Original Article

Evaluation of a multiplex PCR kit for detection of 17 respiratory pathogens in hospitalized patients

Cuiping Zhang[1#], Xiaoyan Chen[1#], Lu Wang[1], Juan Song[1], Chunmei Zhou[2], Xiaohuan Wang[2], Yan Ma[2], Cuicui Chen[1], Wei Guo[2], Yuanlin Song[1,3,4,5,6]^

[1]Shanghai Key Laboratory of Lung Inflammation and Injury, Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, Shanghai, China; [2]Department of Laboratory Medicine, Zhongshan Hospital, Fudan University, Shanghai, China; [3]Shanghai Institute of Infectious Disease and Biosecurity, Shanghai, China; [4]Shanghai Respiratory Research Institute, Shanghai, China; [5]National Clinical Research Center for Aging and Medicine, Huashan Hospital, Fudan University, Shanghai, China; [6]Jinshan Hospital of Fudan University, Shanghai, China

Contributions: (I) Conception and design: W Guo, Y Song; (II) Administrative support: C Chen, C Zhou, W Guo, Y Song; (III) Provision of study materials or patients: C Zhang, X Chen, L Wang, J Song, C Zhou, W Guo, Y Ma, X Wang; (IV) Data collection and compilation: C Zhang, X Chen, L Wang, J Song; (V) Data analysis and interpretation: C Zhang, X Chen, W Guo, Y Song; (VI) Manuscript writing: All authors; (VII) Final manuscript approval: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Wei Guo. Department of Laboratory Medicine, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China. Email: guo.wei@zs-hospital.sh.cn; Yuanlin Song. Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China. Email: ylsong70_02@163.com.

Background: Rapid pathogen identification is critical for optimizing diagnosis and treatment of infectious diseases. Multiplex polymerase chain reaction (PCR) is a sensitive, broad-spectrum molecular detection technique that is simple and rapid to perform. It is capable of simultaneously screening for multiple pathogens within a short time range. Here, we designed and evaluated a multiplex PCR kit for the identification of 17 common respiratory pathogens in clinical samples from hospitalized patients.

Methods: A total of 452 samples from hospitalized patients, including 242 respiratory and 210 non-respiratory samples, were analyzed for 13 bacteria and 4 fungi by a multiplex fluorescent PCR kit. The diagnostic performance of the kit was assessed by considering routine microbiology as the reference standard.

Results: The overall positivity rate of the multiplex PCR kit was 86.9%, much higher than that noted on routine microbiology (56.9%). Furthermore, the co-infection detection rate was also significantly higher than that noted on routine microbiology (69.5% *vs.* 15.0%). Compared with routine microbiology, kit sensitivity was >90% for detection of most target bacteria, with a negative predictive value (NPV) of >99%, especially for detection of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli*. The kit was noted to be particularly superior in identifying *Stenotrophomonas maltophilia* and *Streptococcus pneumoniae* as compared to routine microbiology. The multiplex PCR kit was noted to be less sensitive (33.3–59.6%) and more specific (93.9–100.0%) for detection of mycobacteria and fungi.

Conclusions: Our multiplex PCR kit offers a rapid and sensitive diagnosis of common bacterial pneumonia, although sensitivity for mycobacteria and fungi warrants enhancement. Further optimization includes minimizing false positivity and increasing relevance to clinical application.

Keywords: Multiplex polymerase chain reaction (PCR); pathogen detection; rapid diagnostics; molecular diagnostics; pneumonia

Submitted Apr 20, 2022. Accepted for publication Aug 19, 2022.
doi: 10.21037/jtd-22-544
View this article at: https://dx.doi.org/10.21037/jtd-22-544

^ ORCID: 0000-0002-9826-4383.

Introduction

Pulmonary infections are the largest cause of human disease burden and carry high morbidity and mortality (1). Crude mortality was estimated at 20–60% for hospitalized patients with severe community-acquired pneumonia (2-4) and 30–70% for hospital-acquired pneumonia (5). Rapid identification of causative pathogens and prompt initiation of effective antimicrobial therapy are crucial for the prognosis of pneumonia patients, with significantly increased mortality reported among patients receiving delayed or ineffective treatment (6). In China, in-hospital mortality rates for diagnosed and undiagnosed patients are 21.7% and 25.9%, respectively (2).

Diagnosis of most bacterial and fungal infections depends on successful microbial or fungal culture, a process that takes about 48–72 h, prior to which patients can only receive empirical therapy. In addition, the low sensitivity of routine microbiology (approximately 44.2%) (7) that frequently limits patients to broad-spectrum treatment not only worsens prognosis but also increases the risk of adverse effects and promotes antibiotic resistance (8,9).

Multiplex polymerase chain reaction (PCR) offers rapid detection and precise identification of a large number of respiratory viruses by amplifying genomic fragments (10). A prior study reported a 30–50% increase in respiratory virus identification when using multiplex PCR as compared to antibody and culture methods (11). However, relatively few studies have investigated the use of multiplex PCR for the diagnosis of bacterial and fungal infections. Here, we designed and assessed a multiplex fluorescent PCR kit capable of detecting 17 respiratory pathogens (13 bacteria and 4 fungi) simultaneously in one PCR panel. We collected 452 respiratory and non-respiratory samples from hospitalized patients to evaluate the clinical performance of this kit and compared the results with those of routine microbiological tests. We present the following article in accordance with the STARD reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-544/rc).

Methods

Multiplex fluorescent PCR kit

The 17 respiratory pathogens targeted for detection by our multiplex PCR kit were as follows: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, *group A streptococcus*, *Haemophilus influenzae*, *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Mycobacterium tuberculosis*, *atypical mycobacteria*, *Candida albicans*, *Mucor*, *Aspergillus* and *Penicillium marneffei*. Target sequences corresponding to these pathogens were searched in the National Center of Biotechnology Information (NCBI); retrieved sequences were aligned using specialized software to select conserved regions with higher homology as targets for pathogen detection and thus ensure greater specificity. Corresponding primer and probe sequences were designed according to relevant pathogen conserved sequences (*Table 1*). The pre-designed multiplex fluorescent PCR kit panel included the primer and probe reaction mixture (4 μL) as well as nucleic acid amplification reaction solution (16 μL). The nucleic acid amplification reaction solution used as quantitative real time PCR (qPCR) reaction premix consisting of Tris (0.05–0.1 M), KCl (100–200 mM), $MgCl_2$ (1–5 mM), dNTPs (0.1–0.8 mM) and Taqman (0.1–1 U/μL).

The plasmid used as a positive control for kit sensitivity evaluation was purchased from Shanghai Generay Biotech Co., Ltd. Target pathogen plasmids were mixed and diluted with Tris-EDTA to 100 copies/μL, 10 copies/μL, 2 copies/μL, 1 copy/μL, and 0.5 copies/μL, and then detected using the PCR kit. For sensitivity testing, a positive result was determined if the cycle threshold (Ct) value was ≤38 and the lowest copy number for which both tests were positive was considered as test sensitivity. Results revealed the minimum copy number of each target pathogen detected to have been 0.5 copies/μL and 1 copy/μL (*Table 2*).

Samples

According to the number of samples required for positive validation and negative controls, a total of 483 samples (452 of them eligible) were discontinuously collected from infectious disease patients admitted to the Department of Pulmonary Medicine at Zhongshan Hospital, Fudan University between December 2020 and October 2021. Specimens of sufficient volume for two repeated PCR tests (>1 mL) and had at least one available test result on routine microbiological testing were included. The 452 eligible samples included 242 respiratory samples and 210 non-respiratory samples. Respiratory samples consisted of 206 sputum and 36 lower respiratory tract samples (endotracheal aspirates, bronchoalveolar lavage fluid and lung tissue). Non-respiratory samples consisted of 62 excrement (feces

Table 1 Primers and probes used for the detection of 17 pathogens

Pathogen	Forward primer (5'–3')	Reverse primer (5'–3')	Probe (5'–3')
Bacteria			
Pseudomonas aeruginosa	TCGCAGACCAAGGACAAGCT	TTGCCCATCTCCTGTTCCA	TCTCCTCCGAGGTGAAGACCGCG
Klebsiella pneumoniae	TCCCCTTTGCCGTGAATAATC	GCGGCATACGCTGCTGTAT	CCCCGGTGGTCACCATTTCGG
Acinetobacter baumannii	TGCGACACAACTCGACGTTT	AATCTAGCACGACCTGACCA TAGAC	TTTAAACCGATTGATTTGTCGCCGA TCTTT
Staphylococcus aureus	TGATGGCTTTGAAGTAGTTTT	CACGATTCGAATAGTAAACATAA	TGCAGCAAGCCTTTTCTCTAAAATT
Stenotrophomonas maltophilia	CATGGCCAACGAAGAAAAGC	TGCAGGGTGTGGGTCACTT	TACGGCGTGCAGTTCCACCCG
Streptococcus pneumoniae	CCTCGTTGAAGCAATGGTGC	CCCAACAAGTGAATCACCAACA	TGGCATGGGCATGGTTGGTTTGGT
Group A streptococcus	CTCCTGGTGATCCCATACCAA	CTCCTGGTGATCCCATACCAA	TCCCACAAAGTCAGCACTGCTTA GACCA
Haemophilus influenzae	CCCAACAAGTGAATCACCAACA	TTCGCACATGAGCGTCAGTA	ACCGAAGGCGAAGGCAGCCCC
Enterococcus faecium	CAATGCTGCTTTGATACGAGTGT	AAGTTCTTGTCCGTGTTGA CTTCA	CAGTGATCACGCCGTCTTTCAA AGGAA
Enterococcus faecalis	ACTTTGGTGTTGTTGAAGGTT TAATG	CCTTTAGGATGTGGTCCGTCTAA	CTATCCACGCTTACACAGGTGACC AAATGA
Escherichia coli	GCTGCTGTGGCGTCAAACT	GACCTACATGAGTGATTGC CTGAA	TTTTCACCAGGCGCAGACTTGCTGT
Mycobacterium tuberculosis	GCATCTGGCCACCTCGAT	GCCGCCAACTACGGTGTTTA	CCCTCACGGTTCAGGGTTAGCCACA
Atypical mycobacteria	TCCCGGGCCTTGTACACA	CCACTGGCTTCGGGTGTTA	CGCCCGTCACGTCATGAAAGTCG
Fungi			
Candida albicans	ACCTGAAGTTTTACAATCAG CAACA	TGCTCGTAGCATTATCTATG CCTTA	TTACCAGCAGAATCAAAATGCACTT GACCA
Mucor	AGTACTTTGAAAAGAGAGTTA AACAG	GCTGATTAACTTCAAGTCAGTCT	TCGCAACCGACTCCATTAAGAA CACCA
Aspergillus	GGCCGACAACAGCGTCAT	TCTGCTTGGCGGTGATGTAA	ATGTGAAATTGCCAAGAGGGAAGC ATTTG
Penicillium marneffei	TGAAATTGTTCCTGCTCATGGT	CCACTCCCGTCGTAAATGTGT	TTCCTGGCATCCCTGTCAGCCATT
Internal reference	CTTCAGCATGGCGGTGTTT	CCGCGCAGAGCCTTCA	CAGATTTGGACCTGCGAGCGGG

and urine) and 148 other body fluid and tissue samples (pleural effusion, ascitic fluid, cerebrospinal fluid, bile, pericardial fluid). After sample collection, approximately 1 mL of specimen was aliquoted per sample and stored at −80 ℃ for multiplex PCR testing within one week. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Medical Ethics Committee of Zhongshan Hospital, Fudan University (approval No. B2019-249R) and written informed consent was obtained from all patients.

Nucleic acid extraction

After homogenization, enzyme digestion or sample centrifugation, homogenous samples (300 μL) were transferred to a sample lysis plate and subjected to

Table 2 Sensitivity assessment of the multiplex PCR kit for the 17 pathogens

Pathogen	100 copies/μL		10 copies/μL		2 copies/μL		1 copies/μL		0.5 copies/μL	
	Value 1 (Ct)	Value 2 (Ct)	Value 1 (Ct)	Value 2 (Ct)	Value 1 (Ct)	Value 2 (Ct)	Value 1 (Ct)	Value 2 (Ct)	Value 1 (Ct)	Value 2 (Ct)
Bacteria										
Pseudomonas aeruginosa	28.67	28.54	31.69	30.67	33.44	33.25	34.22	36.31	34.71	35.87
Klebsiella pneumoniae	27.15	27.30	30.51	29.44	32.41	32.17	33.87	33.25	35.38	32.66
Acinetobacter baumannii	29.95	29.88	33.27	32.99	36.06	35.59	35.57	–	37.12	36.71
Staphylococcus aureus	28.89	29.01	32.84	32.04	34.37	34.90	36.73	35.43	36.13	36.17
Stenotrophomonas maltophilia	29.11	29.12	32.23	32.14	34.86	35.65	37.62	35.76	37.67	–
Streptococcus pneumoniae	29.37	29.47	33.16	30.06	34.54	35.46	35.24	37.19	–	–
Group A streptococcus	27.59	27.60	31.32	30.83	33.17	33.28	35.00	33.79	35.71	35.50
Haemophilus influenzae	30.64	30.91	35.48	35.06	38.78	35.12	34.82	–	–	36.95
Enterococcus faecium	30.28	30.43	33.16	34.57	34.79	35.90	–	36.17	36.91	–
Enterococcus faecalis	30.14	30.50	34.95	33.36	34.96	35.23	36.94	36.45	–	–
Escherichia coli	28.97	29.25	32.22	32.37	36.10	34.39	35.91	34.88	–	–
Mycobacterium tuberculosis	28.51	28.52	31.51	32.61	35.18	34.64	34.89	36.10	–	36.82
Atypical mycobacteria	27.91	27.52	30.87	31.05	34.35	34.69	34.46	34.36	33.90	35.97
Fungi										
Candida albicans	26.350	28.848	30.651	33.690	34.477	36.970	36.164	34.976	36.517	36.377
Mucor	27.431	27.457	30.505	31.309	35.603	33.693	35.324	35.225	35.571	36.694
Aspergillus	25.937	25.928	27.874	31.236	32.483	32.921	33.645	34.419	34.821	34.613
Penicillium marneffei	27.053	27.564	32.355	31.663	34.128	33.295	33.570	35.512	36.055	36.943

PCR, polymerase chain reaction; Ct, cycle threshold value.

automated nucleic acid extraction using a nucleic acid extraction kit (Cat. #TQ-BG-001; BioGerm, Shanghai, China) and a BG-Abot-96 automated nucleic acid extraction system (BioGerm) according to manufacturer instructions.

Multiplex fluorescent PCR detection of respiratory pathogens

Nucleic acid extracted from all 452 specimens was subjected to multiplex amplification using our multiplex fluorescent PCR kit for respiratory pathogen detection. All PCR reactions were carried out using 5 μL of resuspended deoxyribonucleic acid (DNA), 4 μL of primer/probe mixture and 16 μL of nucleic acid amplification reaction solution. Optimal cycling conditions for nucleic acid amplification were initially 5 min at 95 ℃ followed by 40 cycles at 95 ℃ for 10 s and 55 ℃ for 40 s using an ABI 7500 (Invitrogen, Waltham, USA) device. It took a total of 80 min to complete pathogen detection. A positive result was defined by Ct values of ≤35 or 35–38 confirmed on repeat testing. Clinical information and routine microbiological tests results were not provided to researchers performing and evaluating multiplex PCR testing.

Routine microbiological result collection

All routine microbiological tests were performed according to requests of clinicians based on relevant standard operating procedures. Routine microbiological tests including bacterial and fungal smears and cultures, mycobacterial acid-fast smear and culture, and *Aspergillus* culture; data were collected from medical records upon conclusion of multiplex PCR testing. Clinical data and multiplex PCR test results were not available to performers

Table 3 Positive rates and numbers of pathogens detected by routine microbiology from respiratory samples or non-respiratory samples

Pathogen	Positive rate		
	All samples (n=452)	Respiratory samples (n=242)	Non-respiratory samples (n=210)
Bacteria			
Pseudomonas aeruginosa	15.8% (70/442)	22.8% (54/237)	7.8% (16/205)
Klebsiella pneumoniae	6.8% (30/442)	8.4% (20/237)	4.9% (10/205)
Acinetobacter baumannii	19.7 (87/442)	23.6% (56/237)	15.1% (31/205)
Staphylococcus aureus	5.4% (24/442)	7.2% (17/237)	3.4% (7/205)
Stenotrophomonas maltophilia	3.6% (16/442)	5.1% (12/237)	2.0% (4/205)
Streptococcus pneumoniae	0% (0/442)	0% (0/237)	0% (0/205)
Group A streptococcus	0% (0/442)	0% (0/237)	0% (0/205)
Haemophilus influenzae	1.4% (6/442)	2.5% (6/237)	0% (0/205)
Enterococcus faecium	3.6% (16/442)	1.7% (4/237)	5.9% (12/205)
Enterococcus faecalis	2.3% (10/442)	1.3% (3/237)	3.4% (7/205)
Escherichia coli	2.0% (9/442)	0.8% (2/237)	3.4% (7/205)
Mycobacterium tuberculosis	6.2% (9/145)	8.6% (8/93)	1.9% (1/52)
Atypical mycobacteria	5.5% (8/145)	8.6% (8/93)	0% (0/52)
Fungi			
Candida albicans	11.9% (48/405)	19.1% (43/225)	2.8% (5/180)
Mucor	0% (0/405)	0% (0/225)	0% (0/180)
Aspergillus	6.5% (6/93)	6.6% (6/91)	0% (0/2)
Penicillium marneffei	0% (0/405)	0% (0/225)	0% (0/180)

of routine microbiological examinations.

Statistical analyses

Statistical analyses were performed using SPSS version 20 (IBM, Armonk, USA) and MedCalc version 20.027 (MedCalc Ltd. Ostend, Belgium). Statistical comparisons were analyzed using the chi-squared test. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were analyzed for each PCR target considering routine microbiology as the reference standard (smears and cultures are the traditional diagnostic criteria for the 17 pathogens detected by our kit) (12).

Results

Routine microbiology results

Conventional bacterial smears and cultures were performed using 442 samples for detection of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, *group A streptococcus*, *Haemophilus influenzae*, *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli* (*Table 3*). Conventional fungal smears and cultures were performed on 405 samples to identify *Candida albicans*, *Mucor*, *Aspergillus* and *Penicillium marneffei* (*Table 3*). A total of 145 samples were evaluated using acid-fast smear and culture for *M. tuberculosis*. *Aspergillus* cultures were performed on 93 samples (*Table 3*). Presence of one or more pathogens was noted in 56.9% (257/452) of all samples; the positivity rate in respiratory and non-respiratory samples was 74.0% (179/242) and 37.1% (78/210), respectively.

Among the 442 samples studied using bacterial smear and culture, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were the most frequently noted pathogens among both respiratory and non-respiratory samples (*Figure 1*).

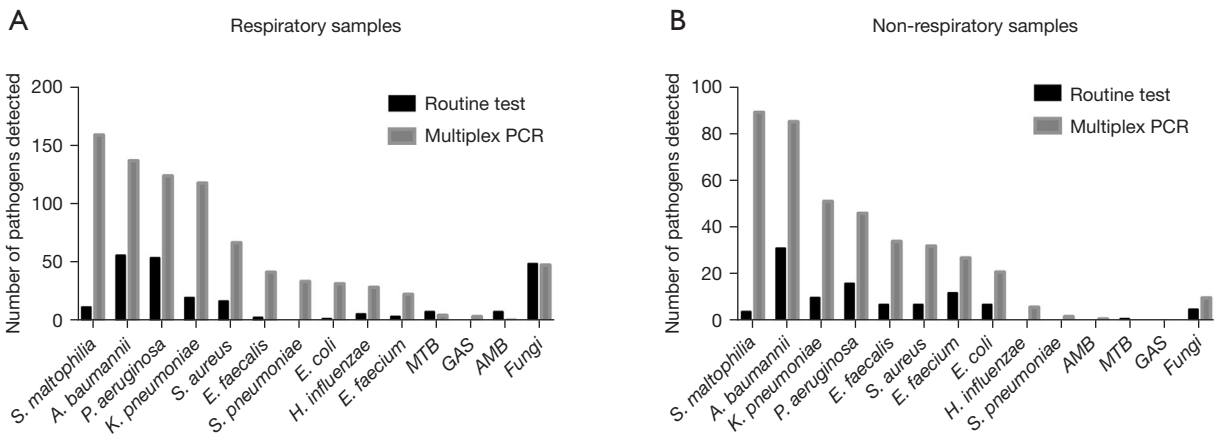

Figure 1 Numbers and types of pathogens detected by routine microbiology or multiplex PCR kit from studied samples. (A) Numbers and types of pathogens detected by routine microbiology or multiplex PCR kit from respiratory samples; n=242. (B) Numbers and types of pathogens detected by routine microbiology or multiplex PCR kit from non-respiratory samples; n=210. PCR, polymerase chain reaction; MTB, *Mycobacterium tuberculosis*; GAS, *group A streptococcus*; AMB, *atypical mycobacteria*.

Acinetobacter baumannii was found in 23.6% (56/237) of respiratory and 15.1% (31/205) of non-respiratory samples, while *Pseudomonas aeruginosa* was found in 22.8% (54/237) of respiratory and 7.8% (16/205) of non-respiratory samples (*Table 3*). *Klebsiella pneumoniae* was the third most frequently detected pathogen (8.3%) in respiratory samples (*Figure 1A*), while *Enterococcus faecium* was the third most frequently detected pathogen (5.9%) in non-respiratory samples (*Figure 1B*). Notably, neither *Streptococcus pneumoniae* nor *group A streptococcus* was noted among the 442 samples evaluated (*Table 3*; *Figure 1*). Similarly, *Haemophilus influenzae* and *atypical mycobacteria* were not detected among the 210 non-respiratory samples (*Figure 1B*). On fungal smear and culture, *Candida albicans* was found in 43 respiratory and five non-respiratory samples, while *Aspergillus* was found in six respiratory samples (*Figure 1*).

Multiplex PCR results

The overall positive rate for multiplex PCR detection exceeded that of routine microbiology (86.9%; 393/452); overall positive rates in respiratory and non-respiratory specimens were 94.2% (228/242) and 78.6% (165/210), respectively. Furthermore, the most frequently detected pathogens on multiplex PCR were *Stenotrophomonas maltophilia* in both respiratory (65.7%; 159/242) and non-respiratory (42.4%; 89/210) samples (*Figure 1*). In respiratory samples, the other most frequently detected pathogens were *Acinetobacter baumannii* (56.6%; 137/242), *Pseudomonas aeruginosa* (51.2%; 124/242), *Klebsiella pneumoniae* (48.8%; 118/242) and *Staphylococcus aureus* (27.7%; 67/242) (*Figure 1A*; *Table 4*). Among non-respiratory samples, the other four most frequently noted pathogens were *Acinetobacter baumannii* (40.5%; 85/210), *Klebsiella pneumoniae* (24.3%; 51/210), *Pseudomonas aeruginosa* (21.9%; 46/210) and *Enterococcus faecalis* (16.2%; 34/210) (*Figure 1B*; *Table 4*). *Streptococcus pneumoniae* was found in 36 samples using multiplex PCR but not routine microbiology (*Tables 3*,*4*).

No *atypical mycobacteria* were detected in respiratory samples and no *Mycobacterium tuberculosis* or *group A streptococcus* were detected in non-respiratory samples (*Table 4*). Multiplex PCR results revealed 48 cases of fungi in respiratory samples, including 44 *Candida albicans*, 3 *Aspergillus* and 1 *Penicillium marneffei*, as well as 10 cases in non-respiratory samples, including 8 *Candida albicans*, 1 *Mucor* and 1 *Aspergillus* (*Table 4*).

Comparison of routine microbiology and multiplex PCR kit pathogen detection

Not only was the overall positive rate of pathogen detection on multiplex PCR significantly higher than that of routine microbiology, but the rate of multiple organisms detection was also markedly greater. As shown in *Figure 2*, 155 respiratory (64.0%) and 66 non-respiratory (31.4%) samples were found to be positive for three or more pathogens on multiplex PCR, significantly greater than the

Table 4 Positive rates and numbers of pathogens detected by multiplex PCR from respiratory samples or non-respiratory samples

Pathogen	Positive rate		
	All samples (n=452)	Respiratory samples (n=242)	Non-respiratory samples (n=210)
Bacteria			
Pseudomonas aeruginosa	37.6% (170/452)	51.2% (124/242)	21.9% (46/210)
Klebsiella pneumoniae	37.4% (169/452)	48.8% (118/242)	24.3% (51/210)
Acinetobacter baumannii	49.1% (222/452)	56.6% (137/242)	40.5% (85/210)
Staphylococcus aureus	21.9% (99/452)	27.7% (67/242)	15.2% (32/210)
Stenotrophomonas maltophilia	54.9% (248/452)	65.7% (159/242)	42.4% (89/210)
Streptococcus pneumoniae	8.0% (36/452)	14.0% (34/242)	1.0% (2/210)
Group A streptococcus	0.9% (4/452)	1.7% (4/242)	0% (0/210)
Haemophilus influenzae	7.7% (35/452)	12.0% (29/242)	2.9% (6/210)
Enterococcus faecium	11.1% (50/452)	9.5% (23/242)	12.9% (27/210)
Enterococcus faecalis	16.8% (76/452)	17.4% (42/242)	16.2% (34/210)
Escherichia coli	11.7% (53/452)	13.2% (32/242)	10.0% (21/210)
Mycobacterium tuberculosis	1.1% (5/452)	2.1% (5/242)	0% (0/210)
Atypical mycobacteria	0.4% (2/452)	0.4% (1/242)	0.5% (1/210)
Fungi			
Candida albicans	11.5% (52/452)	18.2% (44/242)	3.8% (8/210)
Mucor	0.2% (1/452)	0% (0/242)	0.5% (1/210)
Aspergillus	0.9% (4/452)	1.2% (3/242)	0.5% (1/210)
Penicillium marneffei	0.2% (1/452)	0.4% (1/242)	0% (0/210)

PCR, polymerase chain reaction.

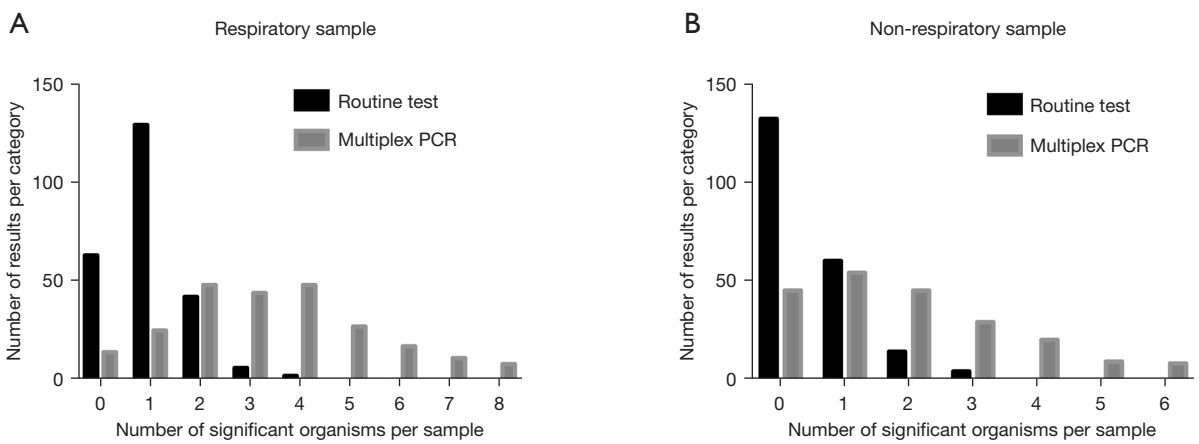

Figure 2 Number of significant organisms detected per sample by routine microbiology or multiplex PCR kit. (A) Number of significant organisms detected per respiratory sample by routine microbiology or multiplex PCR kit. (B) Number of significant organisms detected per non-respiratory sample by routine microbiology or multiplex PCR kit. PCR, polymerase chain reaction.

Table 5 Pathogen-specific performance of PCR tests as compared with routine microbiology as the gold standard in respiratory samples or non-respiratory samples

Pathogen	All samples (n=452)				Respiratory samples (n=242)				Non-respiratory samples (n=210)			
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Bacteria												
Pseudomonas aeruginosa	97.1	72.8	40.2	99.3	98.1	61.7	43.1	99.1	93.8	83.6	32.6	99.4
Klebsiella pneumoniae	96.7	66.5	17.4	99.6	95.0	54.8	16.2	99.2	100.0	79.5	20.0	100.0
Acinetobacter baumannii	95.4	61.1	37.6	98.2	96.4	54.1	39.4	98.0	93.5	68.4	34.5	98.3
Staphylococcus aureus	87.5	81.6	21.4	99.1	88.2	76.8	22.7	98.8	85.7	86.9	18.8	99.4
Stenotrophomonas maltophilia	93.8	46.7	6.2	99.5	100.0	35.6	7.6	100.0	75.0	59.2	3.5	99.3
Streptococcus pneumoniae	NA	92.5	0	100.0	NA	86.9	0	100.0	NA	99.0	0	100.0
Group A streptococcus	NA	99.1	0	100.0	NA	98.3	0	100.0	NA	100.0	100.0	100.0
Haemophilus influenzae	83.3	93.1	14.3	99.8	83.3	89.6	17.2	99.5	NA	97.1	0	100.0
Enterococcus faecium	87.5	91.5	28.0	99.5	75.0	91.4	13.0	99.5	91.7	91.7	40.7	99.4
Enterococcus faecalis	90.0	84.5	11.8	99.7	100.0	83.3	7.1	100.0	85.7	85.9	17.6	99.4
Escherichia coli	100.0	89.8	17.0	100.0	100.0	87.2	6.3	100.0	100.0	92.9	33.3	100.0
Mycobacterium tuberculosis	33.3	100.0	100.0	95.8	25.0	98.8	66.7	93.3	0	100.0	NA	98.1
Atypical mycobacteria	0	100.0	NA	94.5	0	100.0	NA	91.4	NA	100.0	100.0	100.0
Fungi												
Candida albicans	59.6	93.9	56.0	94.6	58.1	90.1	58.1	90.1	75.0	97.7	42.9	99.4
Mucor	NA	100.0	100.0	100.0	NA	100.0	100.0	100.0	NA	100.0	100.0	100.0
Aspergillus	50.0	100.0	100.0	96.7	50.0	100.0	100.0	96.6	NA	100.0	100.0	100.0
Penicillium marneffei	0	100.0	NA	99.3	NA	100.0	100.0	100.0	NA	100.0	100.0	100.0

PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; NA, not available.

eight respiratory (3.3%) and four non-respiratory (1.9%) samples evaluated using routine microbiology. Moreover, no specimens were positive for five or more pathogens on routine microbiology; multiplex PCR revealed 63 respiratory and 17 non-respiratory samples to be positive for five or more pathogens (*Figure 2*).

The overall proportion of agreement (concordant positive/all positive results on routine microbiology) among multiplex PCR and routine microbiology for detection and identification of the aforementioned bacteria was found to be 95.8%. The sensitivity of multiplex PCR was >90% for most target bacteria; NPVs were >99%, especially for *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli* (*Table 5*). Specificity and PPV were lower as multiplex PCR detected more pathogens per sample and revealed more positive samples as compared to routine microbiology (*Table 5*). Test result consistency (Table S1) also revealed that multiplex PCR had higher sensitivity for detection of most bacteria and thus positivity coincident rates were relatively low. *Streptococcus pneumoniae* and *group A streptococcus* were not detected on routine microbiology but were found in 36 and four samples, respectively, on multiplex PCR; as such their relevant specificities were high but sensitivities could not be calculated (*Tables 3-5*). However, lower sensitivity

(33.3–59.6%) and higher specificity (93.9–100.0%) for detection of fungi and mycobacteria was noted using our multiplex PCR kit (*Table 5*). Although the number of fungi detected using multiplex PCR was close to that of routine microbiology, the concordant rate of positivity between the two methods was poor.

Discussion

In recent years, multiplex PCR has emerged as a simple, rapid and highly sensitive method for detection of respiratory pathogens (10,13). Here, we designed a multiplex fluorescent PCR kit for the identification of 17 respiratory pathogens simultaneously and evaluated its performance using 452 clinical samples obtained from inpatients. Sensitivity, specificity, PPV and NPV were analyzed for each PCR target considering routine microbiology as the gold standard.

Based on fluorescent PCR principles, we designed specific primers and Taqman probes for pathogen detection using a fluorescent PCR instrument. Our kit was designed to simultaneously detect 13 bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, *group A streptococcus*, *Haemophilus influenzae*, *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Mycobacterium tuberculosis*, and *atypical mycobacteria*), as well as four fungi (*Candida albicans*, *Mucor*, *Aspergillus*, and *Penicillium marneffei*) in one PCR panel. Compared with routine microbiology testing, which usually takes at least 48–72 h to obtain the culture result and requires higher sample quality, the multiplex PCR kit can complete the detection of 17 pathogens in only 80 min. Our kit is thus useful for the auxiliary diagnosis and epidemiological surveillance of respiratory pathogens.

The overall positive rate of pathogen detection (86.9% *vs.* 56.9%) and the detection rate of multiple organisms on multiplex PCR were both significantly higher than those of routine microbiology. Medical record review revealed detection of relevant pathogens on routine microbiology after significant illness progression; multiplex PCR was capable of detecting pathogens rapidly and with greater sensitivity. Clinical use of multiplex PCR thus offers a greater possibility of earlier diagnosis and treatment initiation.

In this study, clinical data analyzed were obtained from patients suffering nosocomial infections. In agreement with relevant prior literature (14), our routine microbiology findings revealed *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* to have been the three predominant pathogens in respiratory samples, while *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus faecium* were found to have been the three predominant pathogens in non-respiratory samples. However, the pathogen most frequently detected by multiplex PCR in both respiratory and non-respiratory specimens was *Stenotrophomonas maltophilia*, and even it was found in more than half of respiratory specimens. As recent literature has reported the incidence of hospital-acquired *Stenotrophomonas maltophilia* infections to be on the increase (15), particularly among immunocompromised patients (16), its high rate of detection here warrants attention. Although *Stenotrophomonas maltophilia* is not highly virulent, it has emerged as an important nosocomial pathogen associated with a high mortality among patients suffering bacteremia that ranges from 14% to 69% (15,17,18). Inappropriate initial empirical antibiotic therapy is one of the risk factors for poor outcomes in the setting of *Stenotrophomonas maltophilia* pneumonia (18). Thus, the high sensitivity of multiplex PCR in *Stenotrophomonas maltophilia* identification is bound to assist clinicians in formulating more effective treatment regimens earlier and avoiding unreasonable antibiotic administration. Greater detection of *Streptococcus pneumoniae* on multiplex PCR is similarly important. Although one of the most common causes of pneumonia (19,20), the positive rate of *Streptococcus pneumoniae* detection on routine microbiological culture remains relatively low due to its fastidious nature (21,22). None of the 452 samples evaluated in this study were positive for *Streptococcus pneumoniae* on routine culture, but multiplex PCR successfully revealed *Streptococcus pneumoniae* in 36 samples. This advantage of multiplex PCR is bound to help clinicians more accurately establish diagnoses and perform relevant drug susceptibility testing.

Our results revealed that multiplex PCR has a high overall agreement with routine microbiology regarding the detection of bacteria commonly associated with pneumonia. Here, the detection sensitivity for most target bacteria was >90%, with NPVs >99%. One potential challenge of clinically implementing multiplex PCR use is that it detects additional bacteria not reported on routine microbiology (23). The number of bacteria detected by multiplex PCR is more than twice that detected using routine microbiology. Although this phenomenon underscores the high sensitivity of multiplex PCR, it remains necessary to distinguish possible causes of false positive results. First, some of

the discordant results (i.e., multiplex PCR positive but culture-negative) found in this study might have been caused by administration of antimicrobial therapy prior to sampling (13). Because nucleic acids persist *in vivo* for some time, their presence does not necessarily signify ongoing infection. Another challenge posed by nucleic acid diagnostics for respiratory pathogen detection is the difficulty of distinguishing between colonization and infection (13,24). The presence of non-colonizing bacteria may certainly indicate an infectious etiology, but some respiratory pathogens such as *Streptococcus pneumoniae* and *Candida albicans* commonly colonize the upper respiratory tract and thus may present a diagnostic dilemma (25,26). It is thus important to establish a relationship between organism load and clinical context based on factors such as pathogen colonization characteristics to accurately determine etiology (27,28). In the further research plan, we will explore to distinguish infection from contamination or colonization by defining different-level cut-off values.

This study was not without its limitations. Due to insufficient relatively small number of samples, not enough cases of several uncommon pathogens were available for investigation. Thus, a larger-scale study is required to confirm our findings. Furthermore, since routine microbiology testing is not the true gold standard for pathogen detection, considering routine tests as the gold standard for comparison with multiplex PCR may obscure true multiplex PCR effectiveness. Future comparisons based on final diagnoses established by clinicians and documented patient outcomes are warranted.

Conclusions

In summary, our multiplex PCR kit designed to identify 17 respiratory pathogens possesses the advantages of high sensitivity and rapid turn-around. Thus, it is capable of aiding rapid identification of infectious etiologies. Importantly, the lower sensitivities of our kit regarding detection of mycobacteria and fungi require further study and enhancement. In addition, awareness of the clinical significance of potential false-positive results is critical when using our kit in practice. This, in turn, requires comprehensive judgment based on specific clinical manifestations and disease conditions of individual patients to accurately determine both etiology and treatment options (29). A large-scale, prospective study is required to confirm our findings and further explore the clinical effects of our multiplex PCR kit on patient management.

Acknowledgments

The researchers are grateful to all members of Prof. Song's group and the Department of Laboratory Medicine of Zhongshan Hospital for their assistance.

Funding: This work was supported by the National Key R&D Plan (No. 2020YFC2003700), National Natural Science Foundation of China (Nos. 82130001, 82070045), Shanghai Sailing Program (No. 22YF1406100), Science and Technology Commission of Shanghai Municipality (Nos. 20Z11901000, 20DZ2261200, 20XD1401200), Shanghai Municipal Science and Technology Major Project, Clinical Research Plan of SHDC (No. SHDC2020CR5010-002) and Shanghai Municipal Key Clinical Specialty (No. shslczdzk02201).

Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-544/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-544/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-544/coif). WG and YS have a patent of a composition, test kit for detecting respiratory pathogens and their application pending. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Medical Ethics Committee of Zhongshan Hospital, Fudan University (approval No. B2019-249R) and written informed consent was obtained from all patients.

References

1. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2095-128.
2. Qu J, Zhang J, Chen Y, et al. Aetiology of severe community acquired pneumonia in adults identified by combined detection methods: a multi-centre prospective study in China. Emerg Microbes Infect 2022;11:556-66.
3. Cilloniz C, Ferrer M, Liapikou A, et al. Acute respiratory distress syndrome in mechanically ventilated patients with community-acquired pneumonia. Eur Respir J 2018;51:1702215.
4. Lenz H, Norby GO, Dahl V, et al. Five-year mortality in patients treated for severe community-acquired pneumonia – a retrospective study. Acta Anaesthesiol Scand 2017;61:418-26.
5. Torres A, Niederman MS, Chastre J, et al. International ERS/ESICM/ESCMID/ALAT guidelines for the management of hospital-acquired pneumonia and ventilator-associated pneumonia: Guidelines for the management of hospital-acquired pneumonia (HAP)/ventilator-associated pneumonia (VAP) of the European Respiratory Society (ERS), European Society of Intensive Care Medicine (ESICM), European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and Asociación Latinoamericana del Tórax (ALAT). Eur Respir J 2017;50:1700582.
6. Piskin N, Aydemir H, Oztoprak N, et al. Inadequate treatment of ventilator-associated and hospital-acquired pneumonia: risk factors and impact on outcomes. BMC Infect Dis 2012;12:268.
7. Enne VI, Aydin A, Baldan R, et al. Multicentre evaluation of two multiplex PCR platforms for the rapid microbiological investigation of nosocomial pneumonia in UK ICUs: the INHALE WP1 study. Thorax 2022. [Epub ahead of print]. doi: 10.1136/thoraxjnl-2021-216990.
8. Enne VI, Personne Y, Grgic L, et al. Aetiology of hospital-acquired pneumonia and trends in antimicrobial resistance. Curr Opin Pulm Med 2014;20:252-8.
9. van Hecke O, Wang K, Lee JJ, et al. Implications of Antibiotic Resistance for Patients' Recovery From Common Infections in the Community: A Systematic Review and Meta-analysis. Clin Infect Dis 2017;65:371-82.
10. Huang HS, Tsai CL, Chang J, et al. Multiplex PCR system for the rapid diagnosis of respiratory virus infection: systematic review and meta-analysis. Clin Microbiol Infect 2018;24:1055-63.
11. Babady NE. The FilmArray® respiratory panel: an automated, broadly multiplexed molecular test for the rapid and accurate detection of respiratory pathogens. Expert Rev Mol Diagn 2013;13:779-88.
12. Cao B, Huang Y, She DY, et al. Diagnosis and treatment of community-acquired pneumonia in adults: 2016 clinical practice guidelines by the Chinese Thoracic Society, Chinese Medical Association. Clin Respir J 2018;12:1320-60.
13. Lee SH, Ruan SY, Pan SC, et al. Performance of a multiplex PCR pneumonia panel for the identification of respiratory pathogens and the main determinants of resistance from the lower respiratory tract specimens of adult patients in intensive care units. J Microbiol Immunol Infect 2019;52:920-8.
14. Kollef MH, Torres A, Shorr AF, et al. Nosocomial Infection. Crit Care Med 2021;49:169-87.
15. Brooke JS. Stenotrophomonas maltophilia: an emerging global opportunistic pathogen. Clin Microbiol Rev 2012;25:2-41.
16. Looney WJ, Narita M, Mühlemann K. Stenotrophomonas maltophilia: an emerging opportunist human pathogen. Lancet Infect Dis 2009;9:312-23.
17. Zhu L, Wang L, Zhang Y, et al. Fatal hemorrhagic pneumonia in patients with hematologic diseases and Stenotrophomonas maltophilia bacteremia: a retrospective study. BMC Infect Dis 2021;21:723.
18. Velázquez-Acosta C, Zarco-Márquez S, Jiménez-Andrade MC, et al. Stenotrophomonas maltophilia bacteremia and pneumonia at a tertiary-care oncology center: a review of 16 years. Support Care Cancer 2018;26:1953-60.
19. Engholm DH, Kilian M, Goodsell DS, et al. A visual review of the human pathogen Streptococcus pneumoniae. FEMS Microbiol Rev 2017;41:854-79.
20. Feldman C, Anderson R. The Role of Streptococcus pneumoniae in Community-Acquired Pneumonia. Semin Respir Crit Care Med 2020;41:455-69.
21. Varghese R, Jayaraman R, Veeraraghavan B. Current challenges in the accurate identification of Streptococcus pneumoniae and its serogroups/serotypes in the vaccine era. J Microbiol Methods 2017;141:48-54.
22. Ikken Y, Charof R, Elouennass M, et al. The novel biphasic medium for transport, culture and conservation

at an ambient temperature of Neisseria meningitidis, Streptococcus pneumoniae and Haemophilus influenzae. World J Microbiol Biotechnol 2021;37:187.
23. Webber DM, Wallace MA, Burnham CA, et al. Evaluation of the BioFire FilmArray Pneumonia Panel for Detection of Viral and Bacterial Pathogens in Lower Respiratory Tract Specimens in the Setting of a Tertiary Care Academic Medical Center. J Clin Microbiol 2020;58:e00343-20.
24. Murdoch DR, O'Brien KL, Scott JA, et al. Breathing new life into pneumonia diagnostics. J Clin Microbiol 2009;47:3405-8.
25. Murdoch DR. How recent advances in molecular tests could impact the diagnosis of pneumonia. Expert Rev Mol Diagn 2016;16:533-40.
26. Poulain D. Candida albicans, plasticity and pathogenesis. Crit Rev Microbiol 2015;41:208-17.
27. Murphy CN, Fowler R, Balada-Llasat JM, et al. Multicenter Evaluation of the BioFire FilmArray Pneumonia/Pneumonia Plus Panel for Detection and Quantification of Agents of Lower Respiratory Tract Infection. J Clin Microbiol 2020;58:e00128-20.
28. Yugueros-Marcos J, Barraud O, Iannello A, et al. New molecular semi-quantification tool provides reliable microbiological evidence for pulmonary infection. Intensive Care Med 2018;44:2302-4.
29. Monard C, Pehlivan J, Auger G, et al. Multicenter evaluation of a syndromic rapid multiplex PCR test for early adaptation of antimicrobial therapy in adult patients with pneumonia. Crit Care 2020;24:434.

Cite this article as: Zhang C, Chen X, Wang L, Song J, Zhou C, Wang X, Ma Y, Chen C, Guo W, Song Y. Evaluation of a multiplex PCR kit for detection of 17 respiratory pathogens in hospitalized patients. J Thorac Dis 2022;14(9):3386-3397. doi: 10.21037/jtd-22-544

Supplementary

Table S1 Pathogen-specific performance of PCR tests as compared with routine microbiology as the gold standard in respiratory samples or non-respiratory samples

Pathogen	All samples (n=452)					Respiratory samples (n=252)					Non-respiratory samples (n=210)				
	TP	FP	FN	TN	Kappa (P value)	TP	FP	FN	TN	Kappa (P value)	TP	FP	FN	TN	Kappa (P value)
Bacteria															
Pseudomonas aeruginosa	68	101	2	271	0.445 (P<0.001)	53	70	1	113	0.413 (P<0.001)	15	31	1	158	0.416 (P<0.001)
Klebsiella pneumoniae	29	138	1	274	0.203 (P<0.001)	19	98	1	119	0.156 (P<0.001)	10	40	0	155	0.274 (P<0.001)
Acinetobacter baumannii	83	138	4	217	0.357 (P<0.001)	54	83	2	98	0.337 (P<0.001)	29	55	2	119	0.364 (P<0.001)
Staphylococcus aureus	21	77	3	341	0.282 (P<0.001)	15	51	2	169	0.279 (P<0.001)	6	26	1	172	0.267 (P<0.001)
Stenotrophomonas maltophilia	15	227	1	199	0.052 (P=0.001)	12	145	0	80	0.053 (P=0.011)	3	82	1	119	0.031 (P<0.169)
Streptococcus pneumoniae	0	33	0	409	0.000 (P=1.000)	0	31	0	206	0.000 (P=1.000)	0	2	0	203	0.000 (NA)
Group A streptococcus	0	4	0	438	0.000 (NA)	0	4	0	233	0.000 (NA)	0	0	0	205	NA
Haemophilus influenzae	5	30	1	406	0.226 (P<0.001)	5	24	1	207	0.254 (P<0.001)	0	6	0	199	0.000 (P=1.000)
Enterococcus faecium	14	36	2	390	0.391 (P<0.001)	3	20	1	213	0.199 (P<0.001)	11	16	1	177	0.526 (P<0.001)
Enterococcus faecalis	9	67	1	365	0.176 (P<0.001)	3	39	0	195	0.112 (P<0.001)	6	28	1	170	0.250 (P<0.001)
Escherichia coli	9	44	0	389	0.265 (P<0.001)	2	30	0	205	0.103 (P<0.001)	7	14	0	184	0.473 (P<0.001)
Mycobacterium tuberculosis	3	0	6	136	0.484 (P<0.001)	2	1	6	84	0.332 (P<0.001)	0	0	1	51	0.000 (NA)
Atypical mycobacteria	0	0	8	137	0.000 (NA)	0	0	8	85	0.000 (NA)	0	0	0	52	NA
Fungi															
Candida albicans	28	22	19	336	0.520 (P<0.001)	25	18	18	164	0.482 (P<0.001)	3	4	1	172	0.532 (P<0.001)
Mucor	0	0	0	405	NA	0	0	0	225	NA	0	0	0	180	NA
Aspergillus	3	0	3	87	0.652 (P<0.001)	3	0	3	85	0.651 (P<0.001)	0	0	0	2	NA
Penicillium marneffei	0	1	0	404	0.000 (NA)	0	1	0	224	0.000 (NA)	0	0	0	180	NA

PCR, polymerase chain reaction; TP, true positive; FP, false positive; FN, false negative; TN, true negative; NA, not available.

https://dx.doi.org/10.21037/jtd-22-544