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NOTE

Determination of Enteric Bacteria at Microbiologically Risky Points by Multiplex Polymerase Chain Reaction

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The purpose of this research was to test multiplex polymerase chain reaction in investigating the microbiological quality of the risky surfaces in social living places of a military base where over 15 thousand people live together. In 22 samples of 99, there were no bacteria. Only four of the samples contained *Shigella*, and one of them contained *Salmonella*, but 77 of the samples contained thermotolerant coliform organisms. There was no statistically significant difference among the microbiological quality of different sites and different equipment surfaces ($p>0.05$).

Key words: swab, coliform organisms, multiplex polymerase chain reaction, surface contamination

Thermotolerant (fecal) coliforms and *Escherichia coli* are organisms which have been used to investigate the microbiological quality of water and food sources (Conway, 1998). These organisms could also be used to determine microbiological quality of the surfaces of equipment and living places (Natsiashvili, 1970; Schreiber, 1971; Slavin, 1973)

Studies of the contamination level of the surfaces are generally performed in order to determine efficacy of the cleaners which contain disinfectants or to determine the source of an outbreak. In this kind of study, a swab technique for sampling and total bacterial count for evaluation are commonly used (Spicher and Peters, 1976; Pfeiffer *et al.*, 1978; Keswick *et al.*, 1983; Scott *et al.*, 1984; Borneff, 1986; Mafu *et al.*, 1990).

The swab method is one of the most common methods used to investigate the contamination level of surfaces (Pfeiffer *et al.*, 1978; Keswick *et al.*, 1983; Mafu *et al.*, 1990). After collection of the samples from investigated surfaces, one must use appropriate media to culture microorganisms. In order to isolate microorganisms exactly we need more special methods. This isolation process is time consuming.

The polymerase chain reaction (PCR) technique has been commonly used to detect microorganisms in microbiology. Multiplex PCR is a method which you could detect several microorganisms or other nucleic materials by using corresponding primer sets in the same reaction. Especially when used to detect bacteria, the complete procedure could be as short as three or four hours. By using PCR and multiplex PCR, we also could detect "viable but nonculturable (stressed) microorganisms" which can not be isolated by classical culture methods (Roszack and Colwell, 1987).

The purpose of the present study was to test multiplex PCR in order to investigate the microbiological quality of the risky surfaces in social living places of a military base where over 15 thousand people live together. In this way we also intended to evaluate hygienic activities performed in a public living place by PCR.

Study Sites and Sample Collection

Ninety-nine common living places in a military facility were examined in this study. The study type was descriptive. Primarily, the surfaces of taps, dining tables, door handles, equipment from kitchens and dining halls and soap and soap dishes were investigated. To collect samples from surfaces, cotton swabs were prepared and sterilized in an autoclave (at 120°C for 30 min). Transport medium was prepared using peptone (Sigma, USA), lactose (Sigma, USA) and beef extract powder (Sigma, USA) 10, 10 and 6 grams respectively, in one liter distilled water. The pH was adjusted to 7.4 and sterilized as described above. Samples were taken cotton swabs on target surfaces with an average area of 25 cm^2 (21~30 cm^2). The swabs were put into separate transport mediums and transmitted to the PCR Laboratory of the Public Health Department within two hours on ice. Tubes, containing 5

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mL of medium and sample loaded swabs, were incubated for 2 h at 35.0°C (±0.5). At the end of the incubation period, tubes were agitated by vortex and 0.5 mL of medium was transferred into sterile micro-centrifuge tubes for the following procedures.

Recovery of DNA

A freezing and thawing method was used to recover DNA from bacterial cells. Micro-centrifuge tubes with sample were put into a freezer (-20°C) and in a water bath (60°C) for five and ten minutes, respectively; this cycle was repeated five times (Bej *et al.*, 1991). Tubes were centrifuged at 5,000 rpm for 10 min to precipitate PCR inhibiting particles. Supernatants were used as template DNA for PCR amplification.

PCR Amplification

Multiplex PCR was conducted using a DNA thermal cycler (Corbett Research PC-960, Australia). The amplification mix contained 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.5 μM of each primer, and 2.5 units of *Taq* DNA polymerase (Promega Corporation, USA) in final reaction volumes of 50 μL. Ten μL of extracted DNA were used as template. Amplification conditions were denaturation at 95°C (5 min), followed by 30 cycles of 25 sec at 95°C, 30 sec at 55°C and 55 sec at 72°C. A final extension of 10 min at 72°C was used after cycling, followed by cooling to 4°C prior to analysis. The PCR primers (Operon Technologies Inc., USA) are listed in Table 1.

Detection of Amplified DNAs

In order to detect PCR-amplified DNAs, gel electrophoresis was used. The DNAs were separated by using 2% (w/v) horizontal agarose gel. Agarose gels were run in TAE buffer (0.04 M Tris acetate and 0.001 M EDTA [pH 8.0]). The gels were stained in 2×10^{-4} % ethidium bromide solution, visualized with a UV transilluminator (Alpha Innotech Corporation, TMW-20, USA) and photographed (Bej *et al.*, 1990).

Data were analyzed using the Chi-square test on SPSS for Windows (Version 9.0) and the level of significance was 0.05.

Table 2. Data on bacterial isolation

Bacterial Group Detected	Number* of sites
Thermotolerant Coliform Organisms	77
E. coli	47
E. coli + Shigella	3
E. coli + Shigella + Salmonella	1
No Microorganism	22

*Total number is greater than 99.

Table 3. Microbiological status of sample collection sites

Sites		Results		Total
		Microorganism (−)	Microorganism (+)	
Toilet	Number	8	33	41
	(%)	19.5	80.5	100.0
Dining hall	Number	12	28	40
	(%)	30.0	70.0	100.0
Kitchen	Number	2	9	11
	(%)	18.2	81.8	100.0
Bath	Number	–	4	4
	(%)		100.0	100.0
Other	Number	–	3	3
	(%)		100.0	100.0
Total	Number	22	77	99
	(%)	22.2	77.8	100.0

We did not detect any cross reactions between primers during multiplex PCR amplification.

Data on bacterial isolation are given in Table 2. In twenty-two samples, there were no bacteria. Only four of samples contained *Shigella*, one of them contained *Salmonella*, but 77 of the samples contained thermotolerant coliform organisms.

There was no statistically significant difference among the microbiological quality of different sites ($p>0.05$). Also, we did not detect any statistically significant difference among the different equipment surfaces ($p>0.05$). Results are displayed in Tables 3 and 4.

The most contaminated surface (*E. coli* + *Shigella* + *Salmonella*) was a hand soap surface (not liquid). Each bacterial group produced specific bands on agarose gel

Table 1. Sequences of primers used and sizes of fragments produced

Primers	Sequence	Fragment Size	Reference
ZL-1675 ZR-2025	5'-ATGAAAGCTGGCTACAGGAAGGCC-3'(24mer) 5'-GGTTTATGCAGCAACGAGACGTCA-3'(24mer)	326 bp	Bej *et al.*, 1990
ual 754 ual 900	5'-AAAACGGCAAGAAAAAGCAG-3'(20mer) 5'-ACGCGTGGTTACAGTCTTGCG-3'(21mer)	147 bp	Bej *et al.*, 1991
SalA 1144 SalB 1650	5'-ACGGTTGTTTAGCCTGATAC-3'(20mer) 5'-CTGGATGAGATGGAAGAATG-3'(20mer)	526 bp	Roll, 1997
ShigA ShigB	5'-TTGACCGCCTTTCCGATAC-3'(19mer) 5'-ACTCCCGACACGCCATAGA-3'(19mer)	408 bp	Roll, 1997

Table 4. Microbiological status of the surfaces of equipments

Equipments		RESULTS		Total
		Microorganism (−)	Microorganism (+)	
Door handles	Number	5	11	16
	(%)	31.3	68.8	100.0
Tap	Number	2	26	28
	(%)	7.1	92.9	100.0
Dining tables	Number	8	12	20
	(%)	40.0	60.0	100.0
Soap dishes	Number	2	5	7
	(%)	28.6	71.4	100.0
Equipments from kitchens	Number	1	7	8
	(%)	12.5	87.5	100.0
Equipments from dining halls	Number	3	12	15
	(%)	20.0	80.0	100.0
Other	Number	1	4	5
	(%)	20.0	80.0	100.0
Total	Number	22	77	99
	(%)	22.2	77.8	100.0

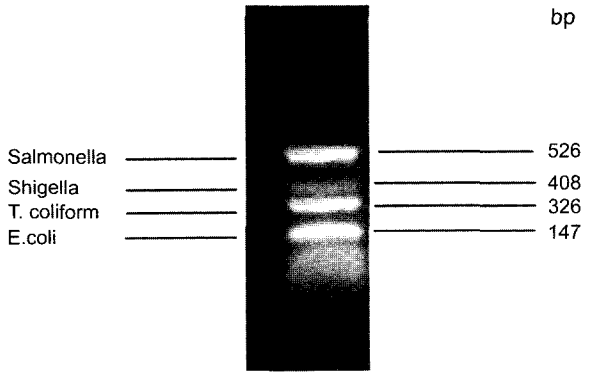

Fig. 1. We isolated all four microorganisms in soap dishes.

(Fig. 1).

Surface contamination studies are generally performed in order to determine efficacy of the cleaners which contain disinfectants or to determine the infection source during an outbreak; we did not find any studies about surface contamination performed in a large population (except hospitals) like ours in MEDLINE (Pfeiffer *et al.*, 1978; Keswick *et al.*, 1983; Mafu *et al.*, 1990).

The primer sets used in this study were checked for their specificity and published by different authors (Bej *et al.*, 1990; Bej *et al.*, 1991; Roll, 1997).

In previous studies, duplex PCR for total coliform and *E. coli* and for *Salmonella* and *Shigella* were performed separately (Bej *et al.*, 1991; Roll, 1997), but multiplex PCR for all of the above microorganisms has not been performed. In this research, we tested all of the organisms separately and together and there were no cross reactions between primers. Also, multiplex PCR by using these PCR primers could be performed for water and food analysis, too.

It is clear that a military facility is one of the most hygienic and cleanest places around us, generally because of discipline in their life styles and also in cleaning. But our findings indicated that surfaces of our study area were largely contaminated with fecal coliforms, in other words about 78% of the examined surfaces were polluted with feces; we concluded that this occurred because of insufficient and inappropriate hand washing.

Of the examined surfaces, the most polluted one was a soap dish (for bars of soap, not liquid) and we isolated *Salmonella* and *Shigella* in addition to *E. coli*. This solid kind of soap could be an infection source, and liquid soaps should be used instead.

Study results showed that all of the surfaces people touch may be an infection source; of course, most of the organisms are not infective for most people, but newborns, immunosuppressive patients and the elderly are at risk. We have to teach people how to do appropriate hand washing and how to maintain environmental hygiene and we may encourage people to use antiseptic handkerchieves in public sites like public toilets.

This kind of study must be performed on public areas every six months routinely and the cleaning procedures must be organized according to results.

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