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***FemA* gene in Indian isolates of methicillin resistant *Staphylococcus aureus* – isolation and amplification through real time PCR**

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ABSTRACT

Staphylococcus aureus is a Gram positive bacterium, which is naturally present in nasopharyngeal region of human beings and animals. *S. aureus* had developed drug resistant capacity to against all penicillin class antibiotics after severe administration since 1941. Firstly, in 1961, Methicillin Resistant *Staphylococcus aureus* (MRSA) were isolated. In this research, Methicillin Resistant *S. aureus* (MRSA) were isolated from clinical pus samples. Isolates were grown on Luria Bertani (LB) Agar containing Oxacillin antibiotic by traditional methodologies. Genomic DNA was isolated from the isolates and electrophoresis was performed by using standard criteria. *Fem A* gene amplification was performed with Real Time PCR and with suitable primers. The amplified gene was produced 510 bp at Agarose Gel Electrophoresis.

Key words: Methicillin Resistant *Staphylococcus aureus* (MRSA), LB Agar, Real Time PCR, *FemA* gene, Agarose Gel Electrophoresis.

INTRODUCTION

Staphylococcus aureus is one of the most commonly found pathogenic bacteria and is hard to eliminate from the human and animals environment. It is responsible for many nosocomial infections, besides being the main causative agent of food intoxication by virtue of its variety of enterotoxins [1]. The toxic shock syndrome of humans and animals is caused by the presence of *S. aureus* [2]. It is the commonest of all clinical isolates and responsible for several suppurative types of infections. *S. aureus* is causing a wide variety of diseases including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis and toxic shock syndrome [3][4], carbuncles more serious device and wound related infections, to life threatening conditions, such as bacteremia, necrotizing pneumonia, and endocarditis. *S. aureus* produces different types of virulence factors that facilitate attachment, colonization, cell-cell interactions, immune invasion, and tissue damage [5]. They have a differential ability to spread and cause outbreaks in hospitals. However, treatment of these infections has become problematic due to development of methicillin resistance in *S. aureus* isolates. Methicillin resistance first appeared among nosocomial isolates of *S. aureus* in 1961 [6]. Worldwide, an estimated 2 billion people carry some form of *S. aureus*, of these up to 53 million (2.7% of carriers) are thought to carry MRSA. As of early 2005, the number of deaths in United Kingdom attributed to

MRSA has been estimated by various sources to lie in the area of 3000 per year [7]. The incidence of methicillin resistant *S. aureus* (MRSA) in India ranges from 30 to 70% [8]. *S. aureus* is often found passively colonizing human skin and nasal passages. As an opportunistic pathogen, the bacterium can also cause infections that vary widely in their severity and in their susceptibility to antibiotic treatment. This variability is caused by differences in gene content among strains. One source of exposure to novel, potentially more virulent strains of *S. aureus* is recreational swimming areas, since *S. aureus* is readily shed from humans into water [9]. Most people are passive carriers of *S. aureus*, which is often found colonizing the nasal passages [10], and in some cases, an infection may arise from self-inoculation of a wound. *S. aureus* infections have been a major problem in hospitals for decades, but the incidence of community-acquired infections has also been increasing [11][12]. The type as well as severity of *S. aureus* infection and its response to antibiotic treatment is dictated by the specific suite of virulence and antibiotic resistance associated genes carried by the strain of *S. aureus* causing the infection. There are over 40 virulence-associated genes identified among

various strains of *S. aureus*, many of which are encoded by mobile genetic elements. Over the last few decades, there has been an enormous increase and emergence of *S. aureus* strains [13]. The intrinsic resistance to these antibiotics is attributed to the presence of *mecA*, whose product is a 78kDa protein called penicillin binding protein 2a [14]. Identification of *mecA* in such MRSA strains has led to some knowledge regarding the use of the antibiotic vancomycin [15]. The *femA* gene encodes a factor which is essential for methicillin resistance and is universally present in all MRSA isolates. The *femA* gene product, a 48 kDa protein, has been implicated in cell wall metabolism and is found in large amounts in actively growing cultures. Phenotypic methods for detection of methicillin resistant *S. aureus* (MRSA) have been compared with the gold standard which, as of now, is by the detection of *mecA* gene and *femA* gene by polymerase chain reaction (PCR) [16].

MATERIALS AND METHODS

Bacterial strains and culture media: A total of Nine MRSA strains were used in this study. These strains were isolated from pus samples. All strains were stored on 1% Oxacillin containing LB medium. Strains are grown with Brain heart infusion broth and incubated for 12 hrs.

DNA isolation: Total DNA was isolated from 0.5 ml of brain heart infusion broth culture grown 12 hrs for all the MRSA strains used in this study. An aliquot (0.5ml) broth culture was pelleted by centrifugation (5,000 rpm for 5 minutes). The bacterial pellet, resuspended in 300ml of lysis buffer (50mM Tris HCl [pH8.0], 100 mM EDTA, 150 mM NaCl, 1% [V/V] (Sodium Dodecyl Sulfate) containing 100 mg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) and 100 mg of RNase, was incubated at 37.80 C for 30 min. Lysis was achieved by incubation at 37.80 C for 30 min in the presence of 200 mg of Proteinase K. Samples were treated with 1 volume of Phenol – Chloroform – Isoamyl alcohol (25:24:1) and then with 1 volume of Chloroform – Isoamyl alcohol (24:1) prior to precipitation of the aqueous phase in 2 volumes of 95% ethanol – 0.2 M NaCl for 1 h at 2208C. DNA was pelleted by centrifugation (12,500 rpm for 10 min), washed with 80% ethanol, air dried. DNA samples were dissolved in Tris - EDTA buffer (10 mM Tris chloride, 1 mM EDTA [pH 8.0]), and the concentration was determined as micrograms per milliliter according to A260 values. Template DNA in amounts ranging from 10 to 1,000 ng was used in this study.

Polymerase Chain Reaction : The polymerase chain reaction (PCR) is an *in vitro* DNA amplification of target DNA with a pair of primers and a DNA polymerase, resulting in several million fold amplification of the target sequence within few hours. 50µl of DNA sample was taken for the amplification. Then the reaction mixture prepared, which contained 5µl of 10x reaction buffer (500 mM KCl, 15 mM MgCl₂, 100 mM Tris HCl pH 8.3, 0.1% w/v gelatin). Template DNA was added at a concentration of 200ng/µl. Added 1µl of each primer (Forward Primer 5'AAAAAAGCACATAACAAGCG3', Reverse primer 5'GATAAAGAAGAAACGAGCAG3'). The primers were used at a final concentration of 100 M. Add 3 µl of 10 mM dNTP mixture. The four deoxyribonucleotide triphosphate (dNTP, dTTP, dGTP, dCTP) were used at a concentration of 100 µl each. To each sample, 1 µl of Taq polymerase (3U/l) was added. The remaining volume made up to with sterile water. Amplification was carried out in a Thermal cycler. Initial denaturation at 94 °C for 1 minute followed by 30 cycles, each cycle consisted of denaturation of DNA for 30 seconds at 94 °C, annealing of the primers for 30 seconds at 50 °C and elongation at 72 °C. The PCR products were analyzed by gel electrophoresis in a 1% agarose in Tris Acetate buffer (pH 8.3).

Agarose gel electrophoresis: Agarose gel electrophoresis is employed to quickly determine the yield and purity of DNA isolated, or check product of PCR reaction, check progression of restriction enzyme digestion and to size fractionate DNA molecules which then could be eluted from the gel. Cleaned the gel casting apparatus and sealed

both ends of the gel casting tray with an adhesive tape. Prepare 1% agarose gel. Agarose dissolved in 1X TAE buffer. Boil till agarose dissolved completely. Pour the agarose gel solution to the tank when the temperature reaches approximately 55°C. Avoid air bubbles formation. The thickness of the gel should be around 0.5 to 0.9 cm. Keep the gel at room temperature for the agarose to solidify. Remove the comb carefully, ensuring the wells remains intact and peels of the tape from the gel casting tray. Placed the gel in horizontal electrophoresis apparatus containing 1X TAE electrophoresis buffer in the reservoirs. The buffer level should be such that is just covers the agarose gel. 10µl of DNA sample taken and mixed with 3µl of gel loading dye and loaded into the gel. Then connected the electrophoresis apparatus with 100 V DC current and allowed it to run for 30minutes. Stained the gel with Ethidium Bromide (EtBr) solution of 0.5 µg/ml concentration for 10 minutes. Transfer the gel onto U V transilluminator. Observe the results and note it carefully.

RESULTS AND DISCUSSION

The present study successfully isolated genomic DNA molecules from MRSA isolated strains. Clear bands were seen in gel kept on U V transilluminator. The present study, *femA* gene band showed at 510 b p region. All MRSA isolated strains were produced 510 b p band (**Fig 1**).

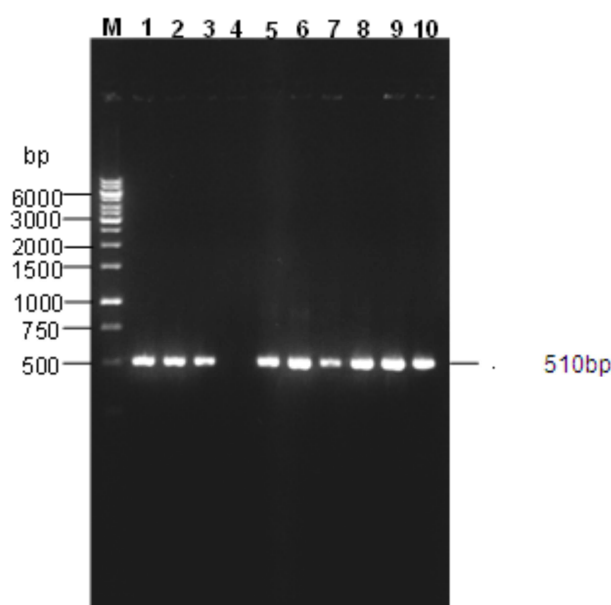


Fig 1 Agarose (1%) Gel Electrophoresis showing the PCR amplification of *femA* gene of MRSA isolates (M – Marker; 1, 2, 3, 5, 6, 7, 8, 9 and 10 – MRSA isolates; 4 – Blank)

MRSA now accounts for an important cause of nosocomial infections. MRSA and MSSA are equally pathogenic and capable of causing the same spectrum of nosocomial infections. Initially, there were sporadic reports of methicillin resistance amongst nosocomial *S. aureus* isolates, but later MRSA became a well established hospital acquired pathogen with few reports on increased prevalence of community acquired MRSA with different factors compared to the earlier investigations from Detroit which first reported the community acquired MRSA. The epidemiology of MRSA is also changing over the past few decades. Nearly 51.6% of *S. aureus* isolates were found to be resistant to methicillin in the present study while in the previous studies the incidence was found to be 32.8% in 1994, 24 % in 1996 , 32 % in 1997 . This implies that the incidence of infection by MRSA isolates keeps changing every year and it is on the rise when compared to last few years. Worldwide, an estimated 2 billion people carry some form of *S.*

aureus, of these up to 53 million (2.7% of carriers) are thought to carry MRSA. As of early 2005, the number of deaths in United Kingdom attributed to MRSA has been estimated by various sources to lie in the area of 3000 per year.

The resistance of MRSA to a wide range of anti-bacterial is well documented. This makes the empirical use of antibacterial effective against MRSA imperative. Vancomycin has been clearly shown to be the drug of choice for the treatment of MRSA infections. The virulence genes *mecA* and *femA* were examined because they are believed to be the major contributors to methicillin resistance. Previous studies have assessed the feasibility of the PCR approach either for the identification of *S. aureus* strains or for intrinsic methicillin resistance on the basis of *femA* gene amplification. A probe corresponding to the 39 - end of amplified fragments of the *femA* determinant hybridized to the PCR products of DNAs of *S. aureus* isolates, confirming the *femA* gene origins of these amplified products. Analysis of the *femA* product indicated that this protein is associated with the expression of high levels of methicillin resistance without affecting PBP-2 production. The significance of the *femA* genes in the mechanism of methicillin resistance was supported by the demonstration that an *S. aureus* strain with *femA* gene inactivated lost the methicillin resistance trait, but with the transduction of *femA* genes restored the resistance. Another biochemical analysis suggested that *femA* product may be involved in the metabolism of cell wall synthesis.

CONCLUSION

The present study described in this paper provides detailed information about MRSA and *fem A* gene. *fem A* is one of the toxin producing gene. This gene is very essential to give methicillin resistant nature of MRSA strains. This PCR assay will help to provide the information required for appropriate therapy and infection control during outbreaks of MRSA. It is important to recognize that this technique only can be used to identify strains harboring the toxin genes and is independent of the expression and secretion of the toxin. To verify toxin production by any given isolate, time- and labor-intensive immunological methods may be used to detect the excreted toxins. Considering the low cost and much shorter time required to detect the genes of MRSA by multiplex PCR, this is to be a powerful tool for studying the genotypes of staphylococcal isolates. This procedure was specially developed to fit into the daily work pattern of a routine clinical laboratory, since genotypic detection of drug resistance and the presence of toxin genes is becoming an important component of the diagnostic inventory of such laboratories.

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