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Detection of Pathogenic Bacteria in the Air by Culture Techniques in Combination with Multiplex Polymerase Chain Reaction

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Abstract

Respiratory infections are among important infections in human. The causative agents are diverse group of bacteria, virus, fungi and parasites. This research focuses on the detection of microbial contaminants and respiratory pathogens in indoor air of university environment by application of a molecular method. Seven pathogens including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Legionella pneumophila* were screened. Twenty-eight air samples were analyzed by conventional culture method and the multiplex polymerase chain reaction (mPCR) that detected those seven pathogens simultaneously within three reactions. The conventional culture method showed the average total count of large lecture rooms at 3.4 CFU plate^{-1} h^{-1}, small lecture rooms at 5.2 CFU plate^{-1} h^{-1}, meeting rooms at 4.3 CFU plate^{-1} h^{-1}, office rooms at 3.0 CFU plate^{-1} h^{-1}, canteen rooms at 31.5 CFU plate^{-1} h^{-1} and service vans at 5.5 CFU plate^{-1} h^{-1}. Contaminants comprised Gram-positive bacteria, Gram-negative bacteria and molds. The genus compositions were *Staphylococcus* spp. (62.50%), *Micrococcus* spp. and *Kytococcus* spp. (58.33%), *Bacillus* spp. (41.64%), *Moraxella* spp. (29.17%), *Corynebacterium* spp. (25.00%) and *Pseudomonas* spp. (20.83%). MultiplexPCR was able to detect *Staphylococcus aureus* and *Streptococcus pyogenes* at 16.67% and 4.17% of air samples, respectively, whereas conventional method did not. These two bacteria are important pathogens of human and are common cause of wide range of infections. The results suggested that mPCR was a useful supplement to the conventional method to monitor indoor air microbiological quality and should be introduced in guidelines of indoor air quality monitoring.

Keywords: Airborne bacteria, respiratory infections, multiplex polymerase chain reaction
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1. Introduction

Respiratory infections are among most important infections of the world population. Large budgets have been spent by many countries to deal with these diseases. However, they tend to be more severe and widely spread, hence efficient and urgent controls are needed. Starting from understanding the sources and important factors concerning human respiratory infections is agreed to be a reasonable approach [1, 2].There are large variety of respiratory infectious diseases which may be acute or chronic, and examples include influenza, pneumonia, tuberculosis, bronchitis, and the common cold. The causative agents can be bacteria, virus and fungi with a big diverse group and pathogenicity level. These microbes can be transmitted by aerosols from infected human oral and respiratory fluid, causing widely spread of diseases [3-5]. Exposure to contaminated air is a kind of threat for human health because even generally nonpathogenic microbes can cause infection in hosts that are compromised. The reservoirs of pathogens, such as the antibiotic-resistant *Staphylococcus aureus*, are known to be not limited only to hospitals, but are also found in the indoor environments of normal residential buildings [6, 7]. Understanding of aerosols and particles in the indoor environment tends to be closely concerned with infection control of respiratory diseases [2, 3]. Interestingly, it has been expressed that characterization of airborne bacteria is important for understanding disease transmission from person to person.There were also reports that some bacteria isolated from indoor air can be tracked back to human origin [8, 9].

Human occupants are one of the most important factors concerning respiratory infection from indoor air. The efficient prevention and control of the diseases to provide safety to people who spend most of the time indoors should start from learning about the aerosols and materials from human occupants that may disperse in environmental areas. However, the microbial contents in indoor environment of most universities have not been investigated and the essential information for good management is still needed. The conventional method to determine the microbial content is culture and identification which is the gold standard method. However, some fastidious organisms may not be detected when they are present at low amount. Molecular techniques such as Polymerase chain reaction (PCR) have been accepted to be very helpful for microbial detection in various samples according to their sensitivity, specificity and rapidity [10-13].

The multiplex polymerase chain reaction (mPCR) is a type of PCR technique that has become widespread and very popular for amplification of more than one target sequence in a reaction mixture using multiple primer pairs. This technique is time saving since many species of microorganisms can be detected at the same time [14-16]. Therefore, in this study we aimed to investigate indoor air of a university for the concentration and diversity of microbial contaminants including the seven important pathogens by using culture technique in combination with mPCR. The results show the concentration and composition of microbes including some pathogenic species that may exist. This will be a very useful information for universities to prevent and control the respiratory infections for their students.

2. Materials and Methods

2.1 Sampling environment

Total of twenty eight air samples were collected from six groups of locations in a university including large lecture rooms, small lecture rooms, meeting rooms, office rooms, canteen rooms and service vans.

2.2 Cultivation method

Indoor air samples were collected by using settle plate method with the 1/1/1 (1 h, 1 m from the floor, at least 1 m away from walls) schedule [17]. Various culture media in a 10 cm diameter Petri-dish were used according to the investigated microbes, including Plate count agar (PCA), Blood agar (BA), Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), Chocolate agar for *Haemophilus influenzae* and Legionella BCYE agar for *Legionella pneumophila*. Plate count agar and Blood agar were incubated at 37ºC, Sabouraud dextrose agar and Potato dextrose agar were incubated at 25ºC, Chocolate agar were incubated at 37ºC in candle jar and Legionella BCYE agar were incubated at 37ºC. All plates were incubated for 24-48 h followed by colony examination and identification by conventional method [18]. Levels of contamination were recorded as colony forming unit per plate per hour (CFU plate^{-1} h^{-1}).

2.3 Molecular method

Samples for DNA detection of contaminants were collected by using impactor sampler, Reuter Centrifugal Air Sampler, loading with a 9 cm filter, according to the instruction.The samples were prepared for DNA extraction by cutting each filter into small pieces and suspended in 5 ml buffer peptone water (pH 7.2, 1 l comprising 10 g of peptone, 5 g of sodium chloride, 3.5 g of disodium phosphate and 1.5 g of potassium dihydrogen phosphate). After vortexing for 2 min followed by centrifugation at 12,000 rpm for 5 min, the supernatant was collected for DNA extraction steps. Bacterial DNA from the collected supernatant was extracted using InstaGene^{TM}Matrix according to the manufacturer's instruction and the clear supernatant was collected and stored at -20ºC for using as DNA sample in further steps.

PCR detection of bacterial contaminants in all samples was performed by using primers specific for each of seven important bacterial species including *Streptococcus pneumonia*e (Ply F, Ply R), *Streptococcus pyogenes* (LytA F, LytA R), *Staphylococcus aureus* (sa442 F, sa442 R), *Haemophilus influenzae* (Hae F, Hae R), *Klebsiella pneumoniae* (Pf, Pr2), *Pseudomonas aeruginosa* (PA1, PA2) and *Legionella pneumophila* (Mip F, Mip R), as shown in Table 1.

Table 1. PCR primers, target genes and product sizes from DNA amplification of studied pathogens

Bacterial Species	Target genes	Primers	Primer sequences	Product (bp)/Ref
Haemophilus influenzae	16S rDNA	Hae F	ttg aca tcc taa gaa gag ctc	167/ [19]
		HaeR	tct cct ttg agt tcc cga ccg	
Streptococus pneumoniae	*ply*	Ply F	gaa ttc cct gtc ttt tca aag tc	348/ [20]
		Ply R	att tct gta aca gct acc aac ga	
Klebsiella pneumoniae	16s-23s ITS	Pf	att tga aga ggt tgc aaa cga t	260/ [21]
		Pr2	ccg aag atg ttt cac ttc tga tt	
Pseudomonas aeruginosa	Antigen H	PA1	tcc aaa caa tcg tcg aaa gc	181/ [22]
	16s-23s ITS	PA2	ccg aaa att cgc gct tga ac	
Streptococcus pyogenes	*lytA*	LytA F	gag aga cta acg cat gtt agt a	317/ [19]
		LytA R	tag tta ccg tca ctt ggt gg	
Staphyococcus aureus	*sa442*	sa442 F	tcg gta cac gat att ctt cac	179/ [20]
		sa442 R	act ctc gta tga cca gct tc	
Legionella pneumophila	*mip*	Mip F	acc gaa cag caa atg aaa ga	144/ [19]
		Mip R	aac gcc tgg ctt gtt ttt gt	

The PCR reaction mixture of 50 µl contained 1XPCR buffer, 2.5 mM of $MgCl_2$,1 µM of each primer, 250 µM of dNTPs, 1.25 U *Taq* DNA polymerase and 100 ng of DNA template.Two

amplification profiles of multiplex reactions were used.The first profile for primers Hae F/Hae R, LytA F/LytA R, sa442 F/sa442 R and Mip F/Mip R began with initial denaturation at 95ºC for 30s followed by 40 cycles of denaturation at 94ºC for 15s, annealing at 50ºC for 30s and extension at 75ºC for 30s and then final extension at 72ºC for 10 min. Another profile for the other set of primers including Ply F/Ply R, Pf/Pr2, PA1/PA2 was initial denatured at 94ºC for 10 min followed by 35 cycles of denaturation at 94ºC for 30s, annealing at 57ºC for 20s and extension at 72ºC for 20s before final extension at 72ºC for 10 min. The PCR products were examined by agarose gel electrophoresis and ethidium bromide staining.

3. Results and Discussion

3.1 Microbial diversity and density

From twenty eight air samples of six groups of sampling locations, all were positive for microbial contamination at different levels. The investigation was performed both for total microbial contamination and for seven specified pathogens. Evaluation of total contamination by using conventional culture method showed the average total count on blood agar of large lecture rooms at 3.4 CFU plate^{-1} h^{-1}, small lecture rooms at 5.2 CFU plate^{-1} h^{-1}, meeting rooms at 4.3 CFU plate^{-1} h^{-1}, office rooms at 3.0 CFU plate^{-1} h^{-1}, canteen rooms at 31.5 CFU plate^{-1} h^{-1} and service vans at 5.5 CFU plate^{-1} h^{-1}. The comparison of total aerobic microbial count of those six location groups was shown in Figure 1 and the level of microbiological air quality according to the Standard Index of Microbial Air Contamination (IMA) classes was shown in Table 2. Figure 1 showed the variation of total aerobic microbial count both among location groups and within the same group. The results showed that canteen areas were more contaminated than others, this may depend on many factors such as the number of occupants, their activities, physical factors, sources of contaminants and cleaning practice. It was also found that more contamination was observed in more crowded locations of canteens. So this will be useful data for further improvements. Characterization of microbes demonstrated both Gram-positive and Gram-negative bacteria including some molds. In total of twenty eight samples sites the rates of finding of bacterial genera frequently isolated were *Staphylococcus* spp. (62.50%), *Micrococcus* spp. and *Kytococcus* spp. (58.33%), *Bacillus* spp. (41.64%), *Moraxella* spp. (29.17%), C*orynebacterium* spp. (25.00%) and *Pseudomonas* spp. (20.83%). Figure 2 showed the prevalence rate of each bacterial group in all air samples. These bacteria are generally found in human body and environment. Two of the most prevalent bacteria were *Staphylococcus* spp. and *Bacillus* spp. This corresponded to the fact that *Staphylococcus* spp. naturally lives on skin and surfaces of human and animals whereas *Bacillus* spp. is commonly found in environment such as in air, soil and water and can also be found in human and animals.Thus, it corresponds to the mention that general human activities and shedding of skin can be very large sources of bioaerosols, airborne particles and microbes [2, 9, 23-27]. Investigation of microbiological contamination of indoor air will reflect the quality of indoor air upon utilization by occupants.

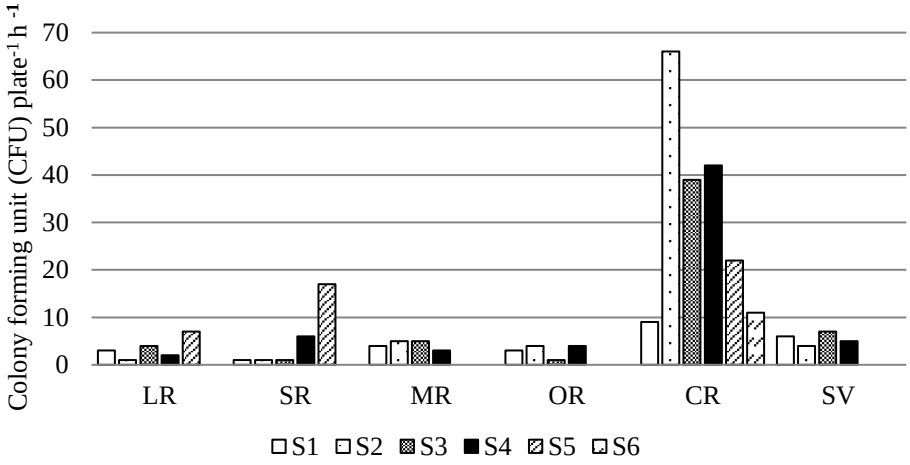

Figure 1. Total aerobic microbial load of indoor air samples from different location groups: Large lecture rooms (LR), Small lecture rooms (SR), Meeting rooms (MR), Office rooms (OR), Canteen rooms (CR) and Service vans (SV). S1-S6 represent contamination level of sampling location 1, sampling location 2, sampling location 3, sampling location 4, sampling location 5, and sampling location 6 within the group

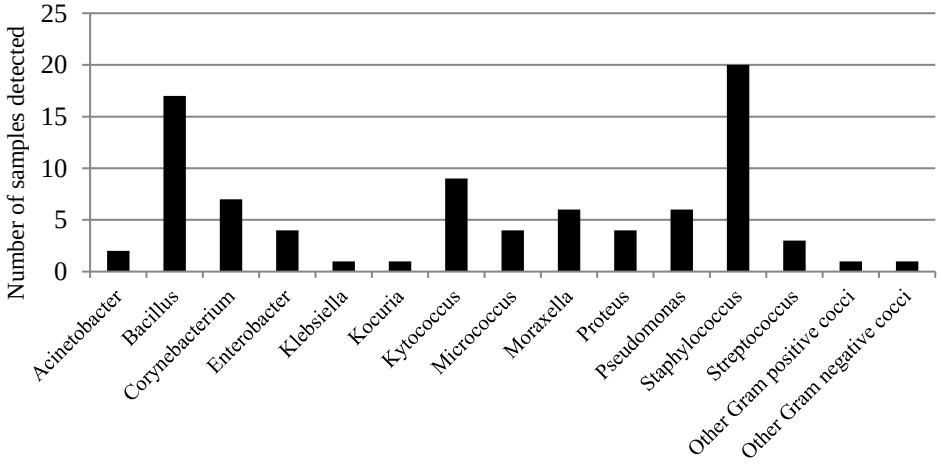

Figure 2. Prevalence rate of aerobic bacterial species in twenty eight air samples of six different sampling locations

Table 2. Microbiological air quality of the six sampling locations according to the Standard Index of Microbial Air Contamination (IMA) classes [17]

Sampling locations	Microbial count (CFU/dm^2/h)	Air quality level (IMA classes)
Large lecture rooms	5.86	Very good (0-9 CFU/dm^2/h)
Small lecture rooms	8.96	Very good (0-9 CFU/dm^2/h)
Meeting rooms	7.33	Very good (0-9 CFU/dm^2/h)
Office rooms	5.17	Very good (0-9 CFU/dm^2/h)
Canteen rooms	54.31	Fair (10-84 CFU/dm^2/h)
Service vans	9.48	Good (10-39 CFU/dm^2/h)

3.2 Detection of specific pathogens by multiplex polymerase chain reaction

Multiplex polymerase chain reaction (mPCR), which is a molecular method, was used to supplement the conventional methods used to evaluate indoor air microbiological quality. As shown in Table 1, seven primers pairs were used to detect seven important pathogens in three PCR reactions with proper optimization for good product at expected length.

Figure 3 showed the electrophoresis result of PCR amplification after proper optimization of DNA from *Haemophilus influenzae* using Hae F/Hae R primers (Panel A), *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* using Ply F/Ply R, Pf/Pr2 and PA1/PA2 primers as multiplex PCR (Panel B) and *Streptococcus pyogenes*, *Staphylococcus aureus* and *Legionella pneumophila* using LytA F/LytA R, sa442 F/sa442 R and Mip F/Mip R as multiplex PCR (Panel C). The length of amplified products specific to each organism was indicated in the illustrations.

By using the mPCR method, *Staphylococcus aureus* and *Streptococcus pyogenes* were detected at 16.67% and 4.17% respectively, of total air samples. The locations of these positive samples were in one small lecture room, one meeting room, two office rooms and one canteen room whereas the conventional method did not detect these pathogens in the same air samples. This corresponded to the fact that the culture method which is known as gold standard method for microbial detection still has some limitations, for example in case of fastidious organisms, viable but nonculturable state and non viable state of microorganisms [28]. *Staphylococcus aureus* and *S. pyogenes* were not observed in the culture process so they were not included in the rate of detection using culture method. This suggested that mPCR could be a helpful tool for detection and monitoring of contaminants since the present of DNA will reflect the presence of that microbe in the area investigated.

Staphylococcus spp. was also reported to be commonly isolated from the air in residential and commercial building [29-33]. It was mentioned that characterization of airborne bacteria can be helpful to determine the source of contaminants and to understand the transmission of microbes from person to person [32-35]. This makes the determination of indoor air contaminants be very important for contamination control.

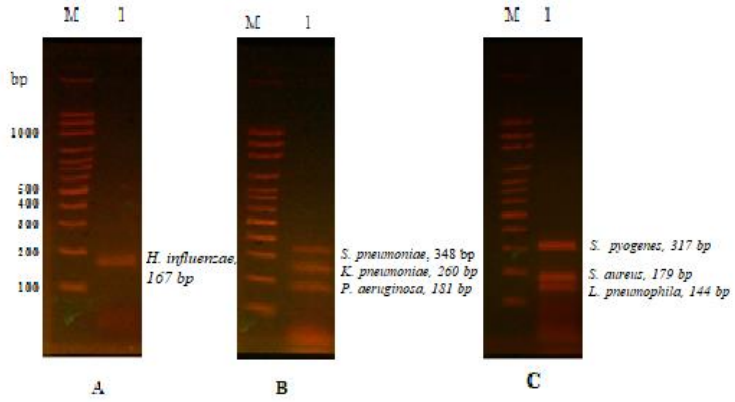

Figure 3. Optimization of mPCR for detection of seven bacterial pathogens as shown by 2% agarose gel electrophoresis of amplification products. (A) *Haemophilus influenzae*, Hae F/Hae R primers, (B) *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, Ply F/Ply R, Pf/Pr2 and PA1/PA2 primers, (C) *Streptococcus pyogenes*, *Staphylococcus aureus* and *Legionella pneumophila*, LytA F/LytA R, sa442 F/sa442 R and Mip F/Mip R primers. M: Molecular size marker, lane 1 in each panel was from PCR amplification

4. Conclusions

The results demonstrated that despite the conventional culture method for determination of microbial contaminations of indoor air, the using of mPCR method can provide early detection of some important pathogens and will lead to early actions to prevent occupants from infections. The mPCR technique was a useful supplement to conventional methods to determine indoor air microbiological quality and should be introduced to indoor air quality monitoring.

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