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Evaluation of three different molecular markers for the detection of *Staphylococcus aureus* by polymerase chain reaction

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Abstract

The aim of this study was to target three genes of *Staphylococcus aureus-fmhA* (coding for a factor of unknown function), catalase and *femA* (coding for a factor essential for methicillin resistance) to establish and validate a PCR assay for the detection of this pathogen. Two pairs of primers were designed for *fmhA* and one pair each for catalase and *femA* genes. The PCR assays were standardized and found to give specific amplicons under similar reaction parameters. Target specificity of the primers was confirmed by DNA sequencing of the amplicons. While the initial inclusivity and exclusivity test reactions were in agreement in case of three of the primer pairs, one pair based on *fmhA* gene produced a non-specific product with a template DNA used in exclusivity test reactions. Forty-five strains of *S. aureus* were subjected to these PCR assays for their evaluation. Three among the four pairs of primers, one against each gene detected all the 45 strains precisely whereas one of the PCR assays using primers targeting the *fmhA* gene did not generate the specific amplicon with several of the strains. Seven unidentified strains of Gram-positive cocci subjected to these PCR assays produced negative results for each culture. Six of the strains were identified as *Staphylococcus haemolyticus* and one strain as *Staphylococcus arlettae* by 16S ribosomal gene analyses. All the three assay systems showed a detection limit of 100 cells per 20 μl reaction assay. For validation of these assay systems, 80 coded samples of 11% skimmed milk spiked with different pathogens were received from NICED (National Institute of Cholera and Enteric Diseases), Kolkata and subjected to these PCR assays. All the three assays could detect *S. aureus* correctly in two of the samples. Amongst 150 raw milk samples, 36 (24%) were found positive for *S. aureus*. We conclude that *fmhA*, catalase and *femA* genes are conserved in *S. aureus* and, therefore, could be used as specific targets for its detection and identification by PCR. The protocols developed herein could be used for rapid and specific detection of this pathogen in food, clinical and environmental samples, especially milk.

Keywords: *Staphylococcus aureus*; Milk; *femA*; Catalase; *fmhA*

1. Introduction

Staphylococcus aureus is one of the most commonly found pathogenic bacteria and is hard to eliminate from the human environment (Perez-Roth et al., 2001). It is responsible for many nosocomial infections, besides being the main causative agent of food intoxication by virtue of its variety of enterotoxins (Iandolo, 1989). Routine detection of *S. aureus* in food is usually carried out by traditional methods based on morphological and biochemical characterization. These methods are time consuming and tedious. In addition, misclassifications with automated susceptibility testing systems or commercially available latex agglutination kits have been reported by several workers (Ruane et al., 1986; Schwarzkopf et al., 1993; Wilkerson et al., 1997; Ribeiro et al., 1999). Consequently, there is a need for methods to specifically discriminate *S. aureus* from other staphylococci as quickly as possible (Roberson et al., 1992; Guzman et al., 1992). Microbial genomes are being sequenced at a staggering rate. Approximately, 10% of the genes of a species in a genus are unique to each organism, and we are now beginning to appreciate the genetic diversity among bacterial strains (Versalovic and Lupski, 2002). This forms the basis for genotypic identification of microorganisms including bacterial and fungal pathogens. PCR techniques have provided increased

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sensitivity, allowed for more rapid processing times, and enhanced the likelihood of detecting bacterial pathogens. In the last 15 years, several detection methods have been proposed for foodborne pathogens to replace the time-consuming classical techniques (Candrian, 1995; Hill, 1996; Olsen, 2000). Specific primers for the detection of *S. aureus* have been directed to the *nuc* gene encoding thermostable nuclease (Wilson et al., 1991; Brakstad, 1992), enterotoxin genes (Wilson et al., 1991; Johnson et al., 1991; Tsen and Chen, 1992; Mantynen et al., 1997; Becker et al., 1998), *tst* gene (shock syndrome) genes coding for exfoliative toxin A and B (*eta* and *etb*, respectively) (Johnson et al., 1991) the 16–23 rDNA spacer region (Saruta et al., 1997), the 23S rDNA (Straub et al., 1999) and *femA* gene (Vannuffel et al., 1995; Mehrotra et al., 2000). In addition to the analysis of foods, PCR has also been successfully applied to detection and identification of pathogenic organisms in clinical and environmental samples (White et al., 1992; Simon, 1999; Olsen, 2000).

Several studies have recently focused on detection of *S. aureus* in milk and milk products including the use of quantitative real-time PCR (Hein et al., 2001; Alarcon et al., 2006). However, due to variability in selectivity of different primers (Klaassen et al., 2003), it is imperative to target new genes for the detection of *S. aureus*, so that infallible assays are developed for its detection and identification. Validation is an important requirement for the development of a PCR-based detection system. The present study describes the comparison of PCR protocols based on two new target genes *fmhA* and catalase, and a previously used target gene, *femA* (Vannuffel et al., 1995; Mehrotra et al., 2000) and consequently the development of a validated and reliable PCR assay for the rapid detection of *S. aureus*. The *femA* gene encodes a factor, which is essential for methicillin resistance and is universally present in all *S. aureus* isolates (Johnson et al., 1995). Catalase is a haem-containing enzyme involved in dismutation of hydrogen peroxide generated during cellular metabolism to water and molecular oxygen (Loewen, 1992). The function of the FmhA protein is not clear, however, it has significant identities to FemA (Tschierske et al., 1999).

2. Materials and methods

2.1. Bacterial strains

A total of 107 bacterial strains were used in this study (Tables 1 and 2). The cultures were grown on Tryptone Soya Agar (TSA) (HiMedia, India) at 37 °C and also maintained in glycerol (50%) normal saline (0.85% NaCl, w/v) at −70 °C.

2.2. DNA isolation for PCR

Templates were prepared from pure cultures of *S. aureus* and other bacterial species by thermal extraction. Strains

Table 1
Bacterial cultures used in the exclusivity test reactions

Species	No. of isolates	Source
Staphylococcus epidermidis ATCC 12228	1	ATCC
Staphylococcus haemolyticus ATCC 29978	1	ATCC
Staphylococcus saprophyticus ATCC 15305	1	ATCC
Streptococcus pyogenes	1	IIIM repository
Enterococcus faecalis ATCC 21777	1	ATCC
Enterococcus faecalis ATCC 29212	1	ATCC
Enterococcus faecalis SP 346(VRE)	1	IIIM repository
Enterococcus faecium 6A	1	IIIM repository
Klebsiella pneumoniae ATCC 75388	1	ATCC
Klebsiella aerogenes MTCC 39	1	MTCC
Klebsiella rhinoscleromatis ATCC 13884	1	MTCC
Pseudomonas aeruginosa ATCC27853	1	ATCC
Bacillus cereus	4	CFTRI
Bacillus cereus	1	NICED
Listeria monocytogenes ATCC 15313	1	ATCC
Citrobacter spp.	2	IIIM repository
Enterobacter spp.	2	IIIM repository
Escherichia coli ATCC 25922	1	ATCC
Escherichia coli O157:H7 ATCC 35150	2	ATCC
Escherichia coli DH5α	1	IIIM repository
Escherichia coli JM109	1	IIIM repository
Escherichia coli JM110	1	IIIM repository
Escherichia coli JM101	1	IIIM repository
Escherichia coli SURE	1	IIIM repository
Escherichia coli (clinical isolates)	5	IIIM repository
Shigella dysenteriae	2	AIIMS
Shigella flexneri	2	AIIMS
Shigella flexneri ATCC 12022	1	ATCC
Shigella flexneri MTCC 1457	1	MTCC
Shigella sonnei MTCC 2957	1	MTCC
Salmonella enterica serotype Typhi	1	IIIM repository
Salmonella enterica serotype Typhimurium	1	IIIM repository
Salmonella enterica serotype Paratyphi A	1	IIIM repository
Other unidentified Gram-negative bacteria	10	IIIM repository

ATCC: American Type Culture Collection.
MTCC: Microbial Type Culture Collection, IMTECH, Chandigarh, India.
AIIMS: All India Institute of Medical Sciences, New Delhi, India.
CFTRI: Central Food and Toxicology Research Institute, Mysore, India.
NICED: National Institute of Cholera and Enteric Diseases, Kolkata, India.
IIIM: Indian Institute of Integrative Medicine (CSIR), Jammu, India.