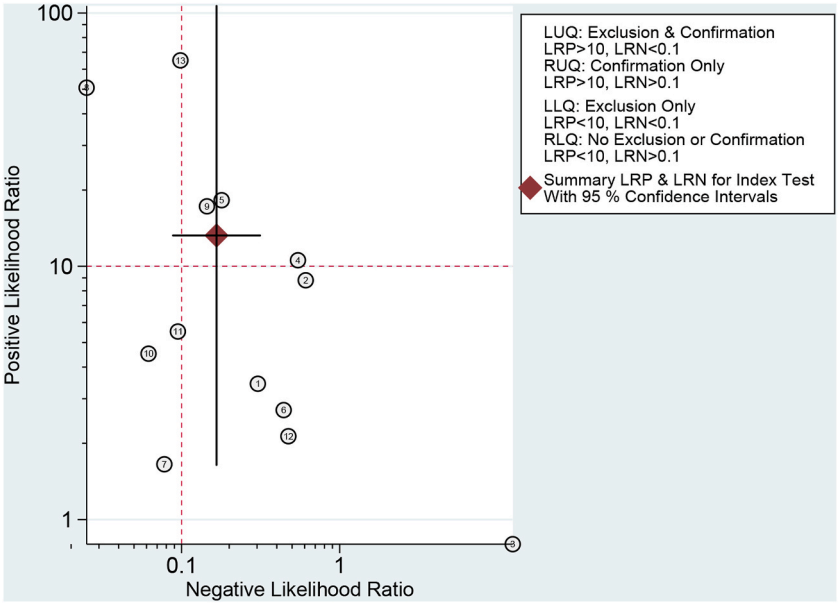

Fig. 6. Plots displaying positive likelihood ratios and negative likelihood ratios can be used to assess the diagnostic significance of a test.

1. At present, there are still certain deficiencies in using bronchoalveolar lavage fluid as a sample to diagnose pathogens of lower respiratory tract infections, the most important reason being host nucleic acid content, which ranges from 10^2–10^9 in BALF [6, 25] (Host nucleic acid: Pathogenic nucleic acid >99:1). Especially when the size of data is small, the possibility of missed detection is relatively high. Depletion of host DNA is an alternative for improving mNGS sensitivity, but it can remove pathogens non-specifically and produce false negatives. The depletion strategy is hence still controversial in sequencing technology. Articles 2 and 3 [9, 10], mentioned in this study, used host DNA depletion techniques during nucleic acid extraction. In addition, the complexity of background bacteria is also high in BALF. Reagent engineering bacteria, introducing sampling or environment or infection of closely related species, may also produce false negatives or positives [19,26].
2. While using mNGS technology, it is necessary to determine the threshold for detecting pathogens, to help distinguish pathogens from background microorganisms. This is because mNGS technology is a self-built laboratory project, and the type and quantity of background bacteria contained in each laboratory and reagent are different [27]. There is currently no unified standard for judgment, and laboratories are mostly required to conduct their own performance verification. In the current research, the bacterial-specific sequences are more than three fragments, and suspicious microorganisms are sorted according to the number of reads in millions or relative abundance [8,9,13,14]. Microorganisms ranked high and had supporting articles demonstrating that the microorganism is significant. The survey [28] showed significant differences in the detection capabilities of various laboratories. The difference in detection performance was mainly reflected in the ability to judge false positives, among which the detection capabilities for fungi and RNA viruses were quite different. Therefore, each laboratory should be mandated to demonstrate a performance verification of experimental pathogens before clinical specimen testing to confirm the threshold of each pathogen. Negative quality control [26] and positive quality control are to be used during the experiment.
3. In the context of severe infections, prompt identification of the underlying microorganisms is of utmost importance for guiding clinical management strategies and administering efficacious antimicrobial therapy [7]. Pathogen virulence and drug-resistance are challenging to detect in sequencing, limiting their impact on the choice of a reasonable antibiotic regime. Some drug resistance genes can be determined by sequencing, but there are many uncertainties in the process, from gene to protein translation and to phenotype. Therefore, a rational selection of antibiotics, guided by sequencing, may bring certain risks to the clinic.
4. It is currently more difficult to distinguish between colonizing and pathogenic microbes. Among our included articles, one of the articles proposed [12] that the fungal load significantly differed between patients with PJP and PJC, while the other articles did not mention it. All the available evidence must be combined with other clinical diagnoses for a comprehensive judgment.

So far, a similar meta-analysis regarding the application of mNGS in pneumonia patients has already been conducted [29] , This analysis was comprehensive and precise, encompassing subgroup analyses for various patient populations. However, our study utilizes a selection criterion whereby the true positive, false positive, false negative and true negative values for the assay can be either directly extracted or calculated from the pertinent studies. That means all 13 included articles in our study contain the comprehensive data. In the aforementioned published paper, only 4 articles were able to analyze specificity. Therefore, our study serves as a complementary effort within the research field.

In addition to this, certain limitations must be highlighted in the present study. The limitations of this study are two aspects. Firstly, the sample size of the RCTs was generally small, with most of the included simple scales consisting of less than 100 subjects. Secondly,

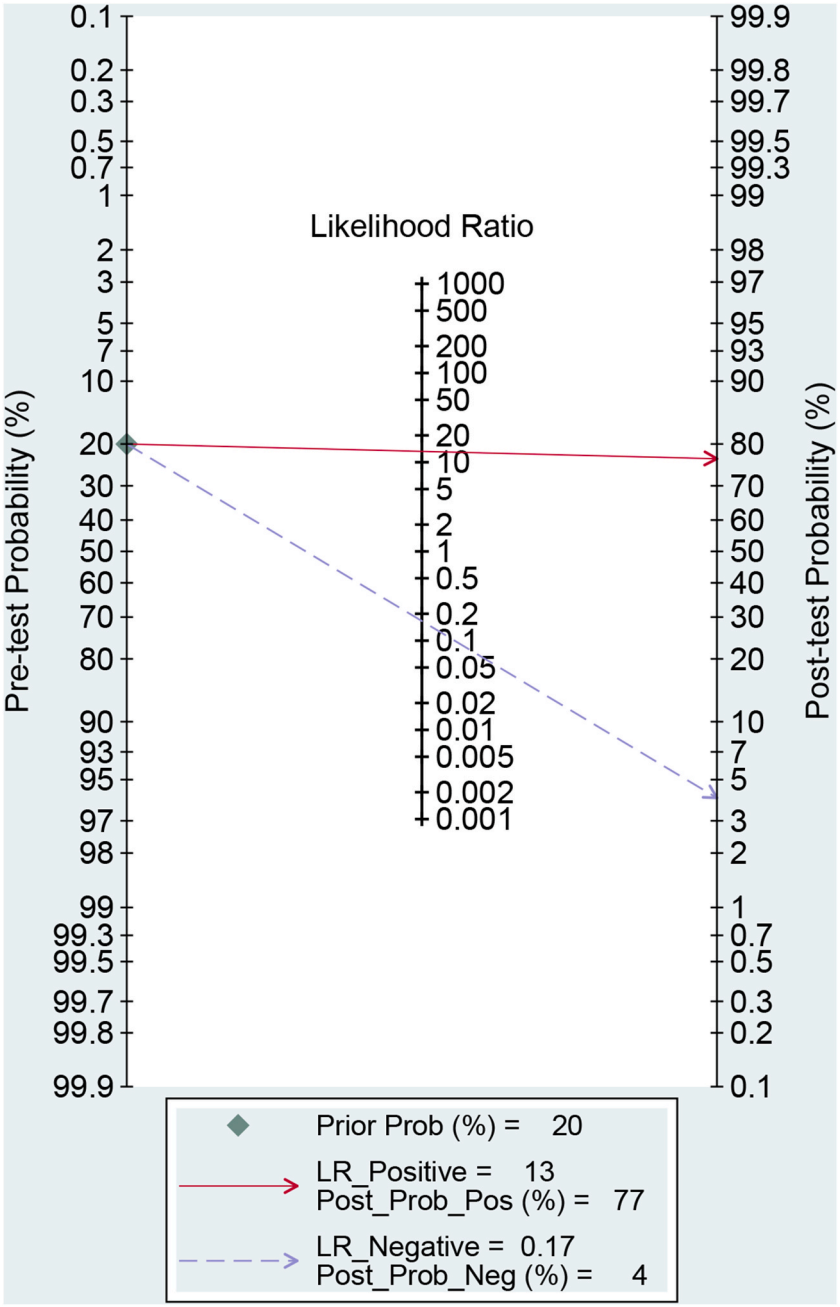

Fig. 7. Pre-probability post-probability plot.

since all the data originated from China, this circumstance could potentially influence the overall findings, introduce systematic bias, compromise result reliability, and might also contribute to the lower occurrence of false positives. Subsequently, it is necessary to continue monitoring this field, incorporating more data from different countries and platforms to analyze the sensitivity and specificity of m NGS in lower respiratory tract infections.

5. Conclusions

As we could observe, mNGS is accurate in diagnosing lower respiratory tract infections, but there is significant variability. Considering the considerably high negative predictive value, a negative result from mNGS should allow clinicians to confidently rule out the possibility of infection. Moreover, as of now, sequencing cannot wholly replace traditional detection technology. It is suggested that a multidisciplinary and multi-experimental technology-based cross-diagnosis is the way to go for the diagnosis of respiratory tract

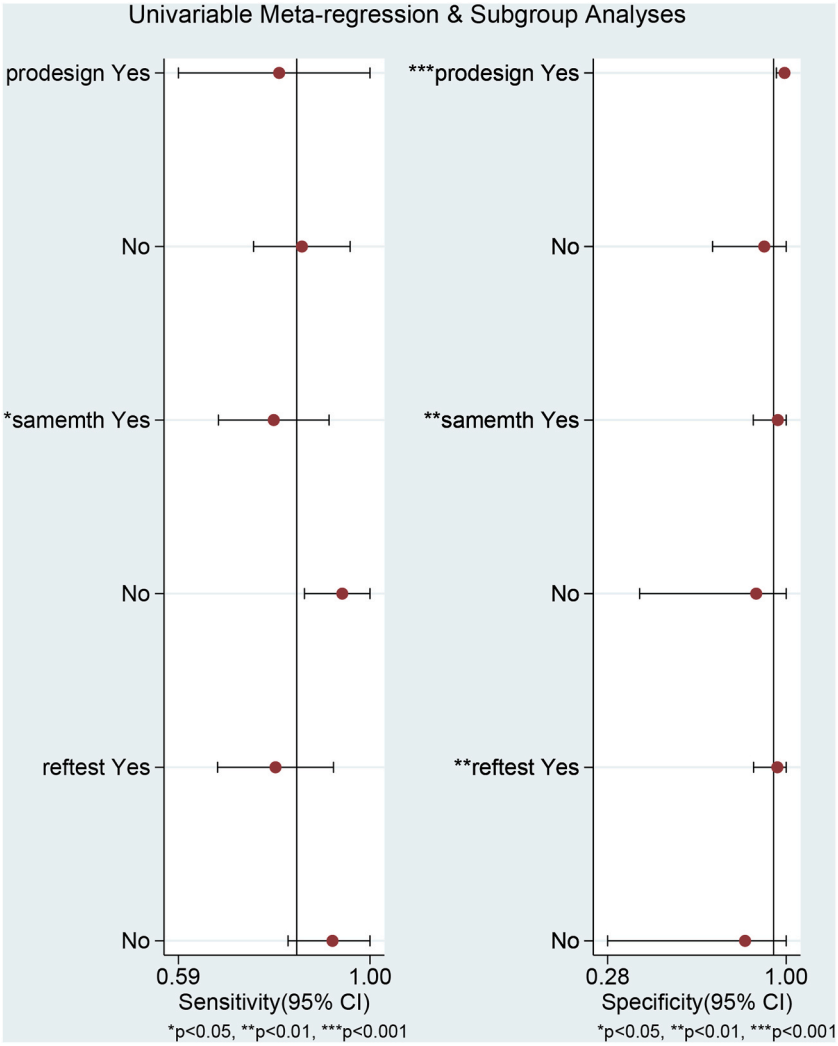

Fig. 8. Plot of univariate regression analysis depicting the analysis of various factors influencing the size of heterogeneity.

infections. The present systematic review offers practical evidence supporting the use of mNGS for clinicians and offers accurate and practical recommendations for diagnosing and treating lower respiratory tract infections. At the same time, large-scale, high-quality articles are warranted for validating our findings and confirming the clinical significance of mNGS among LRTI cases.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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None.

Author contribution

ZSH was responsible for the idea and concept of the paper. XY and GQ built the database and analyzed the data. GQ wrote the manuscript. ZSH critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

CRediT authorship contribution statement

Qiang Guo: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Yang Xiao:** Data curation, Formal analysis, Writing – original draft. **Shihai Zhang:** Data curation, Formal analysis, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23188.

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