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Cloning and Characterization of New *emm* Allele of *Streprococcus pyogenes* Strains Isolated in Kingdom of Saudi Arabia

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Abstract: In the present study, 39 isolates of erythromycin-resistant group A *Streptococcus* (GAS) isolated in the kingdom of Saudi Arabia from 2003 to 2004 were characterized by using phenotypic and genotypic methods. Most strains (94.9%) had similar or highly related pulsed-field gel electrophoresis profiles to different nine *emm* types previously documented. In which, type *emm1* was the most prevalent M type in KSA representing 20.5% of total isolates, followed by *emm3* (15.4%) typing. Two new *emm* sequence types were identified among GAS strains designated SA15 and SA37. The type SA15 carried two resistant *mefA* and *ermTR* genes accounted for 6.3% (1/16) and 50.0% (1/2), respectively. Entire fragment of SA15 was sequenced and expressed in expression vector to be used as a tool for vaccine preparation in this area. This report provides information on new *emm* sequence types firstly detected in KSA GAS isolates as a vaccine candidate antigen in this geographic area which not extensively surveyed, also it contributes to a better understanding of the local and global dynamics of GAS populations and the epidemiological aspects of GAS infections occurring in KSA.

Key words: *Streprococcus pyogenes*, erythromycin resistant isolates, *emm* types

INTRODUCTION

Streptococcus pyogenes is one of the most common and ubiquitous human pathogens and is a significant leading cause of human morbidity worldwide. It is responsible for a wide range of infections in areas where the diseases are endemics or epidemics (Stevens, 2000) with varying from clinically mild infections (pharyngitis, impetigo, scarlet fever, etc.), to severe life-threatening infections (sepsis, necrotising fasciitis, toxic shock syndrome with muli-organ failure), or non-suppurative sequelae such as rheumatic fever, rheumatic heart disease and acute glomerulonephritis (Stevens, 2000). In the late 1980s, reports on the resurgence of severe *Streptococcus pyogenes* frequently referred to as group A *streptococci* (GAS) infection (Hoge *et al*., 1993) resulted in an increased awareness and interest in this organism.

Most of knowledge that has been accumulated concerning GAS epidemiological studies is based on traditional methods of T-agglutination typing (T-typing) and M-precipitation typing (M-typing) that have been used in for many years (Efstratiou, 2000). The M protein, a cell surface protein, (M-typing), is an important virulence determinant in the pathogenesis of suppurative and non-suppurative diseases (Praksachatkunakorn *et al*., 1993) and it is responsible for the antigenic variations which is a basis for serological typing method (Facklam *et al*., 1999). More than 80 different GAS M types have been identified by serological M typing, however, many GAS isolates are nontypeable due to the lack of appropriate type specific antisera or loss of antigen expression (Praksachatkunakorn *et al*., 1993; Pruksakorn *et al*., 2000). In recent years DNA sequencing-based methods for characterizing GAS strains have been used including sequence analysis of *emm* gene-specific PCR products (*emm* typing) of M protein gene that permits the typing of strains which cannot be serologically classified (Beall *et al*., 1996 and 1997). This methodology has allowed the recognition of several previously unknown GSA types in different geographic areas, demonstrating the usefulness of *emm* typing for detecting genetic diversity among GSA isolates and tracing GSA infection. Approximately 150 M protein gene sequence types (*emm*) of GAS have been documented (Fica *et al*., 2003). Some types have seemed to be associated with certain patterns of disease more frequently than others. Historically, types such as M1, M2, M3, M4, M12, M15, M49, M55, M56, M59, M60 and M61 have been associated with post-streptococcal glomerulonephritis, while types M5, M6, M18, M19 and M24 have been linked to rheumatic fever (Bisno, 2001). More recently, types M1 and M3 were also epidemiologically associated with the resurgence of some severe forms of GAS infections, such as toxic shock syndrome and necrotizing fasciffis (Ho *et al*., 2003). A few

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studies have described the distribution of GAS types mainly in Western countries where the epidemiology of GAS may be different. Studies from Malaysia and Thailand, where rheumatic fever is endemic, have indeed a different GAS type distribution in this region (Kaplan *et al*., 2001; Kim and Lee, 2004).

Although penicillin is the drug of choice for the treatment of *S. pharyngitis* (Betriu *et al*., 1993), an increased failure of this treatment due to copathogenicity with β-lactamase-producing microorganisms has been reported (Coleman *et al*., 1993). In these cases and in cases where patients are allergic to penicillin, other antibiotics not subject to inactivation by β-lactamases as amoxicillin-clavulanate, oral cephalosporins, or erythromycin, have been substituted for penicillin (Betriu *et al*., 1993). Resistance to erythromycin remained at low levels among *S. pyogenes* in most countries of the world, however, in the last years a significant increase in erythromycin-resistant isolates in many development countries has been reported (Alberti *et al*., 2003; Cornaglia *et al*., 1996; Martin *et al*., 2002; Seppala *et al*., 1992). Where the erythromycin resistance frequency has increased to more than 16% in the last 10 years, reaching 40% in some regions of Asia (Kaplan *et al*., 1992; Pruksakorn *et al*., 2000). Therefore, epidemiologically and genetically investigations of different and prevalent M protein types in erythromycin resistance *S. pyogenes* causing diseases in local and other parts of the world communities is necessary for formulation and development of a suitable vaccine.

In vaccine development, many studies have defined protective epitopes from the N-terminal and C-terminal regions of the M protein(Brandt *et al*., 1997 and 2000). So identification of predominant M types in certain area would facilitate the development of a vaccine targeted to such population. However, the vast number of isolates from specific regions of endemicity remain largely uncharacterized, with over 80% of isolates being classified as non-M typeable (Kaplan *et al*., 1992; Kim and Lee, 2004), have not yet been characterized (Kim and Lee, 2004). Actually, effective vaccines against GAS would be based upon complex combinations of specific types of M protein components. The formulations for these vaccines would requite knowledge of the types of GAS causing disease in different communities (Kaplan *et al*., 1992; Brandt *et al*., 1997). Recently a vaccine presently under investigation and undergoing clinical trials contains 26 different specific *emm* types of M protein fragments (Hu *et al*., 2002). A similar approach is being used to formulate a vaccine based upon prevalent GAS M protein types observed in the Australian aboriginal population (Brandt *et al*., 2000). It has been estimated that the aforementioned 26-valent vaccine (26VV) represents 78 to 80% of the type distribution seen in pharyngitis and invasive-infection GAS isolates in the United States (Hu *et al*., 2002). The potential coverage of this 26VV for strains causing infections in other parts of the world is unknown.

The objective of the present study was to characterize of GSA M type isolates belonging to erythromycin resistant strains in patients living in KSA for epidemiological studies. These data provide useful information about the prevalent and new *emm* isolates of GAS in KSA, that identified and expressed in a suitable vector to be used in further studies as a vaccine domain.

MATERIALS AND METHODS

Strains: A total of 107 GSA isolates produced beta-hemolytic colonies were randomly collected from 3 different hospital-based laboratories, geographically distributed in different areas in KSA between 2004 to April 2005. Among them 39 erythromycin resistant isolates from different patients infected with *S. pyrogenes* were selected in this study, other samples were eliminated because they were also resistant to other antimicrobial agents. Five strains were blood isolates whereas the remaining strains were throat swabs isolates. Swabs were cultured on blood agar plates, with incubation period at 37°C in CO_2 incubator for 24 to 48 h. All GSA isolates were stored in glycerol stocks (Gherna, 1981) at -80°C until required.

Susceptibility tests and determination of erythromycin resistance phenotypes: MICs of penicillin, erythromycin, tetracycline and clindaroycin (Sigma Chemical Co., St. Louis, Mo.) were determined by the agar dilution method according to the recommendations of the NCCLS (NCCLS, 2000). Resistance phenotypes of erythromycin-resistant isolates were determined by the disk tests (Becton Dickinson, Cockeysville, Md.) as described previously (Seppala *et al*., 1997). The breakpoint used for erythromycin resistance was <1 mg mL^{-1}.

DNA isolation: The organisms were streaked out on blood agar plates and a single colony was used to inoculate 50 mL of Todd-Hewitt broth. After incubation at 37°C overnight, the culture was spun down and the pellet was washed three times with phosphate-buffered saline (PBS; pH 7.0), resuspended in 0.5 mL of a lysozyme solution (100 mg mL^{-1}) and incubated at 37°C for 1 h. Sodium dodecyl sulfate (200 μL of a 20% solution) and Proteinas K (100 μL of a 10 mg mL^{-1} solution) were added and the suspension was incubated at 55°C overnight. One-third volume of a saturated NaCl solution was added and the mixture was incubated at 4°C for 20 min. The

mixture was then centrifuged to sediment the protein, the supernatant was transferred to a new tube and 95% ethanol (3 volumes) was added to precipitate the DNA. The tube was rocked gently until the DNA flocculated. The DNA was then washed once in 70% ethanol and retrieved with a bent-tip pipette, allowed to air dry for 1 min, resuspended in 0.5 mL of Tris-EDTA buffer (pH 7.8) and stored at 4°C until used.

PFGE analysis: Analysis of DNA was carried out by pulsed-field gel electrophoresis (PFGE) analysis by following standard procedures. Briefly, DNA was digested with 10 U of *smaI* (New Eggland Biolabs, Beverly, Mass.), restricted DNA fragments were separated in 1% agarose gel in 0.5X tris-EDTA buffer by using a CHEF-DRIll apparatus (Bio-Rad Laboratories, Barcelona, Spain). Electrophoretic pulses were linearly distributed from 20 to 70 sec for a run time of 22 h. The voltage was 6 V/cm and the temperature of the electrophoresis chamber was kept at 14°C. The gels were stained with ethidium bromide and photographed. The interpretation of restriction fragment patterns was performed in accordance with recent consensus publications (Tenover *et al*., 1995).

Detection of antibiotic resistance genes by PCR: PCR was performed with a volume of 50 μL containing 4 μL of (5 mM) deoxynucIcoside triphosphate mixture, 5 μL of 10X reaction buffer, 0.2 μL (1 U) of Taq polymerasc, 2.5 μL (20 pmol) of each primer, 2 μL of streptococcal genomic DNA and sufficient double-distilled water for the 50 μL total volume. In the thermal reactor, 95°C for 7 min, followed by a total of 35 cycles, comprising denaturation at 95°C for 30s, annealing at 55°C for 1 min and synthesis at 72°C for 1.5 min, were carried out. Sequence of primers used were previously described (Jasir *et al*., 2001) for detection of erythromycin resistance genes (*ermA*, *ermB*, *mefA* and *ermTR*). The expected sizes of PCR products were 208 for *ermA*, 640 bp for *ermB*, 350 bp for *mefA* and 530 bp for *ermTR*. The PCR products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide and photographed with Polaroid film under UV light.

PCR and sequencing analysis of *emm* gene (*emm*-typing): The *emm* gene type of S. *pyogenes isolates* was determined by amplification and sequencing of the *emm* gene as described previously by Beall *et al*. (1999). The forward primer, 5'CAGTATTMMAGAAAATTAAA A3' was derived from leader sequence of the M protein gene (Martin *et al*., 2002). The antisense primer, 5'CCCTTACGGCTTGCTTCTGA3', was derived from the C repeat region of the M protein gene, which is conserved in several GAS isolates (Martin *et al*., 2002). These primers were used in PCR and for cycle sequencing. PCR was done as described above. The product was purified by using the Qia Quick PCR purification kit (Qiagcn) as described by the manufacturer. *emm* sequence was performed directly from the purified product using 6 μL of product per reaction, 4 μL of M forward primer, 8 μL of premix for the ABI 310 automated sequencer, as described by the manufacturer's instruction. The sequences obtained were subjected to homology searches with the nucleotide sequences against all known *emm* sequence of streptococcal M proteins in the GenBank in the National Institutes of Health DNA database with BLASTN (Altschul *et al*., 1997).

***emm* Restriction Profiling (ERP) analysis:** To compare isolates within the same serological M type, the *emm* genes were subjected for restriction endonucleases cleavage analysis as previously documented methods (Facklam *et al*., 2002).

Protein expression: For expression of the encoded protein, the PCR fragment was recovered and digested with *EcoRI* and *BamHI* and ligated into pGEX4T-1 vector. Following transformation of *E. coli* JMI09 competent cells, ampicillin resistant colonies were examined by the cracking gel method to identify colonies with recombinant plasmid. Plasmid DNA was prepared from a positive colony. Orientation and reading frame were verified by sequencing. A single bacterial colony containing the plasmid pGEX-4T-1 was grown in culture and induced with 0.5 mM IPTG. Cells were harvested at 1 h intervals by centrifugation at 3000×g and resuspended in fusion protein extraction buffer (Tris-HCI, 50 μLM; NaCl, 15 mM; EDTA, pH 8.5; Triton-X l.00, 1%; PMSF, 1 mM). Cells were then lysed by sonication for 2-3 min at 20 Khz with 30 sec intervals using 0.2 nm rnicrotipe and centrifuged in a sorvall SS34 rotor at 12000 rpm at 4°C for 10 min. The pellet was then resuspended in the fusion protein extraction buffer and aliquots of the whole unfractionated lysate, supernatent and pellet were analyzed for proteins on 15% SDS PAGE gels (Laemmli, 1970).

RESULTS

Antimicrobial susceptibility: Overall, 4.7% of isolates (5 of 107) were intermediately resistant and 31.8% of isolates (34 of 107) were fully resistant to erythromycin, as determined by the disk diffusion method. The five erythromycin-intermediate isolates were examined further by determination of the MICs. The erythromycin MICs for all five isolates were in the resistant range (MIC 1 to

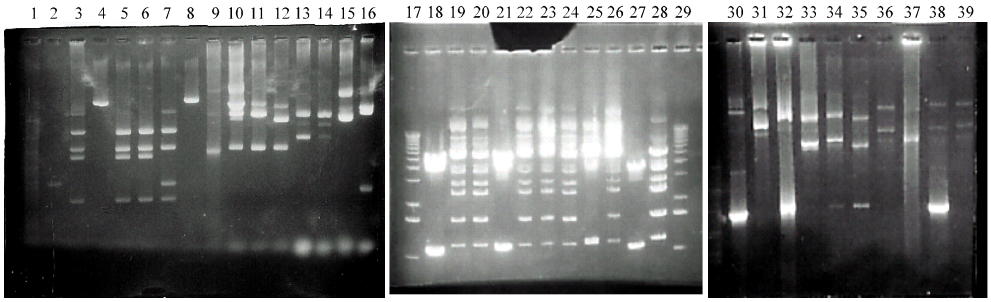

Fig. 1: PFGE profiles of *SmaI* digested genomic DNAs from 39 erythromycin resistant *S. pyogenes* M types

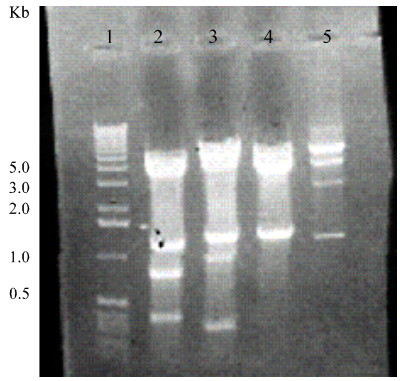

Fig. 2: 1% gel electrophoresis for *emm* gene polymorphism tests of 2 un-typed KSA isolates SA15, in lanes 2 and 4 digested with *Hind III* and SA37, in lanes 3 and 5 digested with *Mbo II* restriction enzymes cleavage respectively. Lane 1 is molecular weight marker, (1kbp DNA ladder; Gibico)

4 μg mL^{-1}). PCR analysis for erythromycin resistance genes was performed for the 39 erythromycin-resistant isolates. Of these, 17 isolates (43.6%) had the *ermA* gene alone, 14 (35.9%) had the *mefA* gene alone and 2 (5.1%) had both the *ermTR* gene and the *mefA* gene. The remaining six isolates (15.4%) had the *ermB* gene alone. The five isolate that had intermediate results in the disk diffusion test all possessed the *ermA* gene as represented in Table 1.

Gene diversity and PFGE: A genomic characterization was carried out by genomic DNA macrorestriction with *SmaI* and PFGE representing profiles of 39 erythromycin-resistant *S. pyrogenes* isolates as shown in Fig. 1. Visual and computerized analysis of the *SmaI* patters revealed 11 different unrelated patterns, where more than two band difference between two patterns was used as a criterion to define a PFGE type (Beall *et al*., 1998). Nine PFGE patterns were previously documented

Table 1: Erythromycin resistant gene determinants by PCR for 39 isolates

		No. of positive PCR strains			
Genotype	Total No.	*mefA*	*ermA*	*ermB*	*ermTR*
emm1	8	0	8	0	0
emm3	6	0	0	6	0
emm4[a]	3	3	0	0	1
emm6	3	3	0	0	0
emm9	4	0	4	0	0
emm12	5	5	0	0	0
emm28	3	3	0	0	0
emm44	2	0	2	0	0
emm75	3	0	3	0	0
$N.D_1$[a]	1	1	0	0	1
$N.D_2$	1	1	0	0	0
Total	39	16	17	6	2

[a] Two strains were positive for both *ermTR* and *mefA*, N.D non determined genotypes

patterns representing (94.9%) among these isolates and remaining two patterns (one isolate each) were unidentified (5.1%). The most prevalent PFGE pattern of the total 39 erythromycin resistant isolates in KSA in this study was represented as pattern D with 8 (20.5%) isolates and pattern G with 6 (15.4%) isolates. A limited clonal heterogeneity was characterized by the identification of these different pulsotypes.

ERP analysis: Among 39 strains tested, PFGE showed two different patterns. Isolates SA15 and SA37 were further genotyping characterization by ERP analyzed by using two different restriction enzymes; *HindIII* and *MboII* as demonstrated in Fig. 2. Three major bands were invariably present with different molecular weights.

Distribution and prevalent *emm* genes: The 39 different strains mentioned above were subjected to *emm* gene sequencing. A total of 9 different M types were found in the 37 invasive and noninvasive isolates Overall, 37 (94.9%) of 39 *S. pyogenes* clinical isolates included in this study had 5' *emm* sequences ≥95% identical to the first 160 bases of one of the *emm* or *emm*-like genes deposited in GenBank. For most of these sequences, 31/37 (83.8%) isolates showed a high level of identity to the sequence of M types which actually extended from 200 to 450 bases

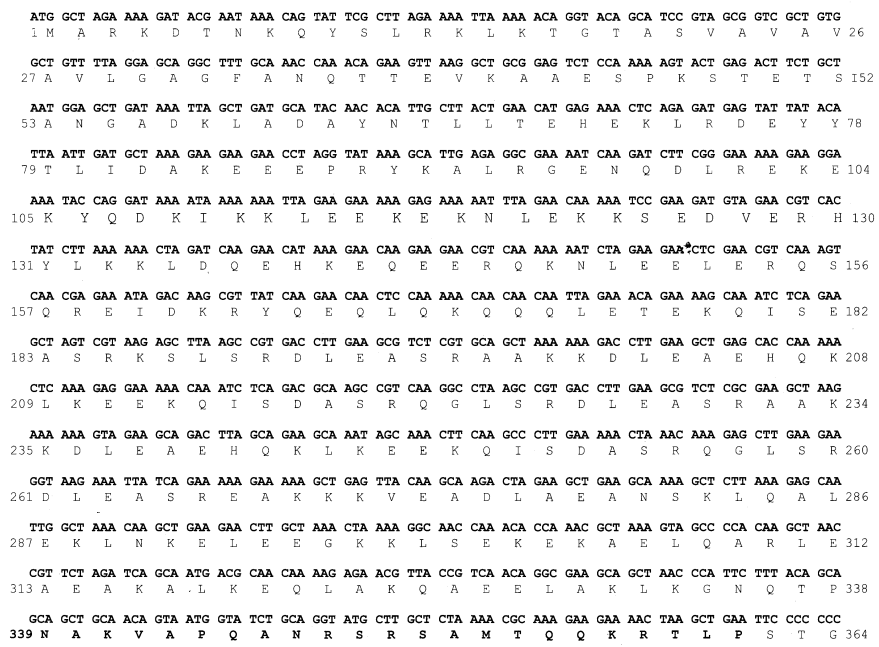

Fig. 3: Nucleotide sequence and deduced amino acid sequence for SA15 clone. The coding nucleotide sequence is shown in the upper case. The start codon ATG is the first condon

without base mutation. The sequences of other 6 (16.2%) isolates were ≥95% identical to the sequence of standard M type reference strain *emm* genes with a point mutation or frameshift for up to five amino acids. The remaining 2 of 39 (5.1%) isolates had an undocumented *emm* gene sequence. This sequence was provisionally designated as SA15 that was only 85% identical to the *emm*28 and SA37 that was only 89% similar sequence over the first 160 bases of *emm*49 type. Different identified *emm* genes were represented in Table 1.

*emm*1, *emm*3, *emm*12 and *emm*9 were the most prevalent *emm* sequences among *S. pyrogenes* isolates susceptible to erythromycin (Table 1). In descending order of frequency, they accounted 8/37 (21.6%) for M1, 6/37 (16.2%) for M3, 5/37 (13.5%) for M12 and 4/37 (9.4%) for M9, respectively, of these isolates. These four M types together 23/37 accounted for 62.2%. Besides these prevalent *emm* sequences, the following most common sequences 14/37 (37.85) were *emm*4, emm*6*, e*mm*28, *emm*44 and *emm75*, each type of which accounted for approximately ≤8% of the erythromycin-susceptible isolates. Three M types (M1, M4 and M12) were the most prevalent isolates from noninvasive group (throat swaps). While, M9 was common in the invasive group (blood isolates) and noninvasive isolates, M28 type was absent from the invasive isolates. Type M44 was more frequent in the invasive isolates than in the noninvasive isolates overall (2 of 5 versus 1 of 32; $p<0.0001$).

Sequence analysis of SA15 and its expression: Remarkably, only two new sequence type was found among the 39 isolates. A complete codoning sequence was carried out for SA15 gene from both sides, the nucleotide sequence and deduced amino acids were represented in Fig. 3. It was found that SA15 (from a pharyngitis patient) shared 85% sequence identity of amino acid sequence with the previously described *emm*28 (GenBank accession number AF091805; Fig. 4a). It shows only about 60% identity over its predicted N-terminal 180 amino acid residues and 64% over its predicted C-terminal 80 amino acid residues. SA37 shows 89% similarities over its predicted amino acid residues for M49 (Fig. 4b). The deduced SA15 sequence sharing very little similarity to other known M proteins within the type-specific region (roughly residues 18 to 92), while sharing a strong similarity within its partial signal sequence (residues 1 to 18) and residues 93 to 240 with corresponding sequences of many other GAS *emm* genes (a comparison with its best overall match with *emm*28 type deduced peptide sequence is shown in Fig. 4a). SA15 differs from M28 in the presence of an additional sixty amino acid residues at the N terminus of the mature protein and a 29-amino-acid deletion. Complete sequence of SA15 was carried out in order to cloned in expression vector and expressed it as a protein for vaccine studies.

In order to study the characteristics of SA15 protein, the coding sequence was subcloned in the expression

```
emm28   1     MARKDTNKQYSLRKLKTGTXXXXXXXXXLGAGFANQTEVKAAESPKSTETSANGADKLAD  180
              MARKDTNKQYSLRKLKTGTASVAVAVAVLGAGFANQTEVKAAESPKSTETSANGADKLAD
SA15    1     MARKDTNKQYSLRKLKTGTASVAVAVAVLGAGFANQTEVKAAESPKSTETSANGADKLAD   60

emm28   181   AYNTLLTEHEKLRDEYYTLIDAKEEEPRYKALRGENQDLREKEGKYQDXXXXXXXXXXXXX  360
              AYNTLLTEHEKLRDEYYTLIDAKEEEPRYKALRGENQDLREKEGKYQDKIKKLEEKEKNL
SA15    61    AYNTLLTEHEKLRDEYYTLIDAKEEEPRYKALRGENQDLREKEGKYQDKIKKLEEKEKNL  120

emm28   361   XXXXXDVERHYLKKLDQEHKEQEERQKNLEELERQSQREIDKRYXXXXXXXXXXXXXXXI  540
              E+KSEDVERHYLKKLDQEHKEQEERQKNLEELERQSQREIDKRYQEQLQKQQQLETEKQI
SA15    121   EKKSEDVERHYLKKLDQEHKEQEERQKNLEELERQSQREIDKRYQEQLQKQQQLETEKQI  180

emm28   541   SEASRKS-----------------------------------LSRDLEASRAAKKDLEAE  615
              SEASRKS                                   LSRDLEASRAAKKDLEAE
SA15    181   SEASRKSLSRDLEASRAAKKDLEAEHQKLKEEKQISDASRQGLSRDLEASRAAKKDLEAE  240

emm28   616   HQKLKEEKQISDASRQGLSRDLEASREAKKKVEADLAEANSXXXXXXXXXXXXXXXXXXX  795
              HQKLKEEKQISDASRQGLSRDLEASREAKKKVEADLAEANSKLQALEKLNKELEEGKKLS
SA15    241   HQKLKEEKQISDASRQGLSRDLEASREAKKKVEADLAEANSKLQALEKLNKELEEGKKLS  300

emm28   796   XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXGNQTPNAKVAPQANRSRSAMTQQKRTL  975
              EKEKAELQARLEAEAKALKEQLAKQAEELAKLKGNQTPNAKVAPQANRSRSAMTQQKRTL
SA15    301   EKEKAELQARLEAEAKALKEQLAKQAEELAKLKGNQTPNAKVAPQANRSRSAMTQQKRTL  360

emm28   976   PSTG   980
              PSTG                                  % identity
SA15    361   PSTG   364                               85%
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Fig. 4a: Alignment of amino acid sequence of SA15 of GAS isolate with M28 amino acid sequence (derived from GenBank), showing 85% homology, X represents missing amino acids, dashes are missing amino acids

```
emm49   1     MARKDTNKQYSLRKLKTGTXXXXXXXXXLGAGFANQTEVKAAESPKSTETSANGADKLAD  180
              MARKDTNKQYSLRKLKTGTASVAVAVAVLGAGFANQTEVKA E   + +++  G +  AD
SA37    15    MARKDTNKQYSLRKLKTGTASVAVAVAVLGAGFANQTEVKATEVKAAGQSAPKGTNVSAD  194

emm49   181   AYNTLLTEHEKLRDEYYTLIXXXXXXDAKEEE  258
               YN+L  E++ LR++    I   + E                          % identity
SA37    195   LYNSLWDENKTLREKQEXXXXXXEYITKIQNE  272                      89%
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Fig. 4b: Alignment of amino acid sequence of SA37 of GAS isolate with M49 amino acid sequence (derived from GenBank), showing 89% homology, X represents missing amino acids

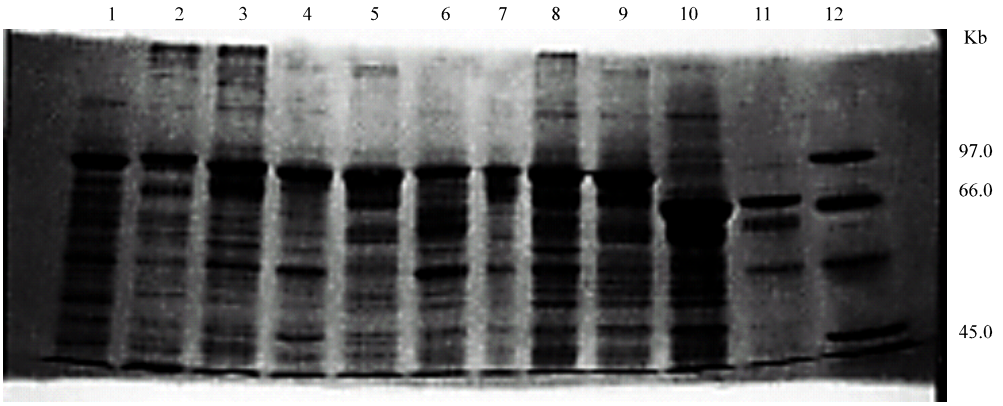

Fig. 5: SDS-PAGE for lysate from induction experiment of clone SA15 in the expression vector pGEX4T-1. Lanes 1-9 show induction 30 min time intervals, lanes 10, 11 show pellet lysate of last two samples, lane 12 is a prestained molecular weight marker; Gibco)

vector pGEX4T-1 and the protein was expressed in *E. coli* as a fusion protein with Sj26. Five hours post induction of pGEX-SA15 bacterial cell lysates, resuspended pellet of lysed cells and supernatant were run on 15% SDS-PAGE. The protein was present mostly in the total cell lysate and in the supernatant fraction. Figure 5 shows a band of ~66 kDa, thus the expected size for a protein of ~40 kDa fused with Sj26.

DISCUSSION

Resistance to macrolids, avocated for GAS infection primarily in case of beta-lactam allergy or intolerance, has been reported from many countries following overuse or abused of these drugs (Seppala *et al*., 1992). Recently, in KSA erythromycin is used in the treatment of many diseases as chlamydial, respiratory tract and mycoplasmal infections rather than streptococcal infections so the rate of consuming this drugs is increasing leading to evoke a new generation of microorganisms that resist to erythromycin antibiotics. Therefore, the rate of erythromycin resistance among Saudi GAS clinical isolates appeared comparatively high 39/107 (36.4%), compared with reported rates of 2.1 to 4.6% in Canada (Weiss *et al*., 2001), 2.6% in the United States (Ho *et al*., 2003; Green *et al*., 2005), 8.6% in Finland (Seppala *et al*., 1993) and 1.8% in Sweden (Jasir *et al*., 2001). Present findings are similar to the overall rate in Italy 25.9% (Cornaglia *et al*., 1996). The pattern of resistance phenotypes in erythromycin-resistant GAS strains in Sweden was markedly decreased (from 12 to 1.8%) of both macrolide consumption and level of erythromycin resistance (Martin *et al*., 2002). A relation between occurrence of the different macrolide resistance phenotypes and total consumption of macrolide antibiotics therefore appears conceivable. The high rates of resistance that found in KSA are probably a reflection of the high level of antibiotic usage in this community, which has also brought about high macrolide resistance rates in pneumococci (Chiu *et al*., 2001).

The erythromycin resistance phenotypes of the 39 erythromycin-resistant isolates were determined by disk test. Thirty-seven (94.9%) isolates were of the erythromycin-resistant M phenotype, 2 (5.1%) isolates were of the constitutive erythromycin-resistant non M phenotype. Genetic determinants of erythromycin resistance were investigated in all erythromycin-resistant isolates by means of PCR experiments with specific primer sets for *ermA, ermB, ermTR* and *mefA*. As predicted, all 39 resistant isolates carried one or more of these resistant genes. The resistance genotypes might show a different chronological distribution. The positive isolates of *ermA* and *mefA* were predominant. Genetic diversity was found among the *mefA* positive isolates, which revealed six PFGE different patterns corresponding to different six *emm* genotyping. Only two unique PFGE patterns were obtained for the 16 *mefA* positive isolates, while other 14 (35.9%) showed four distinct PFGE pattern (Fig. 1 and Table 1). However, 17 (43.6%) of the 39 patients were *ermA* positive isolates and showed four different patterns. Other six isolates (15.4%) were *ermB*-positive isolates showed only one pattern of PFGE. Thus, the predominance of *mefA* was in part due to the prevalence of a genetically related clone (Betriu *et al*., 1993).

This study found that the majority of strains with more than one isolate were isolated from both throat and blood sites. However, types (M1, M4 and M12) were the most prevalent isolates from patients with pharyngeal diseases which were commonly found in noninvasive disease isolates (Jasir *et al*., 2000). Despite this finding, type M1 was disproportionately represented in invasive and pharyngeal isolates, as has been reported elsewhere (Jasir *et al*., 2000). Previous studies suggested that a virulent of M1 clone was responsible for the majority of severe GAS infections that have occurred since the mid-1980s (Jasir and Schalen, 1998). Although type M3 has been reported to be prevalent in the United States, Canada and other countries (Davies *et al*., 1996), particularly for invasive isolates, this M type was second prevalent type observed in the present studying in both invasive and noninvasive isolates (6/37; 16.2%) after type M1 (8/37; 21.6%). As suggested by Green *et al*. (2005) whom showed that the major invasive types, M1 and M3, were equally prevalent in pharyngeal isolates where pharyngeal infections may have served as a reservoir for virulent GAS clones (Kaplan *et al*., 2001; Johnson *et al*., 2002). Furthermore, the M1 and M3 isolates causing invasive infections had PFGE patterns that were identical to those of concurrent pharyngeal isolates. These published results are in agreement with our observations that invasive M1 isolates and noninvasive M1 isolates shared identical PFGE profiles. Moreover *emm4* strains were isolated in Spain, Finland and Great Britain (Seppala *et al*., 1993 and 1997; Alberti *et al*., 2003) as the erythromycin-resistant type and accounted for 20% of the GAS resistant to erythromycin which accounted only less than 6% in present study.

In addition, there was also an increase in severe forms of GAS infection during the mid-1990s (Tang *et al*., 2001). Meningitis, necrotizing fasditis and toxic shock syndrome due to many unknown GAS types. These data revealed that the recent appearance of these severe forms of disease is probably a reflection of changes in the

epidemiology of the prevalent M types. The rapid introduction of new strains into a population is well documented (Viaminckx *et al*., 2005). Therefore, the introduction and dissemination of streptococcal strains with enhanced virulence potential are plausible explanations for this increase in severe forms of infection and are compatible with the disproportionate representation of type M1 in the throat and invasive isolate. Although recent studies involving a comparison of a large number of invasive isolates with control strains, there was no evidence of an association between a particular clone and invasive infection (Pruksakorn *et al*., 2000; Kaplan *et al*., 2001; Green *et al*., 2005).

Antigenic variations in the M proteins compared to published sequences were predominantly due to single base substitutions, small deletions and insertion in the 50 N-terminal residues of hypervariable region representing several new alleles (Beall *et al*., 1997). Previous studies revealed that a number of M family groups showed compensatory frameshift mutations, as in cases of *emm*55, *emm*53, *emm*80, *emm*5, *emm49*, *emm13*, *emm*33 and *emm70* (Beall *et al*., 1997). The translated sequences of the M proteins of isolates under investigation that corresponding to M1, M3, M4, M9, M12 and M75 showed complete homology in the hypervariable region. However, M44, M28 and M6 isolates were differed in sequence by point mutations in the hypervariable region with no more than three amino acid substitutions that predicting a new alleles for those M proteins. This study also showed that the GAS type distribution in KSA might be different from those in Thailand and Malaysia (Pruksakorn *et al*., 2000). In the latter two countries, acute rheumatic fever is still an important health problem, but in KSA this disease has been seen very rarely in the last 20 years. Nonetheless, the serotype distribution in any population is in constant flux (Weiss *et al*., 2001; Green *et al*., 2005). Besides serotype distribution, GAS disease epidemiology is also subject to influence from ethnic, cultural and socioeconomic factors (Kim and Lee, 2004; Sagar *et al*., 2004).

Indeed, the percentage of isolates from patients in Brazil, New Guinea Gambia, Ethiopia and Malaysia with new *emm* gene sequences is much higher than the percentage of such isolates found in this study and certain European countries (Kaplan *et al*., 1992; Coleman *et al*., 1993; Lopardo *et al*., 2005). These recent findings are consistent with a previous statement maintaining that there is a higher percentage of M-nontypeable strains from Africa than from Britain (Coleman *et al*., 1993). Sequence analysis of the 39 isolates used in this study revealed 2 novel-sequence M types of erythromycin resistance among GAS isolates. One exhibited such novel phenotype carried both *ermB* and *mefA* erythromycin resistant genes. The combination of these determinants has only been detected in Italian GAS isolates (Cornaglia *et al*., 1996) and was not identifiable with other published *emm* sequences. This finding shows the diversity of GAS strains found in KSA. These two novel *emm* sequence type, SA15 and SA37, was found in an isolate from patients with pharyngitis. The SA15 sequence type differs from M28, described previously, at two regions in the 5' hypervariable region. Other SA37 isolate shows low homology to M49 (89%) that exhibited a frameshift mutations.

M sequence typing is a useful tool for conducting epidemiological studies of streptococcal infections, particularly in an area where many GAS isolates are non-M typeable by conventional M serotyping methods. It allows not only monitoring of streptococcal carriage within regions of endemicity but also identification of types of circulating *Streptococci* that provides a useful guideline for developing a vaccine in specific area of endemicity. This study indicates that the M protein type distribution within a diverse set of GAS clinical isolates recovered from KSA during 2003 to 2004 is similar to the types distribution found within U.S. invasive GAS isolates (Kaplan *et al*., 1992). In fact, a current 26VV formulated for usage within the United State (Hu *et al*., 2002) would theoretically be effective against 64% of the 37 GAS isolates described here, which represent 7/11 of the M types included in this vaccine. It must be noted here that the type *emm4* isolates (which were the fifth most frequently occurring pharyngeal isolates in this study) together with other two new nontypeable *emm* genes have not been included in this vaccine. Nonetheless, component(s) within the 26VV did elicit bactericidal antibodies against one new type *emm 28* isolates tested (Hu *et al*., 2002).

To achieve the maximum coverage of multivalent, M type-based vaccines within individual countries or regions in the world, different formulations would be based upon specific *emm* sequence types predominant for these areas. Such determinations would optimally entail multiyear surveillance, since changes in serotype distributions do occur over extended time periods and in local communities (Espinosa *et al*., 2003; Sagar *et al*., 2004) very rapid shifts in M type can occur within the same pharyngitis season (Espinosa *et al*., 2003; Lopardo *et al*., 2005). In addition, the data presented here were not necessarily representative of the entire country of KSA. For these reasons, we hope that *emm* typing-

based surveillance is continued and expanded to locations throughout KSA. Such surveillance would be important in evaluations of the feasibility of multivalent M-based vaccines in KSA and would also be required to monitor vaccine effects on GAS populations subsequent to vaccine introduction. With vaccines targeted toward a subset of common M types, there is the possibility that identified untypeable new *emm* genes in this study and that with rarely occurring M types could increase in number. Although most isolate collections have not been population based, it still appears that targeted areas within Argentina (Lopardo *et al*., 2005), Mexico (Espinosa *et al*., 2003), Western Europe (Hollm-Delgado *et al*., 2005), Asia and North Africa (Lopardo *et al*., 2005; Viaminckx *et al*., 2005) share extensive overlap in common *emm* type, distribution with the United States (Kaplan *et al*., 1992; Brandt *et al*., 1997), which makes the concept of multivatent M protein-based vaccines more attractive. However, predominant *emm* types found in clinical isolates within specific areas of New Zealand (Viaminckx *et al*., 2005), Australia (Brandt *et al*., 2000), Chile (Martin *et al*., 2002), Malaysia, India (Sagar *et al*., 2004), Nepal, Egypt and New Guinea overlap less extensively with common *emm* types found in the United States (Brandt *et al*., 1997; Hu *et al*., 2002).

To the best of my knowledges, this study is the first report of the genotypes of GAS associated with erythromycin resistant strains in KSA. In conclusion, Nevertheless, in our area, the total level of erythromycin resistance among GAS is currently high, presumably resulting in increasing usage of macrolides in the treatment of respiratory tract infection. The results show that monitoring of GAS isolate diversity by *emm* gene typing is a useful approach for a better understanding of the epidemiology and origins of specific GAS strains and provide a basis for future studies on changes in the epidemiology of GAS, outbreak investigation and development of preventive measures and of recommendations for vaccine preparation and treatment strategies in KSA.

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