STUDY OF PATHOGENIC BACTERIA DETECTED IN FLY SAMPLES USING UNIVERSAL PRIMER-MULTIPLEX PCR

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ABSTRACT

Filth flies, especially house fly, Musca domestica L., not only is a nuisance pest, but also acts as an important mechanical vector for pathogenic microorganism agents, including bacteria, protozoa, worms, fungi and viruses amongst humans and animals. More than 100 pathogens are associated with the house fly and bacteria have been isolated from feces, vomits, external surfaces, and internal organs of this species (De Vos V, et al., 1998; Dragon, DC, 1995; West, 1951; Markus, 1980; Kasprzak et al, 1981; Akinboade et al., 1984; Iwasa et al., 1999).

The aim of this study was to detect pathogenic bacteria from house fly by UP-M-PCR. In this study, totally 267 house flies were collected and we tried to find a procedure enabling the detection of three pathogens namely, Escherichia coli, Listeria monocytogenes, Salmonella spp and employed for multiplex PCR analysis in house fly. The most common isolated bacteria were L.monocytogenes (132 cases: 49.4%) and another isolated bacteria belong to E. coli (114 case: 42.6%) and Salmonella spp (98 cases: 36.7%). The results of the current study confirm that flies are much more than a nuisance and that they pose potentially serious health risks. The epidemiologic potential of house flies to disseminate pathogenic bacteria may be greater than initially suspected.

KEY WORDS: *Musca domestica, Escherichia coli; Listeriamonocytogenes, Salmonella spp*

INTRODUCTION

The house fly, *Musca domestica L.* (Diptera: Muscidae), is recognized as an important factor in the dissemination of various infectious diseases such as cholera, shigellosis, and salmonellosis. They can also serve as a cross-contamination vector for other food-borne pathogens (Antonio *et al*., 2003). Food borne pathogens are a growing concern for human illness and death. There is continuous development of methods for the rapid and reliable detection of food borne pathogens. *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. are 3 kinds of the mostimportant pathogens spreading through various foods, afflictingpeople worldwide (Chen *et*

al., 2000; Jay 2004;Gillespie *et al.,* 2005; Olsen *et al.,* 2005).

Multiple PCR has potential to detect out multiple pathogenic contaminants simultaneously (Bhagwat 2003). It has been used to detect and identify 1 organism by amplification of more than 1 gene or multiple organisms by targeting different unique sequence of each organism simultaneously (Fratamico 2001), which has also been used to detect *E. coli*, *L. monocytogenes*, and *Salmonella* spp. (Kim *et al.,* 2006; Mukhopadhyay et al., 2007). In this study, we used universal primer-multiplex method to detect the 3 pathogens from house fly.

MATERIALS & METHODS

Study area and sampling of flies

Flies were *Musca domestica* according to standard criteria by the use of taxonomic keys. House flies were captured either by using a fly killer, and frozen with -30 degrees.

Fly DNA extraction

Total genomic DNA was extracted from individual house fly using the Genome DNA Extraction kit. The genomic DNA of pathogens, and carrying flies was extracted by homogenizing the cells or organisms with a plastic homogenizer in 200 µl A Buffer A (0.1 M Tris, pH 9.0; 0.1 M EDTA; 1% SDS; and 0.5% DEPC) and incubating the homogenate for 30 min at 70°C. Next, 44.8 µl of 5 M KoAc was added, and the mixture was cooled for 30 min on ice. After centrifugation at 20,000 g for 15 min at 4°C, the DNA-containing 180 µl supernatant was transferred to a new tube and mixed with 90 µl isopropanol. The solution was centrifuged at 20,000 g for 20 min at 4°C, and the precipitated DNA was collected, rinsed with 70% ethanol, and dried. Each DNA pellet was diluted in 60 µl TE.

Universal primer-multiplex PCR (UP-M-PCR)

Primers

Specific primers for E. coli706-F/R, LM440-F/R, and Sal320-F/R were used to detect the *E. coli*, *L. monocytogenes*, and *Salmonella* spp, respectively (Yanfang Y et al., 2009). Details of primer sequences are shown in Table 1.

Table 1

Primer details

Bacteria	Primer name	Sequence	Product (bp)	Reference
E. coli	E. coli706-F	CCTTCCTTCCTTCCCCCCACCTGC GTTGCGTAAATA	706	Yanfang Y. et al., 2009
	E. coli706-R	CCTTCCTTCCTTCCCCCCGGGCGG GAGAAGTTGATG		
L .monocytogenes	LM440-F	CCTTCCTTCCTTCCCCCCATCATC GACGGCAACCTCGGAGAC	440	Yanfang Y. et al., 2009
	LM440-R	CCTTCCTTCCTTCCCCCCCACCATTCCC AAGCTAAACCAGTGC		
Salmonella spp.	Sal320-F	CCTTCCTTCCTTCCCCCCGTGAAATTAT CGCCACGTTCGGGCAA	320	Yanfang Y. et al., 2009
	Sal320-R	CCTTCCTTCCTTCCCCCCTCATCG CACCGTCAAAGGAACC		
Universal primer	UP	CCTTCCTTCCTTCCCCCC		Yanfang Y. et al., 2009

PCR condition

In this study, the optimized UP-M-PCR system contained 54 mM $MgCl_2$, 200 μmol/l dNTPs and 500 units of Taq DNA polymerase (Biolabs, UK), 300 nmol/l universal primer, 0.5 mM of each compound specific primer. All the PCR reactions were conducted in a 30 μl reaction volume. The final thermal cycling program included an initial 5 min denaturation at 94°C and then 35 cycles of 30 sec at 94°C, 30 sec at 56°C, and 50 sec at 72°C, followed by a final extension for 10 min at 72°C. 20 μl of PCR products were separated by electrophoresis in 20 g/l agarose in TAE. The gels were stained with ethiduim bromide for examination under UV light.

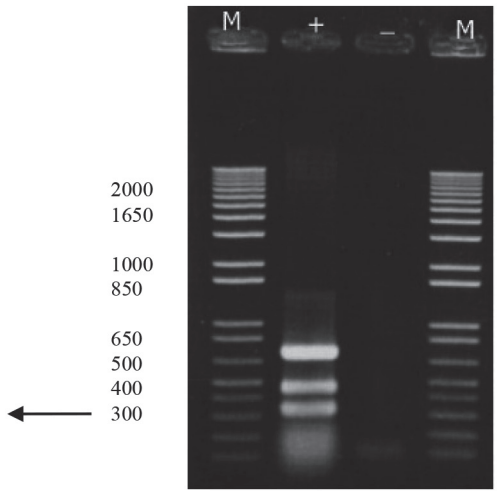
RESULTS

Fly DNA isolation

We extracted a total of 340 DNA (from 360 house flies) as samples. The extraction of the adequate amount of the pure total DNA is a basic requirement in PCR based detection. The purity of the extracted DNA of flies was also in the acceptable range. The ratios of the absorbance at 260 nm to the absorbance at 280 nm were in the range of 1.6 to 1.8 for all extracted DNA samples.

Optimization of PCR condition and Primers

The multiplex PCR method has been used by many scientists to detect different types of pathogenic microorganisms in food and water (Pham et al. 2007; Petra et al. 2007). The specificity of primers used to detect target gene is very important in the detection of pathogenic organisms from sample which could contain a mixture of microorganisms, and this is a major concern in establishment of multiplex PCR protocols. The primers used in this study were tested for their specificities. In this study, Taq DNA polymerase (from Biolabs, UK) and master-mix was used with the optimum conditions based on the reported applications of the same primers (Yanfang Y *et al.,* 2009). The used primers (Table 1) were able to amplify successfully the target genes of the extracted DNA from fly samples (Pic.1), so that no non-specific band was observed in gel electrophoresis.



Picture 1. Detection of universal primer triplex PCR. Bands 706, 440, and 320 bp represented the amplification of target gene. M, 1kb DNA marker; (+), amplification of target gene from E. coli, L. monocytogenes, and Salmonella spp.

Bacterial DNA extraction for positive control

The bacterial DNA extracted from strains, *E. coli* (DH5α-GFP), *L. monocytogenes* (10403c) and *S. typhimurium* (SL1344) respectively, were reserved in our laboratory. Three bacteria separately were able to produce bands of 706 bp, 440 bp and 320 bp for amplification of target gene from of *E. coli*, *L. monocytogenes,* and *Salmonella* spp respectively, while the bacterial DNA extracted from bacterial strains of all of the three bacteria produced the all above three bands (Figure 1). These results indicated the suitability of the above primers in a UP-M- PCR for the simultaneous detection of three pathogens, *E. coli*, *L. monocytogenes,* and *Salmonella* spp. Application of the UP-M-PCR assay for detection of *E. coli*, *L. monocytogenes,* and *Salmonella* spp resulted in successful amplification.

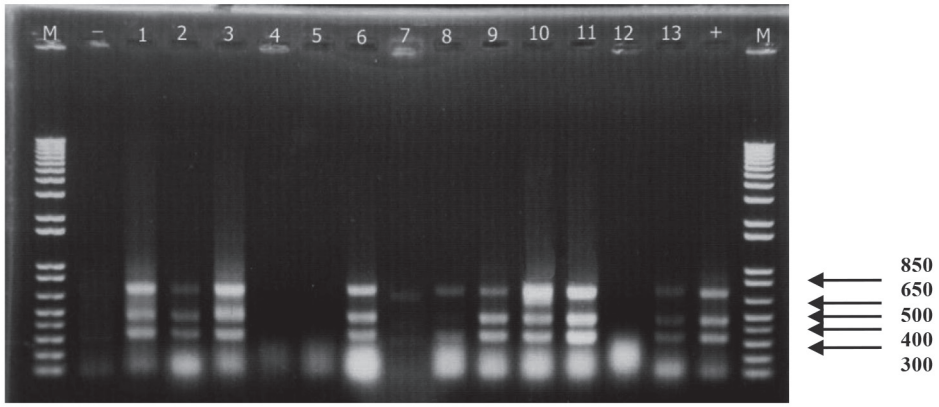
Detection of pathogenic bacteria from house flies by UP-M-PCR

We analyzed a total of 267 fly samples. The most common isolated bacteria were *L.monocytogenes* (132 cases: 49.4%), and anotherisolated bacteria belong to*E. coli* (114 cases: 42.7%), and *Salmonella spp* (98 cases: 36.7%) (Table 4, Pic.2-3).

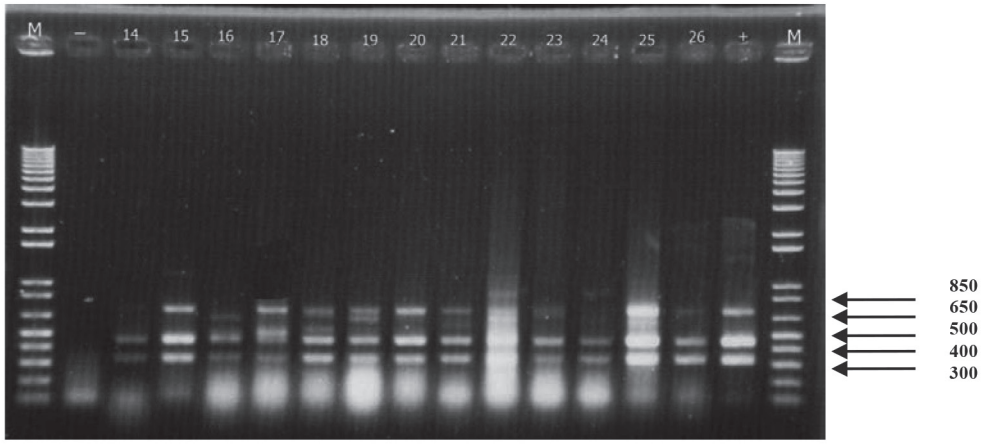
Table 4

Isolated bacteria species

Bacterial species	Number of positive samples	Percentage of positive samples
E. coli	114	42.6
L. monocytogenes	132	49.4
Salmonella spp	98	36.7



Picture 2. Detection of universal primer triplex PCR. Bands 706, 440, and 320 bp represents the amplification of target gene from *E. coli*, *L. monocytogenes*, and *Salmonella* spp., respectively. M, ladders of DNA Marker (1kb); (-), negative control; 1-13, UP-M-PCR with templates and (+), positive control.



Picture 3. Detection of universal primer triplex PCR. Bands 706, 440, and 320 bp represents the amplification of target gene from *E. coli*, *L. monocytogenes*, and *Salmonella* spp., respectively. M, ladders of DNA Marker (1kb); (-), negative control; 14-26, UP-M-PCR with templates and (+), positive control.

DISCUSSION

Previous studies have shown that filth flies can disseminate viable pathogens such as *Helicobacter pylori* (Grubel *et al.,* 1997), *Salmonella* (Greenburg 1965), and *E. coli* O157 (Kobayashi *et al.,* 1999) to other substrates. We identified *E. coli* on 42.6% (114 cases) of the analyzed fly samples.

Other studies have shown that infection of flies by *Salmonella*could be external as well as internal. Sulaiman *et al.,* (2000) isolated a variety of pathogenic organisms from the gut of flies including *M.domestica*. The findings of their study indicate that *M.domestica* can transmit *Salmonella* and *Shigella* species showing that dirty environments can easily attract flies which subsequently deposit pathogenic organisms on food and water. The more evident to find the role of house flies on the

transmission of bacteria is using different insect species and different methods of transmissions.

Universal Primer-Multiplex PCR has been used successfully for rapid detection of *Escherichia coli, Listeria monocytogenes*, and *Salmonella* spp. in food samples (Yanfang et al., 2009). This type of PCR testing is intriguing to the food industry because of its reduction in labor and reagent cost as well as a reduction in testing time for multiple bacterial pathogens (Gasanov*et al.,* 2005)

In the present study, we tried to find a procedure enabling the detection of tree pathogens namely, *E. coli*, *L.monocytogenes*, *Salmonella* spp. and employed for Universal Primer-Multiplex PCR analysis in house fly. It has not yet been investigated in this study whether or not these organisms were carried externally or internally. All detected bacteria from *M. domestica* in the current study are pathogenic. These findings agree with the previous reports of Alam & Zurek (2004), in which *E. coli* O157:H7 was detected in both farm and urban environments and Greenburg (1965), Kobayashi *et al.,* (1999) and Moriya *et al.,* (1999).

In conclusion, we developed an easy and rapid Universal Primer-Multiplex PCR showing high specificity capable of detecting three bacteria. Furthermore, this multiplex PCR can be used to investigate other pathogens in fly.

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