African Journal of Microbiology Research Vol. 7(8), pp. 698-700, 19 February, 2013
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1248
ISSN 1996-0808 ©2013 Academic Journals

Short Communication

Development of a multiplex polymerase chain reaction (PCR) with an internal control method to detect *Yersinia pestis* in the plague foci surveillance

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Accepted 7 February, 2013

To detect *Yersinia pestis* in the foci surveillance rapidly and specially, we developed a multiplex polymerase chain reaction (PCR) with an internal control technique. The specific segments of *Yesinia pestis* were co-amplified by PCR directly from the hosts' organ samples with an internal control to prevent false negative results that might be caused by PCR inhibitors. Comparing the PCR results with that of the bacteria culture, the positive detection rate of PCR method was higher and it was more rapid. So, it is a useful method for the detection of *Yesinia pestis* in the plague foci surveillance.

Key words: multiplex polymerase chain reaction (PCR), internal control, *Yersinia pestis*.

INTRODUCTION

Plague, transmitted primarily by fleas, is one of the most devastating acute contagious diseases. It has caused three worldwide pandemics and killed millions of people in history (Sherman, 2006). The causative agent of plague is *Yesinia pestis*. It is also considered to be a potential bioweapon. There are 12 plague natural foci that covers 19 provinces in China. However, we still used the traditional techniques including indirect hemagglutination, reverse indirect hemagglutination test and bacteria culture methods to monitor the plague cases; they are time-consuming and less sensitive. It is necessary to develop a rapid and special technique to detect *Yersinia pestis*. We know that the PCR technique has been widely used, especially to detect the pathogenic microbe; it is rapid and sensitive (Erlich et al., 1991). So, we developed a multiplex PCR method to detect the *Yersinia pestis* directly from the samples of the hosts' organs. The F1 capsular antigen is the virulence determine factor and one of the special antigens of *Yersinia pestis* (Brubaker, 1972) and the Pla gene coding for the plasminogen activator is also *Yersinia pestis*-specific gene (Lähteenmäki, et al., 2001). Several researchers have used these genes as PCR target genes (Tsukano et al., 1996; Woron et al., 2006; Janse et al., 2010). We also take these two genes as our PCR target genes. PCR always arise false positive or negative results. False negative results is serious for the disease such as plague and this may be potentially life threatening. In order to avoid false negative results, we incorporated internal control to the PCR assays. The internal control was designed in such a way that the same primer pair was used to amplify the internal control and the target DNA which were differentiated by size. This may have important implications for clinical and public health investigations, especially if the investigations involve food and environmental screening.

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MATERIALS AND METHODS

According to the ecotypes of the *Yersinia pestis* and the conditions of the plague monitoring sites, we selected 17 plague monitoring sites distributed in 14 provinces in China to test this technique in the

Table 1. Results of the PCR and bacteria culture.

Culture	PCR		
	Positive	**Negative**	**Total**
positive	95	18	113
negative	167	2244	2411
total	262	2262	2524

foci surveillance, and we also used the bacteria culture method at the same time to compare it. 2524 *Yesinia pestis* hosts' liver samples were collected from the 17 plague monitoring sites. These hosts included *Meriones Unguieulataus*, *Rattus flavipectus*, *Marmot*, *Apodemus agrarius Pallas*, *Rhombomys opimus*, *Microtus. Brandti* and so on. The samples were tested with PCR and bacteria culture methods. We identified the bacteria with *Yersinia pestis* bacteriophage. The PCR primers were F1F 5'-GGAACCACTAGCACATCTGTT-3'; F1R 5'-ACCTGCTGCAAGTTTACCGCC-3'; PlaF5'-ACTACGACTGGATGAATGAAAATC-3'; PlaR5'-GTGACATAATATCCAGCGTTAATT-3'. PCR reactions contained 2.5 µl of 10×PCR buffer, 1.5 µl of 20 mM $Mgcl_2$, 2 µl of 2.5 mM dNTP, 2 µl of 10 pmol/µl primers, template DNA, 1 µTaq polymerase, and 25 µl water sterile distilled was added. Thermal cycling were performed with an initial denaturation step of 5 minat 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and finally 5 min at 72°C. The internal control was co-amplified to prevent false negative results that might be caused by PCR inhibitors. It was designed in a way that the same pair of primer F1 was used to amplify the target DNA and co-amplify the internal control which was constructed by inserting an amplicon to the original target DNA between the two primer sites to produce an internal control longer than the target DNA. We tripsised the liver samples and added appropriate distilled water, and then boiled them in water-bath for 10 min. After that, they were centrifuged and the supernatant was taken as PCR template.

RESULTS AND DISCUSSION

The 2524 hosts' liver samples were tested with PCR and bacteria culture methods. The number of positive results of PCR was 262 and the number of positive results of bacteria culturewas 113 (Table 1). The Positive coincidence rate of the two methods was 92.67%. We concluded that the positive detection rates of these two methods were different, and the positive detection rate of PCR (10.38%) was higher than that of the bacteria isolation (4.48%). (χ^2=682.25, P<0.005).

When plague cases occur, it must be judged correctly and rapidly. After this, we can adopt the corresponsive measurements to deal with it and effectively prevent its dissemination from rodents to human to reduce the public health impact of such highly pathogenic micro-organisms. The traditional techniques that were used to monitor the plague in the foci surveillance in our country included indirect hemagglutination, reverse indirect hemagglutination test and bacteria culture method. Although bacteria culture method was very sensitive, these methods are time consuming, not very specific, involve extensive biosafety measures and some organisms simply resist cultivation. We need to find a rapid and convenient technique. We know that the *Yersinia pestis* culture need at least 2 to 4 days, while detecting it with PCR only need 4 to 6 h. It is much faster than the bacteria culture and is suitable for the rapid diagnosis. Polymerase chain reaction (PCR) methods for detecting *Yersinia pestis* are well documented (Leal and Almeida, 1999; Radnedge et al., 2001), including real-time PCR methods (Janse et al., 2010; Tomaso et al., 2003; Woron et al., 2006; Matero et al., 2009). However, the instruments and reagents used in real-time PCR methods are expensive and it is difficult to develop universally. There are few studies that have applied a multiplex PCR with an internal control to detect the *Yersinia pestis* directly from the hosts' organ samples. We developed this technique because the most obvious origin of PCR inhibitors in endogenous contamination is the compounds present in insufficiently purified target DNA, so we use an internal control to co-amplify. The use of an internal control not only enable the detection of a strong inhibitor within the reaction but also avoid false negative results caused by the action of mild inhibitors that may interfere with specific DNA templates and primer pair interactions (Brightwell et al., 1998).

The multiplex PCR with an internal control method has been proven to be a sensitive, specific, and rapid method for the detection of *Yersinia pestis* in our study, and it is also cost-effective. So it is useful and reliable for the detection of *yersinia pestis* in the foci surveillance.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Special Fund for Health Sector, the People's Republic of China (Award 201202021) and a grant from the national technology key special study program of the twelfth five-year plan (Award 2011zx10004-001).

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