Original Research

Development of a bead-based suspension array for the detection of pathogens in acute respiratory tract infections

Yu-Sheng Chen[1,*], Hong-Ru Li[1,*], Wei Zhang[1], Zhi-Dan Hua[1], Xiao-Hong Lin[1], Meng-Qing Lin[1], Wen-Sen Huang[1], Li-Ping Huang[1], Xiao-Li Yu[1], Neng-Luan Xu[1], Ming Lin[1], Bao-Song Xie[1], Xiao-Na Shen[2], Jian-Feng Xie[2], Yi Wang[3], Meng Huang[2], Yan-An Wu[4] and Xin-Lan Hu[4]

[1]Department of Respiratory and Critical Care Medicine, Fujian Provicial Hospital, Fujian Provincial Medical College, Fujian Medical University, Fuzhou 350001, China; [2]Department of Viral Diseases, Fujian Center for Disease Control and Prevention, Fuzhou 350001, China; [3]Shanghai Tellgen Life Science Co. Ltd, Shanghai 201203,China; [4]Clinical Microbiology Laboratory, Fujian Provincial Hospital, Fuzhou 350001, China

*Joint first authors.

Corresponding author: Yu-Sheng Chen. Email: slyyywb@126.com

Abstract

We developed a high-throughput bead-based suspension array for simultaneous detection of 20 respiratory tract pathogens in clinical specimens. Pathogen-specific genes were amplified and hybridized to probes coupled to carboxyl-encoded microspheres. Fluorescence intensities generated via the binding of phycoerythrin-conjugated streptavidin with biotin-labeled targets were measured by the Luminex 100 bead-based suspension array system. The bead-based suspension array detected bacteria in a significantly higher number of samples compared to the conventional culture. There was no significant difference in the detection rate of atypical pathogensatypical pathogens or viruses between the bead-based suspension array and real-time PCR. This technology can play a significant role in screening patients with pneumonia.

Keywords: Acute respiratory tract infections, unexplained pneumonia, bead-based suspension array, pathogen detection, high-throughput screening

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Introduction

Severe acute respiratory tract infections have historically had a very detrimental impact on human health and social stability,[1,2] and continue to cause devastating outbreaks.[3–5] Although the importance of early and rapid diagnosis is recognized, the complexity of pathogens associated with these infections is a major challenge. Conventional diagnostic methods such as bacterial cultures, immunological tests and real-time PCR assays have the disadvantages of low sensitivity, single detection at a time, and delayed result availability.

Recent molecular techniques used a sensitive and accurate multiplex reverse transcription PCR (RT-PCR) assay for pneumonia and sepsis,[6] and the Respiratory Multicode-Plx Assay system which detected a large number of respiratory viruses with high sensitivity and accuracy.[7] Gene chip technology includes solid phase arrays or loop-mediated isothermal amplification systems and liquid bead-based suspension arrays. A loop-mediated isothermal amplification system developed cooperatively by the Peking University People's Hospital and CapitalBio Corporation in China is currently being tested in clinical trials.[8] The bead-based suspension array (liquid chip) platform developed by the Luminex Corporation in the United States is a rapid, high-throughput system for multi-analyte detection.[4,5,8–11] The system is based on polystyrene microspheres internally labeled with a unique dye combination. The microspheres are coated with thousands of copies of a probe for a specific target which is amplified from clinical samples using 5′-biotin-labeled primers. The fluorescent emission from the microspheres is evaluated using a 635 nm/10 inW red polar laser while a 532 nm/13 inW yttrium aluminum garnet (YAC) laser is used to measure the target analyte by exciting the streptavidin-phycoerythrin (SP-PE) fluorescent reporter bound to the surface of microspheres.[10] Target quantification is done by determining the mean fluorescence intensity (MFI) for each encoded microsphere by high speed computing.[12]

A suspension array-based respiratory virus detection kit (xTAG RVP) developed by the Luminex corporation received FDA approval in 2008.[13,14] This kit does not

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detect various common respiratory tract bacteria or atypical pathogens. Moreover, the kit has not yet been used in China, where significant variation in viruses from different geographical regions contributes to inconsistent detection sensitivity. In the present study, we aimed to utilize the bead-based suspension array technology to develop a high-throughput screening system which can detect common viruses, bacteria as well as atypical pathogens from respiratory tract infections in Chinese patients.

Materials and methods

Patient specimens

This study enrolled 333 subjects who were hospitalized for severe acute respiratory infections at the Department of Respiratory Medicine, Fujian Provincial Hospital between March 2012 and May 2015. Sputum samples were collected from 293 patients and alveolar lavage fluid specimens from 40 patients. This study was reviewed and approved by the Institutional Review Board of Fujian Provincial Hospital (IRB number: K2012-003-01), and informed consent was obtained from the patients.

Inclusion and exclusion criteria

Patients who were able to provide an accurate and complete medical history were included in this study. The inclusion criteria were:

1. Occurrence of fever accompanied by sore throat, stuffy nose, running nose, headache, fatigue, cough, expectoration, difficulty in breathing.
2. Presence of congested and swollen throat, conjunctival congestion, sores and ulcers in the throat, oral cavity or gingiva or fine crackles are heard on auscultation.
3. Pulmonary infection evidenced by presence of spotted, patchy high-density shadows in lungs or disordered and thickened bronchi on chest imaging by chest radiography or CT.
4. Symptoms or physical signs of respiratory tract infections and routine blood tests indicating normal, elevated or lowered white blood cell counts.

Presence of tumors, COPD, bronchial dilation, and other organ-based diseases, such as heart, liver, kidney diseases (including organ failure) were not excluding factors.

Patients with definite or suspected pulmonary tuberculosis, pulmonary fungal infections or non-infectious diseases not accompanied by lower respiratory tract infection were excluded.

Nucleic acid extraction from sputum specimens and reverse transcription

Bacterial concentrations in sputum specimens were calculated as follows: bacterial load in sputum samples (copies/ml) = template concentration (copies/μl) × 100/0.6.[14] Sputum DNA was prepared and quantified as previously described.[15,16] Viral nucleic acids were extracted using the MiniBEST Viral RNA/DNA Extraction Kit (Ver.5.0; TakaRa, Shiga, Japan) and were then reverse transcribed into cDNA with the TUREscipt 1st Strand cDNA Synthesis Kit (Aidlab Biotechnologies Co., Beijing, China). Supplementary Table 1 shows the limits of detection for each pathogen based on serial dilution.

Design of primers and probes

Six kinds of primers and probes corresponding to sequences from the SARS coronavirus, the Influenza A virus, the highly pathogenic avian influenza A H5N1, the human metapneumovirus, and the human bocavirus were designed based on previous studies[6,15,17–21] and optimized for our research purposes. Fourteen pathogen sequences, including human cytomegalovirus transmembrane protein gene (X04650), human adenovirus type 6 hexon protein gene (DQ149613), human parainfluenza virus 1 HN gene (U70942), human parainfluenza virus 2 HN gene (AB367954), human parainfluenza virus 3 HN gene (AB623457), human respiratory syncytial virus nucleocapsid gene (X00001), *Mycoplasma pneumoniae* P1 gene (KF154759), *Chlamydia pneumoniae* rpoB gene (KC305894), *Staphylococcus aureus* tuf gene (HM352930), *Streptococcus pneumoniae* ply gene (GU968401), *Klebsiella pneumoniae* phoE gene (AF009172), *Acinetobacter baumannii* Oxa gene (JQ342838), *Pseudomonas aeruginosa* gryB gene (FJ652722), and *Stenotrophomonas maltophilia* 23S rRNA gene (HE798556), were retrieved from GenBank and aligned with pathogen sequences from patient samples using the Cluster Omega software in order to confirm pairwise identities.

Some primer sequences were obtained from literature,[6,13–15,17,22,23] and optimized to meet our research needs. Primers and probes were designed using Primer Premier 5.0 software and synthesized by Invitrogen Trading (Shanghai) Co., Ltd, Shanghai, China. Primers were labeled with biotin at the 5′ end and all probes were 5′-labeled with NH2C6. The sequences of primers and probes used in our study are shown in Table 1.

Establishment of bead-based suspension array detection system

Development of PCR system

DNA from various samples was amplified with the DBI Bestar Taq DNA Polymerase PCR kit (Shanghai Xinghan Sci&Tech Co., Shanghai, China) using 20 pairs of forward and reverse primers. PCR amplification was performed in a 20 μl reaction volume which included dNTPs (2 μl, 2 mM), Bestar Taq Buffer (4.5 μl 10×), Bestar Taq DNA Polymerase (0.8 μl, 2.5 U/μl), forward and reverse primer pairs (1 μM, 2 μl), and 2 μl template strand DNA. Cycling conditions consisted of one cycle of denaturation at 95°C for 5 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The final extension was done at 72°C for 10 min. Six primer pairs for viruses (HCMV, AD, PIV1, PIV2, PIV3, RSV) were mixed to develop the first set of multiplex PCR amplification reactions and six primer pairs (FluA, H5N1, SARS, HBOV, HMPV) to develop the second set. Three primer

Table 1 Primer and probe sequences for liquid chip arrays of acute contagious respiratory disease pathogens

Pathogen	Name	Sequences	Length (bp)	Reference
SARS	SARS-F1B	Biotin-CTAACATGCTTAGGATAATGG	368	This study
	SARS-R1	biotin-CAGGTAAGCGTAAAACTCATC		
	SARS-P	NH2-TTTTTTTTTTATGCTACAACTGCTTATGCTA		
Influenza A virus	FluA-M-F65	biontin-CCGAGATCGCACAGAGACTTGAAGAT	336	This study
	FluA-M-R400	biontin-GGCAAGTGCACCAGCAGAATAACT		
	FluA-F65/R400-1	NH2-TTTTTTTTTTAAGGGGATTTTAGGATTTGTGTTCA		
Influenza A virus subtype H5N1	H5HA-R1138	biotin- CTCCCCTGCTCATTGCTATG	219	This study
	H5HA-F920	biotin- GCCATTCCACAACATACACCC		
	H5HA	NH2-TTTTTTTTTTATGCCCCAAATATGTGAAATCAAAC		
	N1-F2	biotin-CAAGTGCTTGCCATGATG	367	
	N1-R2	biotin-TCAGGATAACAGGAGCACTC		
Human Bocavirus	HBOV-F	biotin-TAACACTTGGCACGCACAGC	265	This study
	HBOV-R	biotin-TCCCTCGTCTTCATCACTTGGT		
	HBOV-P	NH2-TTTTTTTTTTTCATCAGGAACA CCCAATCAGC		
Human Metapneumovirus	HMPV –F	biotin-GAAGAGCTAACCGTGTACTAAGTGATG	165	This study
	HMPV –R	biotin-CTTTGCTGCCTGTAGAGGATGA		
	HMPV –P	biotin-CTTTGCTGCCTGTAGAGGATGA		
Human cytomegalovirus	HCMV-F	Biotin-AAGTTTGTGCCCCAACGGTA	149	This study
	HCMV-R	Biotin-GCGTGCTTTTTAGCCTCTGC		
	HCMV-P	5′NH2C12-AAACAGCGTGACGATGACCTGC		
Adenovirus	AD-F	Biotin-CGCAGTGGTCTTACATGCACA	295	This study
	AD-R	Biotin-ACGCCGCGGATGTCAAAGT		
	AD-P	5′NH2C6-TTTTTTTTTTGCCTGAATAACAAGTTTAGAA		
Human parainfluenza virus 1	PIV1-F	Biotin-CCTTGGAGCGGAGTTGTTAAG	317	This study
	PIV1-R	Biotin-CCGGTAATTTCTCATACCTATG		
	PIV1-P	5′NH2C6-TTTTTTTTTTGGAAAGACCAAATCTCATCG		
Human parainfluenza virus 2	PIV2-F	Biotin-ATGGAATCAATCGCAAAAGC	234	This study
	PIV2-R	Biotin-GATGATAGATCCCGCTTCCA		
	PIV2-P	5′NH2C6-TTTTTTTTTTGCTGAACTGAGACTTGC		
Human parainfluenza virus 3	PIV3-F	Biotin-CTCGAGGTTGTCAGGATATAG	189	This study
	PIV3-R	Biotin-CTTTGGGAGTTGAACACAGTT		
	PIV3-P	5′NH2C6-TTTTTTTTTTGATCTCTCATACTTTTAACAT		
Respiratory syncytial virus,	RSV-F	Biotin-CAAGTTGTTGAGGTTTATGAATATGC	273	This study
	RSV-R	Biotin-TTCTGCTGTCAAGTCTAGTACACTGTAGT		
	RSV-P	5′NH2C6-TTTTTTTTTTTCAATTTCCTCACTTCTCCA		
M. pneumoniae	MP-F	Biotin-TGCCATCTACCCGCGCTTA	300	Kumar *et al*.[6]
	MP-R	Biotin-GTGATCTGCCCGGTTTGGTC		
	MP-P	5′NH2C6-TTTTTTTTTTTAACAAACCACGTATGAAC		
Chlamydia pneumonia	CP-F	Biotin-AGTTGAGCATATTCGTGAGG	127	Maass *et al.*[17]
	CP-R	Biotin-TTTATTTCCGTGTCGTCCAG		
	CP-P	5′NH2C6-TTTTTTTTTTAGACTTTAACTTGGCGAA		
Staphylococcus aureus	SA-F	Biotin–ATGGAAGTTCGTGACTTATTAAGC	313	Kumar *et al*.[6]
	SA-R	Biotin–AACAGTTGTTTTAGATGTGTCATGT		
	SA-P	5′NH2C6-TTTTTTTTTTTGATTCTGACAAACCATT		
Streptococcus pneumonia	SP-F	Biotin–GTGATATTTCTGTAACAGCTACC	354	Jeong *et al*.[15]
	SP-R	Biotin–GAGAATTCCCTGTCTTTTCAAA		
	SP-P	5′NH2C6-TTTTTTTTTTAAGTGGAAGACCCCAGCAAT		
Klebsiella pneumonia	KP-F	Biotin-CTGGATCTGACCCTGCAGTA	68	Wang *et al.*[18]
	KP-R	Biotin-CCGTCGCCGTTCTGTTTC		
	KP-p	5′NH2C6-TTTTTTTTTTAAAAACGAAGGCCGTGA		
Acinetobacter baumannii	AB-F	Biotin-TCGTGCTTCGACCGAGTAT	248	Nomanpour *et al*.[19]
	AB-R	Biotin-AACCAACACGCTTCACTTCC		
	AB-P	5′NH2C6-TTTTTTTTTTACCATCCCACTTAAATAC		

(continued)

Table 1 Continued

Pathogen	Name	Sequences	Length (bp)	Reference
Pseudomonas aeruginosa	PA-F	Biotin-GGCGTGGGTGTGGAAGTC	185	Lee *et al.*[20]
	PA-R	Biotin-GTGGCGATCTTGAACTTCTT		
	PA-P	5′NH2C6-TTTTTTTTTTGCTTCACCAACAACAT		
Stenotrophomonas maltophilia	SM-F	Biotin-CAGCCTGCGAAAAGTA	532	Whitby *et al.*[21]
	SM-R	Biotin-TTAAGCTTGCCACGAACAG		
	SM-P	5′NH2C6-TTTTTTTTTTGAGGGGAGTGAAATAGAA		

pairs targeting non-viral pathogens (SP, SM, MP) were mixed to develop the third set, and five primer pairs targeting non-viral pathogens (SA, AB, KP, CP, PA) were mixed to develop the fourth set.

Coupling of microspheres to probes

For the coupling reaction, microspheres (50 µl equivalent to 0.623×10^6 beads) were washed thoroughly using sterile water, centrifuged and resuspended in 50 µl MES. The corresponding probes (2 µl of 10 µM) were added to the microsphere suspension, followed by 2.5 µl of freshly prepared 1-Ethyl-3-(3-Dimethyllaminopropyl)- carbodiimide Hydrochloride (EDC). The reaction mix was vortexed and incubated in the dark for 40 minutes at 37°C. Subsequently, 1 ml of 0.02% Tween-20 was added to the coupled microspheres, the mix was centrifuged and the pellet of coupled microspheres was resuspended in 50 µl 1 × Tris-EDTA, and stored in the dark at 4°C.

Cut-off determination of fluorescence for bead-based suspension array and diagnostic criteria for pathogens

Fluorescence intensity values of the resulting hybrids from the amplified DNA products of respiratory tract pathogens were read with a Luminex 100 suspension array analyzer instrument. MFI was defined as the arithmetic mean of the fluorescence signal detected from the encoded microspheres for their respective pathogens. A fluorescence value of $\geq$200 detected from the positive control DNA (10^4 copies/µl) was defined as positive, and a value of $<$150 was defined as negative. Samples with fluorescence values in the grey zone (values $<$ 200 but $>$ 150) were defined as probable positive, and were required to be retested or verified by other methods.[24] The sample was defined as positive if the fluorescence value from a repeat detection was $>$ 150, and negative if the value was $<$ 150. The exact fluorescence levels for each pathogen are given in Supplementary Table 2. In addition, multiplex PCR amplification products from samples in the grey zone were analyzed using agarose gel electrophoresis, and samples were classified as positive based on the presence of pathogen-specific bands.

Detection of respiratory tract pathogens

Products amplified from the same nucleic acid sample using 4 different sets of multiplex PCR were mixed. Twenty kinds of encoded microspheres coupled to their respective probes were vortexed and used to prepare 25 µl of hybridization reaction mixture containing 0.1 µl of each probe-coupled microspheres and 4 µl of mixture comprising four sets of multiplex PCR amplification products. The hybridization mixture was denatured at 95°C for 5 min, and subsequently incubated at the hybridization temperature of 46°C for 60 min. Fluorescent reporter (75 µl of 4 µg/ml solution) was added to the samples in a 96-well plate, the plate was sealed and further incubated at 46°C for 15 min. Fluorescence was measured using a suspension array analyzer.

Choice of the gold standards

Bacterial detection from sputum cultures using the VITEK® 2 microbial ID/AST testing system (bioMérieux, Marcy l'Etoile, France) served as the gold standard for bacteria. For viruses and atypical pathogens (including *M pneumoniae* and *C. pneumoniae* in this study), the gold standard of detection was real-time, fluorescence-based quantitative real-time PCR using the DBI-2040 Bestar Taq DNA Polymerase PCR kit purchased from Shanghai ZJ Bio-Tech Co., Ltd, Shanghai, China.

Statistical analysis

Gender distribution, incidence of severe pneumonia, and results from bead-based suspension arrays, sputum cultures or quantitative reverse transcriptase-polymerase chain reactions (real-time-PCR) were expressed as counts. The accuracy of the bead-based suspension array was tested by calculating the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with 95% confidence intervals (CIs) using the findings from sputum culture and real-time-PCR as the gold standards. The indices were calculated as follows:

$$\text{Sensitivity (true positive rate)} = (\text{number of positive results/total number of results}) \times 100$$

$$\text{Specificity (true negative rate)} = (\text{number of negative results/total number of results}) \times 100$$

$$\text{PPV} = \frac{(\text{number of confirmed positive results}}{\text{/total number of positive results}) \times 100}$$

$$\text{NPV} = \frac{(\text{number of confirmed negative results}}{\text{/total number of negative results}) \times 100}$$

Table 2 Baseline characteristics of 333 patients

Variables	N/mean ± standard deviation	Percentage/ range
Age, years	63.2 ± 16.3	(13, 96)
Male	223	(67.0)
Severe pneumonia	56	(16.8)
*Laboratory test**		
Bacteria (+)	36	(10.8)
Virus (+)	104	(31.2)
M. pneumoniae (+)	10	(3.0)
White blood cell, 10[9†]	8.8 ± 4.3	(1.3, 32.7)
Neurophil cells in total white blood cells, %[†]	69.3 ± 15.2	(6.5, 98.4)
Lymphocytes in total white blood cells, %[†]	20.1 ± 13.1	(0.7, 114.5)
C-reactive protein, mg/L[†]	59.0 ± 74.9	(0.0, 404.0)

*Laboratory tests were done by sputum smear for bacteria, and quantitative reverse transcriptase-polymerase chain reaction for virus as well as *M. pneumoniae*.
[†]Seven patients had missing data for white blood cell counts; 9 patients for N cell counts; 63 patients for lymph node, and 43 patients for C-reactive protein.

A higher value for sensitivity, specificity, PPV, or NPV, reflected good accuracy of the bead-based suspension array in identifying bacteria, virus and Chlamydia. The difference in accuracy between the suspension array and the gold standards was examined using the McNemar's test. Conditional logistic regression was carried to test the effect of different technologies on positive detections of bacteria, viruses, and non-classic pathogens. The significance level was defined as 0.05. All statistical analyses were two-sided, and performed using the SAS version 9.2 software (SAS Inc., Cary, NC, USA).

Results

Patient characteristics

In this study, we integrated the bead-based suspension array system for rapid detection of 20 pathogens associated with acute respiratory tract infections. The study population had 223 males and 110 females (Table 2). The age range of patients was 13–96 years and the mean age was 63.2 ± 16.3 years. Although 17% of the patients reported severe pneumonia, only 10.8% of the sputum smears had positive cultures. A total of 115 patients (34.5%) were positive for viral infections, and 10 patients (3%) were positive for *M. pneumoniae* (In this study, atypical pathogens include *M. pneumoniae* and *C. pneumoniae*. As a result, *M. pneumonia* was detected in 10 cases, while *C. pneumoniae* was not detected among all examined cases by standard methods. So in this study, only the data of *M. pneumoniae* were shown and analyzed.) (Table 2). Of the 39 strains of bacteria detected, 29 (74.4%) were Gram-negative bacilli and 10 (25.6%) were Gram-positive cocci.

Analysis of coinfections

Analysis of bacterial infections indicated 126 positive patients (37.8%) by liquid phase chip detection; 93 of these patients (73.8%) had single infections and 33 (26.2%) had multiple infections. Among the 33 patients with multiple infections, 25 (19.8%) had infections by 2 bacteria (PA + KP: n = 6; PA + SP: n = 4; KP + SP: n = 3; SA + AB: n = 3; SA + SM: n = 2; PA + AB: n = 1; PA + SM: n = 1; PA + SA: n = 1; SP + AB: n = 1; SP + SM: n = 1; KP + SA: n = 1; KP + SM: n = 1), and 8 (6.3%) had infections by 3 bacteria (KP + PA + AB: n = 2; KP + PA + SP: n = 1; KP + AB + SP: n = 1; KP + AB + SM: n = 1; KP + SM + SP: n = 1; PA + SB + SA: n = 1; PA + SP + SA: n = 1).

Analysis of viral infections indicated 115 positive patients (34.5%); 95 of these patients (82.6%) had single infections and 20 (17.4%) had multiple infections (Flu-A + HCMV: n = 10 [8.7%]; Flu-A + AD: n = 2 [1.7%]; Flu-A + RSV: n = 2 [1.7%]; Flu-A + PIV1: n = 2 [1.7%]; Flu-A + N1: n = 1 [0.9%]; Flu-A + PIV2: n = 1 [0.9%]; HCMV + PIV3: n = 1 [0.9%]; HCMV + H5: n = 1 [0.9%]).

Among all 333 patients, we identified diverse infections in 35 samples (10.5%) in which there was evidence of bacteria or atypical pathogenic mircobes with a virus.

Analysis of sensitivity and specificity

The bead-based suspension array detected bacteria in a significantly higher number of samples (126/333; 37.8%) compared to conventional culture (36/333: 10.8%, $P < 0.001$). There was no significant difference in the detection rate of atypical pathogens (means only *M. pneumoniae* here) between the bead-based suspension array and real-time PCR (9/333 (2.7%) vs. 10/333 (3.0%), respectively ($P = 0.657$). There was also no significant difference in the viral detection rate between the bead-based suspension array and real-time PCR (115/333 (34.5%) vs. 104/333 (31.2%), respectively ($P = 0.181$). The bead-based array had a sensitivity of 70.0% and a specificity 99.4% for *M. pneumoniae* compared to the gold standards, respectively. The bead-based array had a sensitivity of 73.1% and a specificity of 83.0% for viruses compared to the gold standards, respectively. The NPV was 87.2% for viruses and 99.1% for *M. pneumoniae*. Although the PPV was not high as the NPV, it was still higher than 65% regardless of the type of microorganism (Table 3).

We compared the sensitivity and specificity of the bead-based suspension array with that of real-time-PCR for eight different viruses (Table 4). There was no significant difference between the two methods in accuracy of detection of PIV2, PIV3, AD, Flu-A, or N1. However, the bead-based suspension array test had a significantly poorer accuracy for HCMV ($P < 0.001$) and RSV ($P = 0.016$) compared to real-time-PCR.

Discussion

In this study, our high-throughput bead-based suspension array had a significantly higher efficiency of detection of bacteria compared to conventional culture. However, there was no significant difference in the positivity rates for atypical pathogens or viruses between the bead-based suspension arrays and real-time PCR assays.

The demanding culture conditions, complexity of culture techniques, and the long detection time for viruses

Table 3 Sensitivity and specificity of the bead-based suspension array as compared with the sputum culture for bacteria, and quantitative reverse transcriptase-polymerase chain reaction (real-time-PCR) assay for virus and *M. pneumoniae*

Tested result	Sputum culture for bacteria*	RT-PCR for virus			RT-PCR for *M. pneumoniae*		
	Positive	Positive	Negative	*P*†	Positive	Negative	*P*†
Positive	28	76	39	0.222	7	2	0.999
Negative	8	28	190		3	321	
Total	36	104	229		10	323	
Sensitivity (95% CI)	77.8%	73.1% (63.5%–81.3%)			70% (34.8%–93.3%)		
Specificity (95% CI)	NA	83.0 (77.5%–87.6%)			99.4% (97.8%–99.9%)		
PPV (95% CI)	NA	66.1% (56.7%–74.7%)			77.8% (40.0%–97.2%)		
NPV (95% CI)	NA	87.2% (82.0%–91.3%)			99.1% (97.3%–99.8%)		

CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; NA: not available.
*Only patients with positive outcome were included in the analysis due to difficulty in obtaining valid sputum culture.
†McNemar test was performed.

Table 4 Sensitivity and specificity of the bead-based suspension array as compared with quantitative reverse transcriptase-polymerase chain reaction (real-time-PCR) assay* for HCMV, PIV1, PIV2, PIV3, RSV, AD, Flu-A, N1†

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
HCMV	**13.0% (4.9%–26.3%)**	**98.6% (96.5%–99.6%)**	**60.0% (26.2%–87.8%)**	**87.6% (83.5%–91.0%)**
PIV1	NA	99.4% (98.6%–100.0%)	NA	100.0% (98.9%–100.0%)
PIV2	100.0% (15.8%–100.0%)	99.4% (97.8%–99.9%)	50.0% (6.8%–93.2%)	100.0% (98.9%–100.0%)
PIV3	50.0% (6.8%–93.2%)	99.7% (98.3%–99.9%)	66.7% (9.4%–99.2%)	99.4% (97.8%–99.9%)
RSV	**12.5% (3.2%–52.7%)**	**100.0% (98.9%–100.0%)**	**100.0% (2.5%–100.0%)**	**97.9% (95.7%99.2%)**
AD	25.0% (6.3%–80.6%)	99.4% (97.8%–99.9%)	33.3% (8.4%–90.6%)	99.1% (97.4%–99.8%)
Flu-A	82.4% (56.6%–96.2%)	97.5% (95.1%–98.9%)	63.6% (40.7%–82.8%)	99.0% (97.2%–99.8%)
N1	50.0% (1.3%–98.7%)	100.0% (98.9%–100.0%)	100.0% (2.5%–100.0%)	99.7% (98.3%–99.9%)

AD: adenovirus; CI: confidence interval; Flu-A: influenza A virus; HCMV: human cytomegalovirus; N1: influenza A virus subtype N1; NPV: negative predictive value; NA: not available (due to no positive event); PPV: positive predictive value; PIV1: human parainfluenza virus 1; PIV2: human parainfluenza virus 2; PIV3: human parainfluenza virus 3; RSV: respiratory syncytial virus.
Bold values indicate significantly different from gold standard, $P < 0.05$.
*McNemar test was performed.

and atypical pathogens have resulted in the emergence of new diagnostic tools such as bead-based suspension arrays which have shown promise in the detection of Vibrio species, human genital papillomaviruses, biothreat agents, and bacterial pathogens implicated in food-borne illnesses.[25–28] The advantages of our present bead-based suspension array include: (1) the ability to simultaneously detect viruses, atypical pathogens as well as bacteria, in contrast with most current detection methods which are used to detect either only viruses or bacteria,[7,28] (2) rapid, high-throughput detection where detection of 96 samples can be completed in 7–8 h, (3) semi-quantitative detection, (4) small amounts of sample required, and (5) economy and ease of use compared to solid-phase arrays.[29]

Our final annealing temperature of 58°C for target DNA amplification was higher than the annealing temperature recommended by the manufacturer. Higher annealing temperatures may cause decreased amplification efficiency, but a higher specificity. Based on the guidelines suggested by Luminex, the best capture probe lengths are 18–20 bp, and can be extended to 22–24 bp under special occasions in order to obtain stronger fluorescence signal.[30] In the present study, we used probes of around 14 to 18 bp, which would generate lower fluorescence signals for some pathogens, but would reduce non-specific signals. The shorter probe sequences used in our study resulted in a hybridization temperature lower than previously described.[31] We used a relatively longer hybridization period (60 min) to provide sufficient reaction time for interaction of probes with their target fragments, as well as for amplified target gene fragments to compete with non-specific sequences for binding with the probes.

Our detection technology was semi-quantitative. Fluorescence values of the same pathogen could be compared between different samples, although fluorescence values could not be compared between groups of different pathogens. The lowest concentrations that could be detected by our technology ranged from $10^0/\mu l$ to $10^4/\mu l$, and detection efficiency was affected by various conditions, including amplification efficiency of primers, binding of the probe and its target sequence, hybridization temperature, the concentration of SA-PE, hybridization time, Tm of the

probe, percentage of GC content in the probe, and the primer binding site on target fragments.

The higher bacterial detection rates of bead-based suspension arrays compared to conventional culture in our study could be because the positivity rates of bacterial cultures are influenced by factors such as prior antibiotic use, shapes of sputum samples, and whether the samples are delivered to the lab in a timely manner. Previous results reported a high incidence of pathogenic Gram-negative bacilli in patients with acute respiratory tract infections.[32] These data were consistent with our study where the majority of bacterial strains detected (68.2%) were Gram-negative bacilli. This could be because most of the hospitalized patients in our study were older, had complications and had reported self-medication with antibiotics. The rate of virus infections detected in our study was higher than previously reported incidence rates of community-acquired pneumonia (5% to 15%). Of the viral strains found in our study, there are 24 strains of Flu-A (46.7%). This could be because our cases mostly comprised hospitalized patients during the winter-spring season who may experience seasonal influenza epidemics more frequently.

The infection rate of HCMV in the normal population was previously shown to be 30%–100%,[33,34] which was consistent with our present data. *M. pneumoniae* have been shown to cause approximately 10% to 15% of all acute upper respiratory tract infections in children and teenagers.[35–38] *C. pneumoniae* causes infections in over 50% of adults (age ≥ 20) while half of the patients were asymptomatic.[31,39] In contrast, a total of five cases were positive for *M. pneumoniae*, four cases were positive for *C. pneumoniae* in our study and this low incidence could be because most of our cases were elderly patients with various complications. However, our low detection rate of atypical pathogens could be due to the insufficient sample size being tested.

We found that PA and KP were the most common pathogens in patients with multiple bacterial infections. In contrast, de Roux et al.[40] reported that multiple infections were usually caused by SP and other bacteria. This difference might be ascribed to our use of SP-sensitive antibiotics before the sample collection. Our results also showed that 17.4% of patients had infections with two viruses, higher than previously reported by Drews et al.,[41] possibly due to the higher sensitivity of our assays. In the present study, Flu-A and HCMV were the most common viruses in the multiple-strain infections. It is possible that an outbreak of influenza A infection weakened immunity, and led to activation of colonized cytomegaloviruses and the replication of viral DNA. Most studies of infections caused by bacteria and viruses regard the viral infection as secondary to the bacterial infection. This indicates that concomitant viral infection should be considered if there is no response after long-term antibiotic therapy.[42]

In summary, we showed that bead-based suspension array technology had a significantly higher sensitivity for bacterial detection and was not inferior to real-time PCR for the detection of virus and atypical pathogens in patients with acute respiratory tract pathogens. The use of bead-based suspension array technology has some limitations. Non-specific binding can cause interference. Moreover, since the Luminex technology platform is an open system, it is possible that PCR products may cause contamination in labs. Our present study also had some limitations. The positive rates for Mycoplasma and Chlamydia detection were not high enough for further evaluation. Furthermore, we did not analyze our data for false-positives. It is important to validate our findings in larger sample sizes in order to facilitate the transition of this technology to a clinical setting.

Authors' contributions: Y-SC: guarantor of integrity of the entire study, definition of intellectual content, manuscript review; H-RL: study concepts, study design, literature research, data analysis, manuscript editing; WZ: experimental studies, statistical analysis; Z-DH: experimental studies, data analysis, manuscript preparation; X-HLin: experimental studies, data acquisition; M-QL: data acquisition; W-SH: data acquisition; L-PH: clinical studies, data acquisition; X-LY: clinical studies, data acquisition; N-LXu: clinical studies; ML: clinical studies; B-SX: clinical studies; X-NS: experimental studies; J-FX: experimental studies; YW: experimental studies; MH: experimental studies; Y-AW: experimental studies; X-LHu: clinical studies.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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