Chapter 60

Multiplex PCR Kit for Respiratory Bacteria

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Abstract

Common bacteria associated with respiratory tract infections (RTIs) are *Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Mycobacterium tuberculosis* and *Haemophilus influenzae*. The high rate of worldwide morbidity and mortality due to RTIs indicates the importance of established rapid and accurate diagnostic tests, not only for providing early efficient treatments, but also to monitor the diseases tendency. However, the Gold standard culture method requires two to five days to fully identify the organisms, which thus delaying the identification of the etiological agents. Therefore, this invention aimed to provide a rapid, sensitive and specific dry-based polymerase chain reaction (PCR) kit incorporated with internal control for the detection of *K. pneumoniae, S. aureus*, *S. pneumoniae, P. aeruginosa, M. tuberculosis* and *H. influenzae*, in a respiratory sample. This invention offers simplified detection method, in which at least one and up to six bacterial pathogens can be detected in a single tube assay by adding the extracted DNA sample to the assay tube. It is also a dry-based PCR reagent, which is thermal-stable at ambient temperature. Thus, this assay will enhance the user's convenience and reducing cost by omitting the use of cold-transport or storage. This invention will be helpful in the management of communicable RTIs, especially under mass gatherings and other challenging conditions. It also has advantages on its rapidity, simplicity, robustness performance and storage condition, which are important criteria for a diagnostic application during Hajj and also in rural, famine and disaster area.

Introduction

Due to the global health awareness and the emergence of "superbugs", in parallel with the need of updated bacterial surveillance system, ideal methods for pathogen detection and identification is truly crucial. Giving example of the burden of respiratory tract infections (RTIs), the high rate of worldwide morbidity and mortality indicates the importance of established rapid and accurate diagnostic tests, not only for providing early efficient treatments, but also to monitor the diseases tendency (Nweze *et al.*, 2012). RTIs can be classified into two major classes based on the site of infections; i) the upper RTIs, i.e., pharingitis, laryngitis, sinusitis, colds, influenza, whooping cough and throat infection, and ii) the lower RTIs, including bronchitis, bronchiolitis and pneumonia, whereby the causal pathogens are mostly bacteria. Although less common in populations, the lower RTIs are more severe and are more likely to cause morbidity and mortality (Bellos *et al.*, 2010), especially during infancy and late-adulthood.

Bacterial respiratory infections are normally associated with a greater risk of death as compared to viral infections, which commonly associate with the upper respiratory tract, are self-limiting, milder and have shorter recovery period (Dasaraju and Liu, 1996). However, when the body immune system is weakened by viral infections, this may lead to secondary bacterial infections that could be more severe. Most of the fatality and severe illness episodes of RTIs are due to pneumonia and other acute lower RTIs. Around 4.2 million deaths of lower RTIs occurred worldwide among all age groups; with 1.8 million of these are children between age 1 to 59 months (WHO, 2008). The commonest bacteria associated with RTIs are *Haemophilus influenzae, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pneumoniae and Pseudomonas aeruginosa*

(Bosch et al., 2013). *S. pneumoniae* and *H. influenzae* are frequently isolated in adult community-acquired pneumonia (Macfarlane *et al.*, 1993). In contrast, *P. aeruginosa*, *S. aureus* and *K. pneumoniae* have being the common pathogens of hospital-acquired pneumonia (Macfarlane *et al.*, 1993).

The majority of RTIs are poorly documented due to the limitations of conventional diagnosis such as sputum Gram-staining and culture, blood counts and serological tests. The conventional bacterial culture followed by confirmation via biochemical tests have being used as the gold standard in current RTIs diagnoses. The method however requires two to five days to fully identify the organisms, which thus delaying the identification of the etiological agents. The delay may urge for the use of broad-spectrum antibiotics, which indirectly results in potentially unnecessary costs, adverse medication effects, and the emergence of multidrug-resistant bacteria. Therefore, rapid and accurate detection of bacteria pathogens from patients with RTIs is important towards effective treatment and prevention of the disease

Detection of respiratory bacteria using Multiplex PCR Kit

The disclosed invention of Multiplex PCR Kit for Respiratory Bacteria provides rapid, sensitive and specific molecular method using polymerase chain reaction (PCR) for detecting the presence of target bacteria in a respiratory sample. For example, a respiratory sample such as sputum, can be analyzed for the presence of DNA from *K. pneumoniae, S. aureus*, *S. pneumoniae, P. aeruginosa, M. tuberculosis* and *H. influenzae*, based on the specific PCR primers designed for this test.

In details, the single-tube of Multiplex PCR Kit for Respiratory Bacteria comprises all basic components for a PCR, such as PCR buffer, magnesium chloride ($MgCl_2$), deoxynucleotides (dNTPs), *Taq* DNA polymerase enzyme and seven sets of primers in a single tube. The PCR components have been optimized and thermostabilized as dried-based PCR reagent. As such, it is a simple and ready to use, whereby the user will just need to add a small amount of water to rehydrate the reagent and consequently add the sample to the tube. The PCR tube is then placed in a thermocycler machine for DNA amplification. The user will analyze the result via gel electrophoresis by simple presence or absence of the target DNA bands.

Detection of the target bacteria in a sample involves initial bacterial cell lysis to expose the cell lysate containing DNA. In example, DNA can be extracted rapidly using commercialized kits for their promising quality in purity and yield. In contrast, DNA can be extracted using conventional methods such as simple boiling method, chemical-based methods such like phenol-chloroform method (Cheng and Jiang, 2006) and chelex-based procedures (Giraffa et al., 2000). Following bacterial DNA extraction, hybridization of any primer pair to the presence target DNA under particular conditions, will directing the target DNA amplification. This can be achieved by using a thermocycler with the cycling condition as shown in Table 1.

Table 1
Thermal cycling program for Multiplex PCR Kit

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 minutes	1X
Denaturation	95	30 seconds	30X
Annealing	58	30 seconds	
Elongation	72	30 seconds	
Final elongation	72	5 minutes	1X

a. Implementation procedure

Implementation procedure of Multiplex PCR Kit is described below:

Step 1: Collect the respiratory sample (i.e. sputum, throat swabs, bronchoalveolar lavage, nasopharyngeal aspirate) into a sterile container.

Step 2: Perform DNA extraction from the sample by using any suitable methods as described above. DNA can be extracted using as simple as boiling method that consumes around 20 minutes. Other methods such like extraction kits usually take about two hours.

Step 3: For the samples, add 18 microliter (µl) of water to each PCR tube to rehydrate the dried-based reagent. Add 2 µl of extracted DNA sample from Step 2 and mix briefly. For the controls, add 2 µl of Positive Control provided in the kit to a PCR tube to serve as positive control. Add 2 µl of water to another tube to serve as negative control. Enter the tubes to a thermocycler pre-set with the recommended cycling program. The PCR amplification will take around 1 hour and 30 minutes.
Step 4: Once the amplification has completed, load directly the PCR product into the wells of an agorose gel of 1.5 to 2% concentration. View the result after one hour of electrophoresis.

b. Benefits of the invention

In addition to the lengthy bacterial culture method, it is also well noted that setting up monoplex PCR reactions for detecting different organisms at one time are tedious. Using this kit, PCR for clinical diagnosis can be run as many as possible by simply adding the respective extracted DNA sample to the tubes. As a pre-optimized multiplex PCR kit, this assay will eliminate the requirement of tedious optimization and calculation, as well as minimize the pipetting steps, such like in conventional PCR. Hence, it may shorten the duration of a PCR set up and reduce contamination. The results can be obtained in approximately four to six hours, starting from the DNA sample preparation to the final gel interpretation.
Moreover, the multiplex PCR will allow the detection of more than one of those bacteria simultaneously, which is important for the diagnosis of patients infected with more than one bacterial pathogen. The molecular-based multiplex PCR assay will provide accurate and rapid detection results as compared to the conventional culture method, which therefore suitable for clinical diagnosis.
Apart from the credits of a PCR assay, most of the PCR reagents conversely require cold chain transportation and cold storage, due to their heat sensitivity. Thus, there will be additional expenses for packaging and storage, plus inconvenience in reaction setting up, down to the freeze-thawing procedure. Out of these, degradation of the reagents' stability will be the major problem because this will lead to inconsistent and irreproducible results. To overcome the limitations, the invention is developed as a thermostabilized multiplex PCR kit, which is stable at ambient temperature, thereby require no conventional cold transport or storage and ready for immediate use.
The invention of this PCR kit is clinically relevant in the diagnosis and treatment of both upper and lower RTIs, especially during a massive gathering situation, such like Hajj pilgrimage that requiring quick decision making and knowledge of the etiological agent. With the restricted duration of Hajj, pack and in-motion pilgrims, outfit laboratories and such inventions are indeed necessary for the rapid diagnosis and identification of the causal pathogens to minimize the risks of death, morbidity, microbial resistance to drugs and prolonged length of hospital stay due to ineffective therapy. Thus, the pre-optimized, dried reagent-based PCR will be helpful in the management of communicable RTIs, especially under mass gatherings and other challenging conditions. This invention will have advantages on its rapidity, simplicity, robustness performance and storage condition, which are important criteria for a diagnostic application during Hajj and also in rural, famine and disaster area.

Conclusion

The invention of Multiplex PCR Kit for Respiratory Bacteria may accelerate the detection of commonest respiratory bacteria based on its simplicity, rapidity, sensitivity and specificity. The kit allows kit allows simultaneous detection of *K. pneumoniae, S. aureus*, *S. pneumoniae, P. aeruginosa, M. tuberculosis* and *H. influenzae* in a single tube reaction.

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