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A comparison of RPLA and PCR for detection of enterotoxins in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in dogs

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Abstract : A multiplex-polymerase chain reaction (PCR) assay was used to detect staphylococcal enterotoxin production by 12 strains of *Staphylococcus aureus* isolated from clinical specimens. To evaluate the efficacy and/or sensitivity of this method, the results were compared to those obtained with the reversed passive latex agglutination kit (SET-RPLA, Denka Seiken, Japan). Of 10 strains positive by PCR were positive by RPLA but two strains, representing high sensitivity of the former method. Enterotoxin B was the most prevalent by the two methods. The kappa index between the two methods was 0.826, indicating a higher agreement and fully reliable for use. These results would suggest that sensitive, inexpensive, and relatively rapid multiplex-PCR technique may be an effective means for the detection of staphylococcal enterotoxin genes as an alternative to traditional methods such as kits or immunological methods, which depend upon the amount of enterotoxin produced.

Key words : *Staphylococcus aureus*, Polymerase chain reaction (PCR), Reversed passive latex agglutination (RPLA).

Introduction

Staphylococcus aureus has been well known as a major pathogen for many years. It causes disease ranging from minor skin infections to life-threatening deep infections such as pneumonia, osteomyelitis, endocarditis, meningitis, septicemia, and toxic shock syndrome. Soon after the introduction of β-lactamase stable penicillins such as methicillin and flucloxacillin in the early 1960s resistance strains of *S aureus* to methicillin (MRSA) was reported as early as 1961[1]. In recent years the treatment of such infections has been complicated because of its characteristics of multidrug resistant traits.

Like other gram-positive bacteria, *S aureus* causes diseases

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chiefly by production of virulence factors such as adhesins, hemolysins, enterotoxins, and toxic shock syndrome toxin that are thought to contribute to the pathogenicity of the organism. Most of study on toxin-typing has focused on general staphylococci, not on MRSA originated from animal sources[2,3]. In addition to conventional methods such as ELISA[4], immunodiffusion[5,6], or agglutination[7,8] for detecting enterotoxin, amplification techniques like polymerase chain reaction (PCR) have been increasingly used[3,9]. Of these methods, reversed passive latex agglutination (RPLA) and PCR using specific primers have been widely used for this purpose.

There is, however, little information on the comparison of these methods used for detection of staphylococcal enterotoxin (se), particularly in relation to MRSA isolated from animal species. Accordingly, the objective of the current study reported here was to employ 12 MRSA isolates to explore the degree of agreement on enterotoxin types determined by the two methods. Such comparison may allow us to gain insight into what extent we can believe and how we interpret the specificity of the results.

Materials and Methods

Bacterial strains : 12 strains of MRSA used in this study were isolated from clinical specimens of hospitalized dogs. Methicillin resistance was confirmed by the oxacillin agar screening test, using Mueller-Hinton agar containing 4% NaCl and 6μg/ml oxacillin and reading after 24h incubation at 37℃[10]. These strains were further identified by the PCR assay using the *mec* A gene specific primers. The collection, growth, and processing of samples have been described fully elsewhere[11]. All reference strains for multiplex PCR are shown in Table 1. These strains were kindly gifted by Dr. Yong-Ho Park (College of Veterinary Medicine, Seoul National University, Korea). The expected PCR product for each toxin are as follows: 121bp for *sea*, 477bp for *seb*, 257bp for *sec*, 318bp for *sed*, and 169bp for *see*[12].

Enterotoxin typing : Production of enterotoxins A, B, C, D, and E was determined by two methods; commercial RPLA kit and PCR. In the former, production and typing of enterotoxins were tested by reversed-passive latex agglutination using sensitized latex with type specific anti-enterotoxins A, B, C, D, or E immunoglobulin (SET-RPLA, Denka Seiken, Japan). All procedures were followed by the manufacturer's instructions.

Preparation of chromosomal DNA for PCR : Total genomic DNA was prepared using modified guanidinium thiocyanate (GuSCN) method as described by Boom *et al*[13].

Multiplex PCR : The PCR was performed in a thermocycler (GeneAmp PCR System 9600; The Perkin Elmer Corp., Norswalk, CT). Specific primers for each toxin have been reported by other researchers[3] and synthesized with a DNA synthesizer (Expedite 8905; Perseptive Co.). Optimal condition of these parameters was established as followings; the reaction mixture consisted of 2.5μl of 10x reaction buffer without $MgCl_2$ (Promega Corp., Madison, W.I.), 400μM of deoxynucleoside triphosphate mixture (dNTP), 3mM $MgCl_2$, 7.5% DMSO, 50 pmol of primers for all enterotoxins, 100ng of template DNA, and brought up to 25μl final volume with distilled water. After hot starting for 5 min at 95℃, the reaction mixture was kept in the ice quickly and 1 unit of *Taq* polymerase (Promega Corp.) was added. And the following program was performed by 30 cycles in the thermocycler: denaturation for 1 min at 95℃, annealing for 2 min at 56℃ (for *sea, sec* and *sed*) or 50℃ (for *seb* and *see*), and ex-

Table 1. Reference strains used in this study

Strains	Enterotoxin types	Product size (bps)
S aureus FRI913	*sea* and *sec*	121 (*sea*), 257 (*sec*)
S aureus MNHOCH	*seb*	477
S aureus FRI472	*sed*	318
S aureus FRI326	*see*	169

tension for 1 min at 72℃ followed by a final extension step of 5 min at 72℃. To analyze the amplified products, 5μl of the PCR product was electrophoresed in a 1.5% agarose gel for 1 h at 120V and examined under UV illumination after staining with ethidium bromide.

Statistical analysis : The agreement of two methods was measured by calculation of kappa index[14]. This value has a maximum of 1.0 when agreement is perfect, a value of zero indicates no agreement better than chance. A value of 0.80 or higher was considered an acceptable level for clinical use.

Results

Table 2 shows the difference in enterotoxigenic types determined when the results from RPLA and PCR methods were compared. Overall, 10 strains (83.3%) and 8 strains (66.7%) of 12 MRSA strains were found to produce one or more enterotoxins in PCR and RPLA, respectively. Of these 8 enterotoxin producing strains in RPLA, seb and sec accounted for 62.5% (5/8) and 37.5% (3/8), respectively. In PCR assays, 60% (6/10) were *seb* and 40% (4/10) were *sec* producing strains. When the individual toxins were analyzed in both methods, predominant type produced by MRSA was

Table 2. Correlation of enterotoxigenic types among 12 strains of MRSA determined by the RPLA kit and PCR method

Strain No.	Enterotoxin	
	RPLA[a)]	PCR
SAH-1	B	B
SAH-2	–[b)]	–
SAH-3	–	–
SAH-4	–	C
SAH-5	C	C
SAH-6	C	C
SAH-7	–	–
SAH-8	C	C
SAH-9	B	B
SAH-10	B	B
SAH-11	B	B
SAH-12	B	B

[a)]Reversed-passive latex agglutination (RPLA, Denka Seiken Co. Tokyo, Japan).
[b)]No toxin detected.

Fig 1. Enterotoxin detection for MRSA by multiplex PCR.
M; Molecular weight marker (123-bp), R1, Reference strain for enterotoxin A and C; R2, Reference strain for enterotoxin D; lanes: A, SAH-1; B-E, MSSA; F, SAH-3; G, SAH-12; H, SAH-2; I, SAH-7; J, SAH-5; K, SAH-6; L, SAH-10; M, SAH-9; N, SAH-8.

Fig 2. Enterotoxin detection for MRSA by multiplex PCR.
M; Molecular weight marker, R1, Reference strain for enterotoxin A and C; R2, Reference strain for enterotoxin D; lanes: Q, S, and T, MSSA; R, SAH-4; U, SAH-11.

Fig 3. Enterotoxin detection for MRSA by multiplex PCR.
M; Molecular weight marker (100-bp), R1, Reference strain for enterotoxin B; R2, Reference strain for enterotoxin E and TSST-1 (350-bp); lanes: A, SAH-5; B, SAH-2, C, E, and J, MSSA; D, SAH-12; F, SAH-9; G, SAH-7; H, SAH-8; I, SAH-11; K, SAH-10; L, SAH-1; M, SAH-3; N, SAH-6.

Fig 4. Enterotoxin detection for MRSA by multiplex PCR.
M; 100-bp ladder molecular weight market, R1, Reference strain for enterotoxin B; R2, Reference strain for enterotoxin E and TSST-1 (350-bp); Lanes: Q, R, T, and S, MSSA; U, SAH-14.

enterotoxin B. As evident from the Table 2, the degree of agreement between the two methods was 0.826. The results of PCR for enterotoxin typing for MRSA and 7 methicillin-susceptible *S aureus* (MSSA) strains are shown in Figures 1~4.

Discussion

With a multiplex-PCR method, 83.3% (10 strains) of 12 MRSA strains were found to carry one of the enterotoxin genes, compared to 66.7% (8 strains) in RPLA. Although enterotoxin production of *S aureus* in animal isolates has been reported in a limited number of studies this percentage of enterotoxin production was comparable to those of many authors, with rates of around 16.8~50%[2,15~19]

There was general agreement between the results of RPLA and those with the PCR for enterotoxins with a kappa index of 0.826; however, there were two exceptions. Strain SAH-2 and SAH-4 were consistently produced toxin with the PCR, but had not detectable with RPLA. In a study[20] on the comparison of four commercial kits for the detection of *se* in food samples reported similar results that compared to the gel immunodiffusion methods as a gold standard, the kappa index for ELISA-B (n = 18) was 0.752, 0.523 for RPLA (n = 18), and 0.533 for ELISA-M (n = 16). The reasons underlying discrepancy (false negative in RPLA) observed between the kit method and the PCR assays may be partly explained by the sensitivity of the RPLA. This multiplex-PCR assays could detect as few as 8.4×10^1CFU/ml for ***sea***, ***sec*** and ***sed***, and as few as 8.4×10^5CFU/ml for ***seb*** and ***see***. Another possibility is that under some circumstances the enterotoxins might not be produced or may be produced to only a level below the detection limit of RPLA. As described by Genigeorgis[21], the production of enterotoxins by staphylococcal strains is affected by several factors including the level of inoculum, the temperature, pH, and water activity.

Considering the clinical implication of detecting enterotoxigenic *S aureus* more sensitive and specific multiplex-PCR technique need to be developed to increase the accuracy of the test. The multiplex-PCR assay described here could offer a very useful technique for the detection of enterotoxin genes as an alternative to traditional methods.

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