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Bacterial pathogens were detected from human exhaled breath using a novel protocol

Yunhao Zheng[a], Haoxuan Chen[a], Maosheng Yao[a,*], Xiaoguang Li[b,*]

[a] State Key Joint Laboratory of Environmental Simulation and Pollution Control, College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China

[b] Department of Infectious Diseases, Peking University Third Hospital, Peking University, Beijing 100191, China

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ABSTRACT

It is generally believed that influenza outbreak is associated with breath-borne transmission of viruses, however relevant evidence is little for that of respiratory bacterial infections. On another front, point-of-care infection diagnostic methods at the bedside are significantly lacking. Here, we used a newly developed protocol of integrating an exhaled breath condensate (EBC) collection device (PKU BioScreen) and Loop Mediated Isothermal Amplification (LAMP) to investigate what bacterial pathogens can be directly exhaled out from humans. Exhaled breath condensates were collected from human subjects with respiratory infection symptoms at Peking University 3rd hospital using the BioScreen. The screened bacterial pathogens included *Streptococcus pneumoniae, Staphylococcus aureus, Methicillin-resistant Stphylococcus aureus* (MRSA), *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia, Haemophilus influenzae, Legionella pneumophila, Mycoplasma Pneumonia, Chlamydia pneumonia, and Mycobacterium tuberculosis.* The results were further compared and validated using throat swabs from the same patients by a PCR method.

Here, human bacterial pathogens such as *H. influenzae*, *P. aeruginosa*, *E. coli, S. aureus* and MRSA were detected in exhaled breath using the developed protocol that integrates the EBC collection and LAMP. For the patients recruited from the hospital, seven types of pathogens were detected from 36.5% of them, and for the remaining subjects none of those screened bacterial pathogens was detected. Importantly, some super resistant bacteria such as MRSA were detected from the exhaled breath, suggesting that breathing might be also an important bacterial transmission route. Results from throat swabs showed that 36.2% of the subjects were found to be infected with *H. influenzae*, *P. aeruginosa*, *E. coli, S. maltophilia*, *S. aureus* and MRSA. For the EBC samples, 33.3% were found to be infected with MRSA, *E. coli* and *P. aeruginosa*. Depending on the initial pathogen load in the sample, the entire protocol (EBC-LAMP) only takes 20–60 min to complete for a respiratory infection diagnosis. For different detection methods and pathogens, the agreements between the EBC and throat swabs from the same patients were found to range from 35% to 65%. Here, we have detected several bacterial pathogens including MRSA from exhaled breath, and the developed protocol could be very useful for the bedside pathogen screening particularly in remote areas where resources are significantly limited or prohibited.

* Corresponding authors.
E-mail addresses: yao@pku.edu.cn (M. Yao), caitlin901@163.com (X. Li).

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1. Introduction

Respiratory infection results in a tremendous toll on humans worldwide every year. Despite of significant progress in medical science, infectious diseases continue to affect millions of lives around the world, especially in low-income countries (Cohen, 2000; Mori & Notomi, 2009). According to WHO (2014), lower respiratory infections (such as pneumonia) was listed as the second killer and one of the top three causes of years of life lost (YLL) in 2012. In addition, Acute Respiratory Infections(ARI) caused 15% of death among children aged < 5 years in 2013 (WHO, 2015). Among them, pneumonia alone accounts for 16% of all deaths of children under 5 years old, killing 920,136 children in 2015 (WHO, 2016a, 2016b). The transmission of airborne pathogens further worsens the situation, e.g., influenza viruses (Fabian et al., 2008; O'Brien & Nonnenmann, 2016; Smith et al., 2009). Studies showed that the airborne transmission of viruses was the main cause for some outbreaks of respiratory infections (Jones & Brosseau, 2015; Pyankov, Pyankova, & Agranovski, 2012). For example, a previous study has detected viable severe acute respiratory syndrome(SARS) virus and its RNA in the ambient air, and the SARS outbreak was shown to be due to the airborne virus transmission (Booth et al., 2005; Yu et al., 2004). Another work also demonstrated that the H7N9 influenza viruses emerging back in 2013 were transmissible in ferrets through the air by respiratory droplets (Zhang et al., 2013). Kim et al. (2016) reported the detection of Middle East Respiratory Syndrome (MERS) Coronavirus in hospital air samples, suggesting possible airborne transmission of MERS. On the other hand, studies also revealed that *Klebsiella pneumoniae* spreading in the air caused high morbidity and mortality (Chandrashekar, Rathish, & Nagesha, 1997; Prazmo, Dutkiewicz, Skorska, Sitkowska, & Cholewa, 2003), and a review from Beggs (2003) concluded that airborne route transmission of infectious agents were both directly and indirectly underestimated, e.g. with respect to *Staphylococcus aureus* and meticillin-resistant *S. aureus* (MRSA), *M. tuberculosis*, *Acinetobacter spp*., *Aspergillus spp*., *Pseudomonas spp*. and *Legionella* spp. and so on. Nonetheless, less is known compared to viruses for breath-borne bacterial pathogen emissions and transmissions.

When infected, it is critical for patients to be diagnosed accurately and timely to receive earlier and proper treatments (Urdea et al., 2006). Currently, clinical doctors often rely on the empirical experiences for diagnosis (Caliendo, 2011), and those approaches often fall short of providing accurate diagnosis (Falsey et al., 2013). On the other hand, colloidal gold immunization method is widely used in the fever clinic to distinguish Influenza A with Influenza B as it only needs less than 30 min. But these methods usually lead to higher rates of false-negative or false-positive results (Singh, Vasoo, Stevens, Schreckenberger, & Trenholme, 2010). In the meantime, a variety of methods are being developed or used for detection of pathogens, including culturing, amplification of nucleic acid (i.e. polymerase chain reaction (PCR), multiplex PCR, real-time PCR and DNA microarray or nucleic acid sequence-based amplification (NASBA)) (Hu, Yu, Crosby, & Storch, 2013; Petric, Comanor, & Petti, 2006; Xu & Yao, 2013; Zaas et al., 2013), immunological-based method (i.e. immunofluorescence or enzyme-linked immunosorbent assay (ELISA)) (Shen et al., 2012; Usachev, Agranovski, Usacheva, & Agranovski, 2015; Wu, Shen, & Yao, 2010), serologic testing (i.e. cytokine makers, C-reactive protein or procalcitonin) (Falsey et al., 2013; Haran, Buglione-Corbett, & Lu, 2013) and biosensor-based methods (optical, electrochemical and mass-based biosensors). Although some of these methods are effective, they take a longer time or are cost prohibitive for detection, e.g., the time needed for isolating and culturing pathogens. Recently, a new nucleic acids amplification method, the loop-mediated isothermal amplification (LAMP), has attracted great attention as a result of being highly specific for the target sequence (Notomi et al., 2000). Some LAMP commercial kits were already approved by Food and Drug Administration (FDA) (Ratliff, Duffy, & Waites, 2014), e.g., for detecting Mycobacterium tuberculosis complex (MTBC) by WHO (WHO, 2016a, 2016b). Importantly, the LAMP detection results can be simply validated using naked eyes due to higher LAMP product concentration. This opens up an outstanding opportunity for those remote areas without access to modern facilities to screen infectious agents. In addition to nasal swabs, bronchoalveolar lavages, nasopharyngeal aspirates or sputum samples, exhaled breath condensate (EBC) on another front is increasingly being used for disease diagnosis and virus detection (Kostikas et al., 2011; Shen et al., 2012; Teng et al., 2011). Despite of these new developments, affordable point-of-care diagnostic methods are still lacking at the bedside (Niemz, Ferguson, & Boyle, 2011).

Here, we developed a new protocol that integrates an exhaled breath condensate (EBC) collection device (PKU BioScreen) and Loop Mediated Isothermal Amplification (LAMP) and further used it to investigate what bacterial pathogens can be directly exhaled out from humans. The detection results from exhaled breath were then compared and validated using the throat swab samples collected from the same patients together with a gold standard molecular method-qPCR. The results from this work contribute not only to our understanding of human emission of infectious bacteria via breathing, and but also to the development of rapid pathogen screening protocol that could be potentially made available in remote areas where resources are significantly limited or prohibited.

2. Materials and methods

2.1. Clinical specimens

2.1.1. Patients and sample collection

Subjects involved in this research were recruited as respiratory tract infection patients who visited a respiratory clinic of Peking University Third Hospital, Beijing. Those patients were diagnosed with respiratory tract infections if they had at least one of these symptoms: 1) fever > 38 °C; 2) cough; 3) pharyngalgia. A total of 150 specimens comprising 100 throat swabs (IDs: 1–100, and 50 exhaled breath condensate (EBC)) samples (IDs: 1–36 and 87–100) were collected from 100 patients (ID numbers 1–100; their White Blood Cell count and Neutrophil (%) as well as other information are listed in Table S1) to compare the respective efficiencies of qPCR and LAMP method. Throat swab and EBC collection methods are both non-invasive, however the samples were used to study different health problems, e.g., EBC for lower airway inflammation (Cathcart, Love, & Hughes, 2012; Kostikas, Papatheodorou, Psathakis, Panagou, & Loukides, 2003) and throat swabs for upper respiratory tract infection (Thornton, Hay, & Redmond, 2017).

The exhaled breath condensate samples were collected using an exhaled breath collection device (PKU BioScreen) developed in our laboratory. Similar procedure for the device was used as described in a previous study (Xu et al., 2012). For the subjects (IDs: 1–36 and 87–100), they were asked to continuously exhale at a normal breathing rate for five minutes towards the device about 20 cm away from the subject. And then the exhaled breath can quickly form into tiny droplets on the hydrophobic surface on the top of the cooled unit. We collected all the droplets into a 1.5 mL centrifuge tube for analysis by dragging a DI water droplet over the film. The collection device somehow works like an impactor, but with a positive airflow (not a vacuum) and also a large collection surface compared to the straw(the impactor jet). Therefore, the collection efficiency should be very high given the large pathogen containing exhaled droplet, and the standard impactor equation cannot be used here to calculate the d50. In our previous study, we have collected and detected influenza viruses such as H3N2 using the same device (Shen et al., 2012). Accordingly, the d50 for the device could be very small or at least it is able to collect breath-borne viruses. Even for different subjects with different exhalation velocities, they won't affect the performance much. Typically, we obtained about 200–300 μL EBC within 3 min collection for all subjected involved. In our work, the detection of pathogens was most intended for qualitative analysis, thus the absolute physical collection efficiencies of the pathogens played a minor role in our data analysis. Nonetheless, it is certainly interesting to characterize the physical collection efficiencies of the EBC collection device. In our work, if we used a device without the cooling step, the amount of EBC collected was very limited compared to that with cooling. Typically, one cooled unit can last for 3–4 subjects for EBC collection without substantially losing much of the EBC collection efficiency.

For the throat swabs collection, we used the common bacteria collection tube of Youkang Biotechnology (Beijing) Co., Ltd. (item NO. MT0401). We wiped the retropharyngeal wall of each patient (IDs: 1–100) using a sterile swab, and then transfer the swab into buffer quickly. Pathogens can be extracted into the buffer solution by vigorous vortexing for 15 min at a vortex rate of 3200 rpm (Vortex genie-2, Scientific Industries Co., Ltd.). 1 mL extracting solution was transferred into a 1.5 mL centrifuge tube for detection. For each subject, we also collected the clinical data, including gender, age, health conditions, blood routine examination results and maximum body temperature (Table S1). As quality controls, we have also used a high volume sampler to take about 20 m^3 of the hospital room air which was further subjected to the LAMP method analysis in addition to blanks (DI water) as negatives. The EBC device utilizes a one-time use of hydrophobic film that can prevent cross-contaminations between patients. To further eliminate such a possibility, an upgraded version of the BioScreen is now equipped with an internal separator that sits between the collection wall of the device and the exhaled breath from the mouth. The separator is also a onetime consumable, and it can be changed for every patient. However, here we used the first version of the device that was reported in Xu et al. (2012). Human sample collections were approved by the Institutional Review Board of Peking University (PU IRB, approval #: IRB00001052-15012), and informed consent forms were attained from all patients prior to the experiments described above.

2.2. Specimens processing and DNA extraction

Total nucleic acid was extracted from a whole EBC sample (< 1 mL) or a 1 mL throat swab sample. Firstly, we centrifuged the samples at 10,000 rpm for 1 min (Centrifuge 5804 R, Eppendorf). And then, total DNA was extracted from specimens with the bacteria DNA extraction kit (TIANamp Bacteria DNA Kit, Tiangen Biotech (Beijing) CO., LTD) as described in the manufacturer's instruction. The DNA extraction kit utilizes silica membrane technology and special buffer system with lysozyme for a wide range of both Gram-negative and Gram-positive bacteria (Tiangen, Beijing). The DNA was eluted in 100 μL of TE buffer, which was supplied with the kit.

2.3. LAMP amplification for the bacterial pathogens

In this study, we aimed to test four types of bacteria pathogens including *Haemophilus influenzae*, *Pseudomonas aeruginos*, *Staphylococcus aureus*, *Methicillin-resistant Stphylococcus aureus* (MRSA), and one virus type, *Influenza A* using our protocol. The primers used for the LAMP detection for these pathogens were listed in Table 1 with references, which were described and used in previous studies. All the primers used in this work were synthesized by Sangon Biotech (Shanghai) CO., Ltd. For the bacteria detection, the LAMP assays were carried out with a 25 uL reaction mixture containing 2 μL template DNA, 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.4 μM each of LF and LB (if it was designed), 8U of the *Bst* DNA polymerase large fragment, 12.5 μL 2*RM (1.8 mM dNTP mixture, 8 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M Betaine) (Eiken Chemical CO., Ltd, Tochigi, Japan) and the remaining was ddH_2O added. The mixture was incubated at 63 °C for 60 min for *H. influenzae*, *P. aeruginosa* and MRSA; 64 °C was for *S. aureus*, and then heated at 80 °C for 5 min to terminate the reaction, by the Loopamp realtime turbidimeter (LA-500, Kyoto, Japan).

When detecting *Influenza A virus*, we used a one-step reverse transcription-loop mediated isothermal amplification (RT-LAMP) assay for the rapid detection. The PT-LAMP reaction was performed with a Loopamp RNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan). A 25 μL reaction mixture containing 1.6 μM each of primers FIP and BIP, 0.2 μM each of the outer primers F3 and B3, 12.5 μL of 2× reaction mix, 1.0 μL of amplification mixture containing *Bst* DNA polymerase and avian myeloblastosis virus reverse transcriptase, 2.0 μL of RNA sample was incubated at 60 °C for 120 min and then heated at 80 °C for 2 min to terminate the reaction using a real-time turbidimeter (LA-500, Kyoto, Japan).

The real-time amplification by LAMP assay was monitored through spectro-photometric analysis by recording the optical density at 400 nm every 6 s with the help of a Loopamp realtime turbidimeter (LA-500, Kyoto, Japan). A sample is treated as positive for a specific pathogen if the turbidity value exceeds the set threshold, e.g., at 0.1, at a certain time (Tt, in minutes). Positive and negative controls were included in each run, and all necessary steps were performed to prevent possible cross-contaminations.

Table 1
Primers used for LAMP assays for different bacterial pathogens for the protocol proposed in this work.

Target bacteria	Target gene	Primer	Sequence (5′ – 3′)	Reference
H. influenzae	*P6*	FIP	ACTTCTTTACCAAAGGCATCATTTTGCGTTTGTTGACGCCAAATTCTGG	(Torigoe, Seki, Yamashita, Sugaya, & Maeno, 2007)
		BIP	CTGATGATATGGGTACATCTGTTCGCGAAGAATGAGAAGTTTTGTGG	
		F3	CGCCAATACATTCAACAAGA	
		B3	CGTATGGGGTTTGTGCA	
		LF	GCAGACGACCAAAGGTATCTTG	
P. aeruginosa	*oprL*	FIP	GTTGTCACCCCACCTCCGGGCGGCAACGTTCCTCC	(Goto et al., 2010)
		BIP	CTCCGTGCAGGGCGAACTGCAGGCGAGCCAACTC	
		F3	GCGTTGCCGCCAACAATG	
		B3	CATGCGGGCAACCTCTC	
		LF	ACCTGCCGTGCCATACC	
		LB	GTTCATGCAGCTCCAGCAG	
S. aureus	*nuc*	FIP	CGTTTACCATTTTTCCATCAGCATATTTGACAAAGGTCAAAGAACT	(Yang, Ma, Zhang, Wang, & Zhang, 2011)
		BIP	TCAAGGCTTGGCTAAAGTTGCTTATTCGCTTGTGCTTCACTT	
		F3	TGCAAAGAAAATTGAAGTCGA	
		B3	CGTTGTCTTCGCTCCAAAT	
		LF	ACGCTAAGCCACGTCCATAT	
		LB	CAAACCTAACAATACACATGAACA	
MRSA	*spa*	FIP	GCTCTTCGTTTAAGTTAGGCATGTTTGCGCAACAAAATAAGTTCA	(Misawa et al., 2007)
		BIP	AAGTCTTAAAGACGATCCAAGCCTTCGGTGCTTGAGATTCG	
		F3	AATGACTCTCAAGCTCCAA	
		B3	CTTTGTTGAAATTGTTGTCAGC	
		LB	AGCACTAACGTTTTAGGTGAAGC	
Influenza A virus	*M gene*	FIP	TGCTGGGAGTCAGCAATCTGTTACAGGATGGGGGCTGTGACC	(Poon et al., 2005)
		BIP	AGGCAAATGGTGACAACAACCTGTAGTGCTGGCTAGAACC	
		F3	TGGTGCACTTGCCAGTTG	
		B3	CCAGCCATTTGCTCCATAGC	

2.4. PCR amplification of bacterial pathogens

In the PCR amplification reaction, primers and probes used for amplifying the *P6* gene in *H. influenzae*, the *Coa* gene in *S. aureus* cells and the *ecfX* gene in *P. aeruginosa* cells are listed in Table 2 according to previous published studies and they were synthesized in this work by Sangon Biotech (Shanghai) CO., Ltd. For *H. influenza,* PCR assays were carried out in 50 μL volumes containing 5 μL DNA template, 125 nM of each primer, 800 μM dNTP, 2.5 U Taq polymerase, 1*Taq buffer and the remaining was ddH_2O added. PCR amplifications were performed using the following cycling parameters: denaturation at 94 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min and a final elongation step of 72 °C for 7 min. PCR products were visualized by electrophoresis using a 2.5% agarose gel stained with ethidium bromide.

For *P. aeruginosa*, the PCR reaction mixture (total volume was 50 μL) included 5 μL DNA template, 0.5 μM of each primer, 200 μM dNTP, 2.5 U Taq polymerase, 1*Taq buffer and the remaining was ddH_2O added. PCR amplification was performed with a Bio-Rad S1000 thermal cycler (Bio-Rad, USA) with an initial denaturation step of 5 min at 95 °C, 35 cycles of 45 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were visualized by electrophoresis using a 2% agarose gel stained with ethidium bromide.

For *S. aureus*, the PCR amplification was carried out in a final reaction volume of 50 μL. The mixture consisted of 5 μL DNA template, 0.2 μM of each primer, 200 μM dNTP, 0.625 U Taq polymerase, 1*Taq buffer and the remaining was ddH_2O added. The amplification was performed in a Bio-Rad S1000 thermal cycler (Bio-Rad, USA). The PCR cycles consisted of preheating 45 s at 94 °C, denaturation at 94 °C for 20 s, annealing at 57 °C for 15 s, and extension at 70 °C for 15 s. The amplification was performed for 30 cycles with a final extension step at 72 °C for 2 min. PCR products were visualized by electrophoresis using a 1.5% agarose gel containing 0.5 mg of ethidium bromide per milliliter.

Table 2
Primers used for PCR assays for different bacterial pathogens in this work.

Target bacteria of PCR assay	Target gene	Primer	Sequence (5′ – 3′)	Reference
H. influenzae	*P6*	FP	TTGGCGGWTACTCTGTTGCT	(Strålin, Bäckman, Holmberg, Fredlund, & Olcén, 2005)
		RP	TGCAGGTTTTTCTTCACCGT	
P. aeruginosa	*ecfX*	FP	ATGGATGAGCGCTTCCGTG	(Lavenir, Jocktane, Laurent, Nazaret, & Cournoyer, 2007)
		RP	TCATCCTTCGCCTCCCTG	
S. aureus	*Coa*	FP	ATA GAG ATG CTG GTA CAG G	(Ahmadi, Rohani, & Ayremlou, 2010)
		RP	GCT TCC GAT TGT TCG ATG C	

Table 3
List of 13 common respiratory pathogens involved in the multiple-detection chip.

No.	Names of pathogens	Abbreviation index
1	*Streptococcus pneumoniae*	*S. pneumoniae*
2	*Staphylococcus aureus*	*S. aureus*
3	*Methicillin-resistant Stphylococcus aureus*	*MRSA*
4	*E. coli*	*E. coli*
5	*Klebsiella pneumoniae*	*K. pneumoniae*
6	*Pseudomonas aeruginosa*	*P. aeruginosa*
7	*Acinetobacter baumannii*	*A. baumannii*
8	*Stenotrophomonas maltophilia*	*S. maltophilia*
9	*Haemophilus influenzae*	*H. influenzae*
10	*Legionella pneumophila*	L. *pneumophila*
11	*Mycoplasmal pneumonia*	*M.Pneumonia*
12	*Chlamydia pneumoniae*	*C. pneumoniae*
13	*Mycoplasmal tuberculosis*	*M.tuberculosis*

2.5. Multiplexing detection of 13 common respiratory pathogens

In this study, we also screened 13 common respiratory pathogens of the clinical samples (as shown in Table S1) to investigate the major pathogens responsible for the respiratory infections. We used a commercial multiple-detection chip detection system (RTisochipTM-A, CapitalBio Tech CO., Ltd, Beijing) for clinical sample pathogen detection of the 13 pathogens. It combined the LAMP method with a microfluidic technique which purifies the sample before analysis, and can simultaneously screen 13 different pathogens in a single run. The names of these pathogens were listed in Table 3. The screenings were conducted for the EBC samples (IDs: 9,19–32) and throat swabs (IDs: 9, 19–86) as listed in Table S1.

2.6. Statistical analysis

Experimental data were analyzed using SPSS21.0 statistical package (©IBM Corporation 2012). To compare the LAMP and PCR results, we have conducted Kappa tests and McNemar's tests. Guidelines characterized kappa (κ) values over 0.75 as excellent, 0.40–0.75 as fair to good, and below 0.40 as poor. All comparisons were made to the same group but with different analysis techniques (PCR or LAMP) respectively for EBC or throat swabs collected from the same subject. In this work, those selected pathogens were found below the detection limits for the air samples up to 20 m^3 collected from the same hospital rooms where the EBC and throat swabs were collected. In this work, a p-value of 0.05 indicates a statistically significant difference.

3. Results

In this work, we have recruited a total of 100 patients with flu symptoms from November 2014 to May 2015 (their information is shown in Table S1). Among them, throat swabs were collected from all patients (IDs: 1–100), and 50 of them (IDs: 1–36 and 87–100) were further asked to collect their EBC samples using the PKU BioScreen. The EBC volume for each of the patients was more than 100 μL, which was adequate for further LAMP analysis. Overall, we have shown that certain bacterial pathogens can be exhaled out, and the developed pathogen screening protocol that integrates EBC and LAMP can be used as one non-invasive rapid respiratory pathogen screening method.

3.1. Bacterial pathogen detection in exhaled breath using the EBC-LAMP protocol

In this work, we totally screened 20 EBC specimens (IDs: 7–26) and 30 throat swabs (IDs: 1–30) that were collected from the same recruited patients as listed in Table S1 for detecting a common bacterial pathogen *H. influenza* using both qPCR and LAMP. The PKU BioScreen was shown to be able to collect 300–500 μL EBC within 3–5 min. For the patients (IDs: 7–26), *H. influenzae* was detected in six samples (IDs: 7, 8, 9, 10, 11, 12) out of 20 EBC samples (IDs: 7–26), while only four corresponding throat swab specimens (IDs: 7, 8, 9, 11) were found positive with the bacteria as shown in Fig. 1, and the remaining results, i.e., the detection time) are listed in Table S1 (Supporting Info). As shown in Table S1, for the patients (IDs: 7–26) using the LAMP for detecting *H. influenza* there was a 35% agreement (7/20) (negative or positive) between the EBC and throat samples collected from the same patients. To evaluate the performance of the LAMP method, the same samples were also analyzed using qPCR assay when detecting *H. influenzae*. As shown in Table S1, there was a 65% (13/20) agreement if the qPCR method was used between two different samples. However, the advantage of EBC-LAMP protocol is that use of EBC might not need a sample pre-treatment.

As seen in Fig. 1, an interesting thing was that, for all the 6*H. influenzae*-positive EBC samples, the threshold times (Tt value) using the LAMP method were shorter than those of the corresponding throat swabs from the same subjects. This finding implies that EBC could somehow serve as a better sample for LAMP analysis of bacterial pathogens in terms with detection time possibly resulting from its diluted medium, which is less complex than the throat swab. For example, it only took 20.6 min for the EBC sample (patient ID_7) with the highest concentration, saving 7.4 min compared to the use of the corresponding throat swabs from the same subject. For all

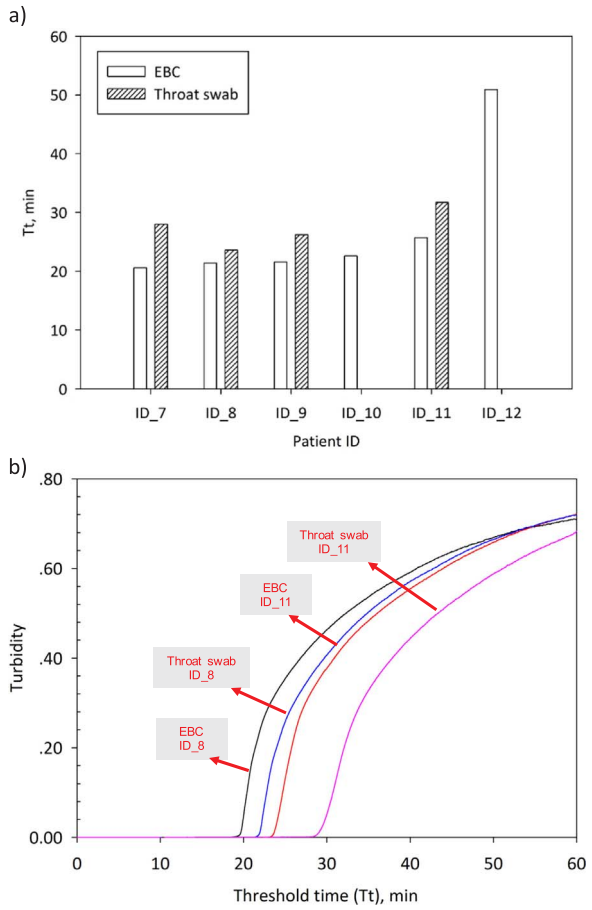

Fig. 1. a) Examples of *H. influenzae* pathogen detection in exhaled breath samples by real-time turbidity assay in EBC and throat swabs collected from selected 6 patients (IDs:7–12). The grouped bars represented the samples of same patients, and the horizontal axis showed the ID number of those patients; no show of bars means bacteria were not detected; b) The LAMP amplification curves (Turbidity vs Detection time) for corresponding samples that were analyzed for *H. influenza* in both EBC and throat swabs collected from the same subjects (IDs: 8 and 11).

the six samples, the average detection time of *H. influenza* for the EBC and throat swabs are 27.1 min and 31.0 min, respectively (These data are listed in the Supporting Information, Table S1). However, there were also several throat swab samples (IDs: 13, 15, 16, 18–21, 23–26) which were detected to be positive with *H. influenzae* while none of this was detected for the corresponding EBC from the same subjects as shown in Table S1. Possible reason could be due to lower concentration level of the pathogen in the EBC sample compared to that of the throat swabs from the same subjects. This could arise from the variations in pathogen concentration during the EBC collection process among different patients. For these patients who were infected by *H. influenzae*, the proportion of neutrophilic granulocyte ranged between 52.0% and 90.0% as listed in Table S1, and it is thus difficult to make a decision based on the empirical threshold 75% rule of neutrophilic granulocyte. Because there were mixed impacts from various factors for the clinical presentation of respiratory infections, it is rather difficult, if not impossible, to reliably predict the pathogen based on clinical signs and symptoms (Caliendo, 2011). Nonetheless, to compare the performances of EBC and throat swabs in detecting pathogens we still need more samples.

As a simple and non-invasive method, EBC is increasingly being utilized for early diagnosis of diseases, e.g., lung cancer, asthma, influenza and some other respiratory infections (Kostikas et al., 2011; Papaioannou et al., 2011; Teng et al., 2011). EBC has demonstrated great potential and advantages in disease screening and diagnosis when combined with gold standard methods such as qPCR and ELISA methods (Ahmadzai et al., 2013). However, in addition to the false-negatives for low level pathogens, the qPCR method takes a longer time that could be up to several hours for detection (Falsey et al., 2013). On the other hand, the LAMP method for detecting various pathogens has been attracting great attention recently owing to its higher sensitivity and lower detection limit. Compared to commonly used clinical specimens such as nasal swabs, bronchoalveolar lavages, nasopharyngeal aspirates or sputum, exhaled breath sample can be more easily collected. Accordingly, use of EBC samples together with LAMP (a relatively inexpensive solution) could be applied to rapid pathogen screening. Overall, results from Fig. 1 and Table S1 show that bacterial pathogens such as *H. influenzae* can be emitted into the environment via human breath, and EBC samples can be used for screening pathogens using both LAMP and qPCR with reasonable detection rates.

Here, this work was also designed to detect other three types of bacteria and one virus type using our LAMP protocol. Supporting

Table 4
Detection of bacterial pathogens in EBC and Throat Swabs by using the commercial LAMP device (RTisochipTM-A).

Types of specimen	No. of samples	Total positive cases		Positive rate
		Cases for single pathogen[a]	Cases for multiple pathogens[b]	
Throat Swabs	69	20	5	36.2%
EBC	15	4	1	33.3%

[a] single pathogen, only one type of pathogen was detected in one sample.
[b] Multiple pathogens, more than one type of pathogen was detected in one sample.

File S1 shows the detection results of *P. aeruginosa*, MRSA and *Influenza A* virus. As for detecting *P. aeruginosa* in throat samples from the patients, the results from EBC-LAMP and qPCR methods as shown in File S1 agreed at a rate of 95%, including 5 positive cases (IDs: 33–36, 88). For detecting MRSA in EBC samples, the results from LAMP and qPCR agreed at 99.9%, including only positive case (ID: 29). This high agreement was partially due to non-presence of MRSA in most EBC samples collected. When detecting FluA in throat swabs, four positive cases (IDs: 8, 10, 18, 22) were detected. Here, the main objective of this work was to evaluate the performances of EBC-LAMP and EBC-qPCR protocols for qualitative analysis of bacterial pathogens in different samples, not on their detection limits. However, in another work, we have fully evaluated the detection limits of qPCR and LAMP methods under controlled conditions for selected pathogens including those tested in this work. Overall, we found that the LAMP method tended to have lower detection limits than the qPCR method.

3.2. Further validation of the EBC-LAMP protocol for breath-borne pathogen detection using a commercial multiplexing LAMP pathogen detection method

To further validate the EBC-LAMP protocol, both EBC and throat swab samples were tested with a commercial LAMP detection unit (RTisochipTM-A, CapitalBio Tech CO., Ltd, Beijing) as shown in Table S1. The unit is able to simultaneously screen thirteen kinds of pathogens for a given sample. A total of 69 throat swabs and 15 EBC specimens (The detail medical information of the corresponding patients was shown in Table S1) were screened using the device. As shown in Table S1, Table 4 and Fig. 2, for the exhaled breath condensate samples, four were detected positive with *E. coli* (66.7%), one for *P. aeruginosa* (16.7%) and one for MRSA (16.7%) respectively, while for one subject both MRSA and *E. coli* were detected in EBC. In addition, 25 (36.2%) out of 69 throat swabs specimens were tested positive for at least one type bacterial pathogen, including 5 (19.0%) specimens had two or three types of pathogens. Among them, *H. influenzae* showed obviously higher positive rates than other pathogens, which suggests that *H. influenzae* was the dominant bacteria responsible for upper respiratory tract infections. In addition to *H. influenza, P.* aeruginosa, *E. coli, S. maltophilia, S. aureus* and *MRSA* were also detected both in the EBC and throat swab samples. For the same patients for whom both EBC and throat samples collected, there was a 53.3% agreement using the commercial LAMP method.

As shown in Fig. 2, the screening of pathogens using the commercial LAMP device showed different results from EBC specimens and throat swabs as listed in Table S1. Similar to the results from the EBC-LAMP protocol developed in this work, the *H. influenzae* was again detected as the dominant pathogen with a positive rate 50.0% by the commercial unit, but none of the EBC samples (IDs:

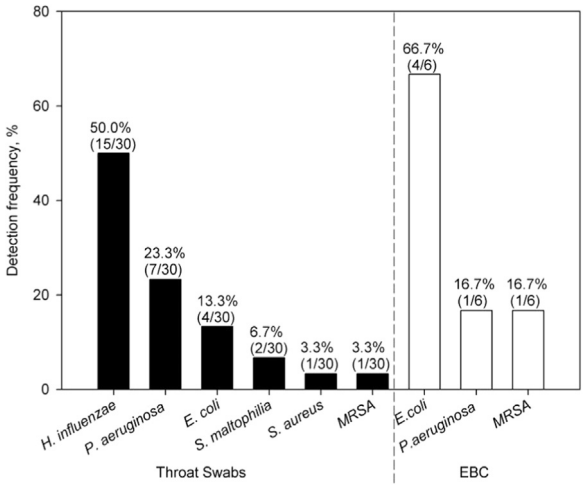

Fig. 2. Bacterial pathogens detected in both 6 EBC and 30 throat swab samples using a commercialized LAMP device. The symbol on each bar was shown as positive rate for the respective pathogen type shown at the x-axis, which was calculated as the ratio of positive number of this type of pathogen over the number of total positive rate for all bacteria. The percentages and also #/# on the figure indicates the frequencies of the selected six pathogens that were detected in 25 throat swabs and 5 EBC samples shown in Table S1.

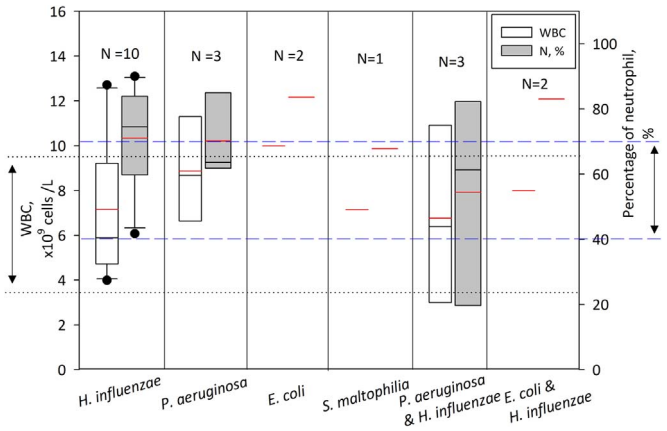

Fig. 3. Boxplots of white blood cell count (WBC) and neutrophil levels for the patients who were found to be infected by single or multiple bacterial pathogens such as *H. influenzae*, *P. aeruginosa*, *E. coli, S. aureus, S. maltophilia* and MRSA using the EBC-LAMP and also the commercial LAMP device. The symbol on each group is the number of patients who were classified in this group; WBC-White blood cells; N%-Percentage of neutrophils from routine blood tests shown in Table S1 (Supporting Information).

19–32, 9) was found positive result with *H. influenzae*. As discussed above, *H. influenzae* was detected in EBC samples using the EBC-LAMP protocol developed in our work. One possible reason is that pathogen levels appeared to be lower in EBC samples than those in the throat swabs, thus the commercial device used here might not be able to detect them due to its detection limit. Besides, the commercial LAMP device (without disclosing their primer information) might have used different primers sets than what we have used as listed in Table 1 in our EBC-LAMP protocol. It is noted that we have also detected MRSA both in the EBC and the throat swab samples using the commercial device. At the same time, MRSA was also detected using the EBC-LAMP assay developed here. S. *aureus*, especially methicillin-resistant S. *aureus* (MRSA) is now one of the most studied pathogens in community-acquired and hospital-acquired infections associated with high mortality, causing great concerns (Misawa et al., 2007). Previous study has evaluated the airborne culturable MRSA in the Chinese public building, which demonstrated that MRSA could transmit in the ambient air (Li et al., 2015). Nonetheless, the results from the commercial device further showed that different types of bacterial pathogens such as Pae, E. coli, and MRSA can be exhaled out.

In this study, we further analyzed the white blood cell (WBC) and neutrophil levels of patients who were found to be infected by bacterial pathogens using the EBC-LAMP and also the commercial LAMP device. Results are shown in Fig. 3, and patients were grouped together if they were either infected by the same single bacterial pathogen or a group of pathogens, such as *H. influenzae*, *P. aeruginosa*, *E. coli, S. aureus, S. maltophilia* and MRSA. Clinical doctors often rely on the empirical diagnosis, with the normal reference ranges of white blood cells (WBC) and percentage of neutrophils are 3.5–9.5*10^9 cells/L and 40–75%, respectively. As seen in Fig. 3, for all patients infected by bacterial pathogens except one patient infected by *E. coli* all their average WBC levels appeared to be in the normal reference range. Even for the leading pathogen *H. influenza*, all the patients (N = 10) had normal WBC levels. However, in clinical settings, e.g., in a respiratory clinic, these patients were generally treated as viral infections because of the normal WBC level. This means that a lot of flu patients could have been mis-treated in the hospitals where the WBC-infection-type rule as discussed are used. On the other hand, it seems that average neutrophils values appeared to be slightly higher than normally expected, e.g., for those infected by the leading pathogen *H. influenza, P. aeruginosa, E. coli, S. maltophilia* and also the combination of *E. coli* and *H. influenza*. The results from Fig. 3 indicate that empirical experiences, e.g., use of WBC for diagnosis of infection type (i.e., higher for bacterial infections), could be leading to wrong decisions with respect to the diagnosis and treatments. Nonetheless, this work was intended to evaluate which protocols are more efficient in terms with rapidly screening respiratory pathogens, but not on the pathogen infection mechanisms.

4. Discussion

In this work, we developed and validated a new pathogen detection protocol (the EBC-LAMP protocol), which integrates the EBC with the LAMP method. Using the protocol, we have shown that certain bacterial pathogens such as *H. influenzae*, *P. aeruginosa*, *E. coli, S. aureus, S. maltophilia* and MRSA can be exhaled out from the infected patients. Using the EBC device, we were able to obtain 300–500 μL EBC samples within 3–5 min, which was adequate for LAMP analysis. The device adopts a one-time use of hydrophobic film and also an internal exhalation separator from an upgraded version of the device that can prevent the cross-contaminations among the patients during the EBC collection process. The BioScreen method developed in our laboratory holds promise for extending the EBC sample analysis for disease diagnosis in many different fields since it is portable and fast without external use of power and little cross contamination in contrast to other currently available devices.

Here, we have found that *H. influenza* was the leading bacteria responsible for the respiratory infections of the patients we have recruited in this study. Similar to our work, Kang et al. (2012) reported that the most common respiratory bacterial infections are caused by *H. influenzae*, *S. pneumoniae*, *respiratory syncytial virus* and so on. Similar studies also demonstrated that the *S. pneumoniae,*

H. influenzae, M. pneumoniae and C. pneumoniae were often predominant in both outpatients and inpatients with respiratory disease (Strålin et al., 2005). The *H. influenza* bacterium was first isolated by Pfeiffer during the influenza pandemic of 1890, and is a Gram-negative opportunistic bacterium which appears to be coccobacilli. In this work, in addition to *H. influenza*, we have also found that *S. aureus*, MRSA, *P.* aeruginosa and *E. coli* can be also exhaled out by the infected patients. The results from this work and other studies indicated that these pathogens could be emitted and transmitted through the exhaled breath, thus adding to additional risks of infectious disease transmission. In other studies, airborne and/or droplet transmission were identified as the major routes for spreading human and animal bacteria, such as *E. coli, S. aureus, anthrax, K. pneumonia, and P. aeruginosa* (Ijaz, Zargar, Wright, Rubino, & Sattar, 2016; Knibbs et al., 2014; Li et al., 2013; Pyankov et al., 2012). The WHO used a pathogen size of 5 μm to distinguish between droplet and airborne (smaller than 5 μm) transmission of exhaled particles (WHO, 2007). In a previous work, it was found that a bacterial concentration up to 7000 CFU/m^3 was detected in exhaled breath, including both viable and dead cells of various types (Xu et al., 2012). Among other bacteria, *Sphingomonas paucimobilis* and *Kocuria* variants were found dominant in EBC samples collected from the healthy subject using a VITEK 2 system (Xu et al., 2012). In their work, SEM images revealed that most bacteria in exhaled breath are detected in the size range of 0.5–1.0 μm (smaller particles), which accordingly poses greater risk of airborne transmission once exhaled out especially for pathogens such as MRSA. In another work, it was shown that humans could emit millions of bacterial particles per min, among which 17% was from the exhaled breath with a fluorescent bioaerosol concentration level of $(1.93 \pm 1.83) \times 10^5$ particles m^{-3} and a peak size at 1.5 μm (Xu, Wu, & Yao, 2017). Besides, human activities could also re-suspend a huge amount of bioaerosol particles. Once the pathogens exhaled to the ambient air, they would be suspended in air for long periods of time, accordingly traveling to greater distances and increasing the respiratory infection transmission risk (Wurie, Lawn, Booth, Sonnenberg, & Hayward, 2016). All these emissions either from nature or infected individuals represent important infection risks under certain conditions.

However, most often even in the developed countries, the diagnosis of the infection type, the health professionals empirically rely on the white blood cell (WBC) and neutrophil levels from a routine blood test and accompanying clinical symptoms such as headache, cough, and arthralgia (WHO, 2016a, 2016b). In principle, this practice lacks the scientific evidence. Our results suggest that use of higher WBC value (e.g., if the WBC is normal or not) as an indicator for bacterial infection might not be accurate in terms with identifying respiratory infection type (bacteria or viruses). In practical scenarios, physiological conditions vary significantly with individuals that could have different immune status, age, medical history, genetics and infections by multiple agents (both bacteria and viruses). It is rather difficult to judge the infection type purely through routine blood examination and also some clinical symptoms. This problem has been going for many years without active solutions that have been developed. The lack of the solution often leads to abusive use of antibiotics. Here, the developed EBC-LAMP together with throat swabs was found to be useful and rapid for identifying the bacterial pathogens. As its applications, the developed method has already been used for identifying the bacterial pathogens in two medical cases which involved two children hospitalized in ICUs. For a child aged at 4 who was diagnosed as severe pneumonia and hospitalized in an ICU in an Inner Mongolia hospital, we have successfully detected *Legionella pneumophila* in his throat swabs using the method we have described here. And for another hospitalized –less-than-6-month baby in pediatric intensive care unit (PICU) in Beijing, MRSA, *S. aureus* and S. *pneumophila* had been detected in his throat swab sample. It only takes 40 min for the LAMP detection while the culturing method needs at least 3 days. The detection results could help the doctors make the appropriate decisions about the medical treatments, particularly for certain agents such as L. *pneumophila* and MRSA. For these two types of pathogens, most medications available might not work better, e.g., MRSA is now resistant to many antibiotics. Timely and accurate diagnosis of the respiratory pathogens could play a vital role in helping the patients recover from the illness. Overall, this work provides a feasible protocol for the rapid detection of respiratory pathogens, especially useful in some under-developing areas.

Our EBC-LAMP protocol was further validated and compared with the standard method qPCR. The results demonstrated that the EBC-LAMP method might be more sensitive than the qPCR. This was possibly due to the inhibition problems with the qPCR from the sample matrix, and samples often need to be several-fold dilutions to eliminate the impact before the amplification. In addition, the LAMP method uses two additional loop primers for detecting pathogens which not only accelerate LAMP reaction, but also further improve the kinetics and sensitivity of the LAMP reaction (Notomi, Mori, Tomita, & Kanda, 2015). In addition to measuring the turbidity using an instrument, the detection results from the LAMP method can also be made to be judged and visualized by naked eyes due to the production of high concentration amplification product. For example, the white magnesium pyrophosphate precipitates generated during the strand displacement auto-cycling reaction could be easily seen at the bottom of microfuge tubes. Adding fluorescent dye SYBR Green I into the reaction tube could lead to the eventual color change. For example, reaction tube appears to be fluorescent color by the ultraviolet radiation for a sample that is positive for a specific pathogen; while for negative control or samples without pathogens the color won't change. In this way, it provides an easy way to identify the reaction results if the turbidity instrument is not available. The EBC samples are relative simple compared to the throat swabs that often need sample pre-treatment before PCR or LAMP analysis. Besides the LAMP method, we have also developed a new DNA extraction solution (involved in our LAMP method for onsite bacterial screenings) that does not require any re-agent and efficiently obtains the bacterial DNA within 5 min. The detailed method has been filed for a patent and will be reported elsewhere. Currently most studies focus on breath-borne viruses, but our study as one of fewer studies reports the release of bacterial pathogens from the exhalation. In general, when pathogens are exhaled out they become dried up immediately, thus possibly transmitting to other places. Our data were from a real clinical setting that has important impacts on the healthcare providers. In addition, our results contribute to the understanding to the hospital infections and also breath-borne pathogen transmission literature. Overall, the developed EBC-LAMP protocol has the great potential of being readily made available in remote or less developed regions where resources are very limited or prohibited.

5. Conclusion

By integrating a commercialized EBC collection device (PKU BioScreen) with the LAMP method, we have developed a rapid and efficient protocol for screening respiratory bacterial pathogens. The results from LAMP assay were shown to be generally consistent with those from the qPCR assays for the same samples, and the sensitivity of LAMP was found to be slightly higher than the PCR amplification. Using the protocol, we have shown that many pathogens such as *H. influenzae*, *P. aeruginosa*, *E. coli*, *S. aureus* and MRSA could be emitted out from the infected patients via exhaled breath. Similar to other studies, our work also demonstrated that *H. influenzae* is the leading pathogen responsible for the common respiratory infections. However, their viability and infectivity after the airborne exposure need to be further investigated. Overall, the protocol is a rapid, effective and noninvasive method in diagnosis of infections pathogens, and has been successful in identifying bacterial pathogens for two hospitalized child in ICU. In the future, the protocol can be made to be portable and available for bedside use. Nonetheless, the results here using the protocol could be negatively impacted by the number of patients involved and also the distribution patterns of patients' gender, age and medical history. In addition, regardless of the methods tested, use of throat swab samples resulted in high positive rates for selected pathogens compared to EBC samples, which might be due to different sources of pathogens or different types of respiratory infections (lower or upper respiratory tract infections). And in our future work the EBC-LAMP protocol will be also tested for screening breath-borne viruses in addition to increasing the sample size of the patients for further improvements. Besides, the transport and the transmission of the exhaled pathogens could be also further explored using an aerosol monitor together with a molecular method or a biosensor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jaerosci.2017.12.009.

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