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PCR amplification of virulence factors (*emm*, *sagA*, *speA*, *speB*, *scpA*, *slo*, *hylA* and *ska* genes) of *Streptococcus pyogenes* isolated from tonsillitis and pharyngitis patients in a local population of Lahore, Pakistan

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Abstract

Tonsillitis and pharyngitis are infections often caused by *Streptococcus pyogenes*, a member of the group A *Streptococcus*. The aim of this study was to isolate *S. pyogenes* and identify the virulence genes associated with its pathogenesis in a local population of Lahore, Pakistan. The samples (n=110) were collected from patients suffering from tonsillitis and pharyngitis. They were proceeded on 5 % sheep blood agar and crystal violet agar for the subsequent isolation and purification of *S. pyogenes*. The biochemical characterization, determination of antibiogram and serum opacity factor (SOF) was performed by using standard procedures. The amplification of the selected virulent genes was carried out by PCR. Results demonstrated β- hemolytic colonies of *S. pyogenes* in 18.8 % (n=20) of the samples, which was also confirmed by coagulation with specific antisera. The 62.5 % of the strains were notably tested positive for serum opacity factor (SOF), whereas 37.5 % of the strains were tested negative. The antibiogram of *S. pyogenes* revealed sensitivity to cefotaxime (62.5 %), gentamicin (37.5 %), penicillin G (87. 5 %), clindamycin (87.5 %) and erythromycin (100 %). All strains were found to be resistant to tetracycline. PCR amplification yielded product sizes of 700 bp, 290 bp, 780 bp, 820 bp, 510 bp, 409 bp, 550 bp and 580 bp for *emm*, *sagA*, *speA*, *speB*, *scpA*, *slo*, *hylA* and *ska* genes, respectively. The presence of these virulent genes confirmed their incidence in the local population, which can be studied further to gain deeper insights into the molecular mechanisms of *S. pyogenes* pathogenicity.

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Introduction

Acute pharyngitis is a medical condition that presents itself as an acquired infection, with one of the main characteristics being sore throat in affected patients. Tonsillitis is the inflammation of the tonsils which can also occur in acute and chronic stages. Sore throat, a self-limiting form of pharyngitis, and tonsillitis do not often require antibiotic treatment because of their viral origins, but most of their episodes are increasingly attributed to Group A *Streptococcus* (GAS) infection, which accounts for more than 25 % and 10 % of the pediatric and adult cases, respectively (Dao *et al.*, 2019). *Streptococcus pyogenes*, also called as Group A *Streptococcus* (GAS), is a Gram-positive bacterium that can either survive as a commensal or also remarkably be accountable for causing a multitude of human diseases, with varying degrees of morbidity (Carapetis *et al.*, 2005). Most of the GAS diseases are reported to be mild, ranging from conventional pharyngitis (sore throat), tonsillitis, to impetigo (skin sores). However, the dissemination of GAS infecting strains to deeper tissues can result in rare but grave invasive infections such as necrotizing fasciitis and Streptococcal toxic shock syndrome (STSS) (Buckley *et al.*, 2018). Although the use of antibiotics is crucial for the prevention of complications, it has sparked serious concerns in the rise of antibiotic resistance. However, the differentiation of bacterial pharyngitis from other infections is deemed to be difficult (Sykes *et al.*, 2020).

The pathogenicity of *S. pyogenes* is accountable to a number of factors such as extracellular toxins, superantigens and proteases. *sagA* (SLS-associated gene A) (Molloy *et al.*, 2011) is the first gene in *sag* operon which encodes SLS (streptolysin S) (Nizet *et al.*, 2000), a potent cytotoxin and a characteristic feature of GAS (Biswas *et al.*, 2001). The streptococcal pyrogenic exotoxin A (SpeA) is a member of the superantigen family of staphylococci and streptococci, which has been reported to play a role in the onset of pathogenesis, which leads to toxic shock syndrome. Moreover, it is also said to be associated with successful infection of the nasopharynx with *S. pyogenes* (Kasper *et al.*, 1992). The gene that encodes SpeA protein, *speA* is present in majority of *S. pyogenes* strains that are responsible for deep tissue streptococcal disease and toxic shock syndrome, and in few of the strains that cause non-invasive diseases (Sriskandan *et al.*, 1999). Drifting from its name, streptococcal exotoxin B (SpeB) is not an exotoxin, but rather an extracellular cysteine protease, being one of the first which were identified in group A streptococci (Yu and Ferretti, 1991). SpeB is encoded by the chromosomal *speB* gene, a highly conserved gene present in almost all of the GAS strains (Shumba *et al.*, 2019). Although the SpeB sequence is reported to be highly conserved, the expression of the *speB* gene can vary greatly among all GAS strains (Carroll and Musser, 2011). The streptococcal C5a peptidase, ScpA, is a specific serine protease enzyme that is present on the surface of *S. pyogenes* (Wexler and Cleary, 1985; Terao, 2012). It is a highly conserved proteolytic enzyme which is reported to be localized to cell surfaces of almost all GAS strains that inhibit phagocytic activity at the GAS colonization site. The gene that encodes this enzyme, *scpA*, is found to be present among clinically isolated GAS strains and is located within the *mga* regulatory operon (Cleary *et al.*, 1992; Walker *et al.*, 2014).

GAS strains are generally categorized on the basis of their cell surface protein called M protein, which is a major virulence factor primarily encoded by the *emm* gene (Shea *et al.*, 2011). *slo* gene of *S. pyogenes* is responsible for the production of exotoxin streptolysin O which is cytolytic mode of action (Savic and Ferretti, 2003). The production of hyaluronidase by *S. pyogenes* and other group B/C/G streptococci has been revealed to be an important virulence factor in the spread of disease. Hyaluronic acid (HA), the substrate of hyaluronidase, is a polymer comprised of N-acetylglucosamine and glucuronic acid residues.

It is present in the connective tissues of humans and is also a major component of the capsule of GAS strains. The presence of a hyaluronic acid capsule, as well as the hyaluronidase enzyme which is capable of degrading the capsule is a remarkable feature of *S.*

pyogenes. The gene encoding the extracellular hyaluronidase enzyme, *hylA*, is responsible for the expression of HylA. Although all of the GAS strains possess the *hylA* gene, very few numbers of clinical isolates are responsible for the production of HylA when it is experimented upon *in vitro*, which marks its dependency on the function of the serotype and the type of strain (Hynes *et al*., 1995; Starr and Engleberg, 2006). *ska* gene expresses streptokinase which is an antigenic protein resulting in complement activation by inflammation (Molaee *et al*., 2013). The study aimed to determine the presence of virulence genes of *S. pyogenes* from patients affected by pharyngitis and tonsillitis in a local population in Lahore, Pakistan.

Materials and methods

Place of work

The research work was conducted in the Microbiology laboratory, at the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore.

Sample collection, isolation and biochemical identification of S. pyogenes

Samples were collected by the help of sterile swabs (Amies®) from 110 patients suffering with tonsillitis and pharyngitis from a tertiary care hospital of Lahore, Pakistan from October 2017 to December 2017. The swabs were pressed firmly along the tonsils and were proceeded within 72 hours.

The samples were streaked primarily on blood agar and placed in into the incubator for 24-48 hours in 5 % CO_2 at 35-37 ºC (Gera and McIver, 2013). After incubation, media was observed for the growth. The strains were further confirmed to be *S. pyogenes* by β- hemolysis on crystal violet blood agar. The presumed *S. pyogenes* colonies were then streaked on nutrient agar for the performance of Gram staining and biochemical tests (Cheesbrough, 2002), bacitracin sensitivity and co-agglutination with Group A antisera (Perez-Trallero *et al*., 2007). *S. pyogenes* was finally confirmed by commercially available identification system Rapid ID 32 STREP® kit (Liang *et al*., 2008).

Establishment of antibiogram

All isolates were tested for the antibiotic resistance by Kirby Bauer disk diffusion method in accordance with the standards recommended by Clinical and Laboratory Standards Institute (CLSI, 2011). McFarland solution (0.5 %) was used to inoculate the Mueller Hinton agar (MHA) with 5 % sheep blood. *S. pyogenes* pure colonies were streaked on the plates which were then administered with penicillin G (10 IU), erythromycin (15 µg), cefotaxime (30 µg), clindamycin (2 µg), gentamycin (30 µg) and tetracycline (30 µg). After incubation of 24 hours at 37 ºC, the zones of inhibition (mm) were measured and compared to the CLSI manual standards for measuring sufficient resistant (R), sensitive (S) and intermediate (I) isolates, respectively.

Serum opacity factor by microwell plate method

The determination of serum opacity factor (SOF) was performed by using microwell plate method. The 100 µl of inactivated horse serum was added into each well of the 96-well microtiter plate, which was followed by the addition of 10 µl culture supernatant from each strain in the wells. The plate was then placed in the incubator for 24 hours at 37 ºC. After incubation, 100 µl of normal saline was added to each well before the test results were examined (Courtney *et al*., 2003).

Isolation of genomic DNA and PCR amplification of virulence genes

Extraction of genomic DNA was carried out by cetyl trimethylammonium bromide (CTAB) method of Wilson (2001), as described in Ausubel *et al*. (2003). Extracted genomic DNA was suspended in 50 µl of TE buffer and stored at -20 ºC until it was used as a template for PCR. For the detection of virulence genes of *S. pyogenes*, the PCR reaction mixture (25 µL) contained 2.5 µL of 10x Taq Buffer (Thermo Scientific), 0.25 µL of 5 U/µL DNA taq polymerase (Thermo Scientific), 1.25 µL of 0.5 mM dNTPs (BioShop Canada), 3 µL of 1.5 mM $MgCl_2$ (Thermo Scientific), 14 µL of nuclease free sterile water, 2 µL of DNA template, 1 µL of primer F (100 pmol/ µL) (Macrogen) and 1 µL of primer R (100 pmol/ µL)

(Macrogen). The details of the primers for seven virulence genes *emm*, *sagA*, *speA*, *speB*, *scpA*, *slo*, *hylA* and *ska* are given in Table 1.

The amplification reaction was performed in a PCR thermal cycler (Veriti 96-Well Thermal Cycler; Applied Biosystems) with 30-cycle reactions for *emm*, *sagA*, *speA*, *speB* and 35-cycle reactions for *scpA*, *slo*, *hylA*, *ska* genes respectively, where the initial denaturation was at 94 °C for 5 minutes, subsequent denaturation at 94 °C for 30 seconds, annealing temperature (Table 1) for 30 seconds, initial extension at 72 °C for 1 minute and the final extension at 72 °C for 20 minutes, which was followed by a 4 °C hold. A volume of 5 µL of PCR product was analyzed by using agarose gel electrophoresis (Sambrook and Russell, 2001). The 1 % gel was run in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), with 0.5 µg/ mL ethidium bromide at 80 V for about 1 hour using a Mini-Sub Cell horizontal gel apparatus (Bio-Rad). Gel was visualized under UV light using Molecular Imager Gel Doc XR+ System (Bio-Rad).

Statistical analysis

All experiments were performed in triplicate, where the mean, standard error and standard deviation was calculated. The significance of the data ($p \leq 0.05$) was evaluated using SPSS (v. 23.0).

Results

Out of 110 samples, 20 samples showed β- hemolysis and were considered to be positive while 90 samples were negative for *S. pyogenes*. The samples were collected from males and females of 0-50 years who were suffering from tonsillitis and pharyngitis.

The patients of chronic tonsilitis were under antibiotic treatment for one week prior to sampling.

Table 1. Primer sequences for virulence genes of *S. pyogenes*.

No.	Virulence genes	Primer sequence 5'-3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
1.	*emm*(F)	GGGAATTCTATTGCTTAGAAAATTAA	51°C	Variable	Musser *et al.* (1995)
	emm(R)	GCAAGTTCTTCAGCTTGTTT			
2.	*sagA*(F)	AATTGAGCTAGCCTTGTCCTTG	54 °C	20-25	Fontaine *et al.* (2003)
	sagA(R)	TTTACCTGGCGTATAACTTCC			
3.	*speA*(F)	ATGGAAAACAATAAAAAAGTATTG	51 °C	708	Musser *et al.* (1995)
	speA(R)	TTACTTGGTGTTAGGTACCTTC			
4.	*speB*(F)	GTTGTCAGTGCAACTAACCGT	54 °C	1.484	Musser *et al.* (1995)
	speB(R)	ATCTGTGTCTGATGGATATGCTT			
5.	*scpA*(F)	CAATCCCCAAAAAACCATCACC	56 °C	416	Musser *et al.* (1995)
	scpA(R)	CATACATCGTTGCTGCTGAAGC			
6.	*slo*(F)	TGGATGATATGCTTAACTCTAACGA	56 °C	Variable	Zhu *et al.* (2017)
	slo(R)	TCAGTGTGATCTTCTTCGCTCT			
7.	*hylA*(F)	ATGGGATCCATGTATGAACACGCT	56 °C	Variable	Hynes *et al.* (2000)
	hylA(R)	AACAAGCTTTATTTTGTTTCCTAAGATA			
8.	*ska*(F)	AACCTTGCCGACCCAACCTGT	58 °C	513	Musser *et al.* (1995)
	ska(R)	GTGAACAGTTTCAAGTGACTGCGAT			

The biochemical characterization of *S. pyogenes* is given in Table 2. The presence of *S. pyogenes* was confirmed by coagulation with specific antisera. After 1 min of mixing the reagent A, agglutination occurred which showed positive reaction (Fig. 1). In a microtiter plate, the strains which were clearly visible (opaque or white) showed strongly positive serum opacity reactions, whereas others with transparent appearance showed the absence of serum opacity factor (Table 3). Based on CLSI standards, the results for the antibiogram were elucidated. All isolates were observed to be resistant to at least one of the antibiotics tested. None of the isolates were found to be sensitive to tetracycline (Table 4). 87.5 % and 12.5 % of the *S. pyogenes* strains were found to be sensitive and resistant to penicillin G. All *S. pyogenes* strains were erythromycin sensitive.

Clindamycin sensitivity was very high (87.5 %). All strains of *S. pyogenes* were resistant to tetracycline

and gentamycin (Table 4). The isolation of genomic DNA for all eight strains of *S. pyogenes* is shown in Fig. 2, which demonstrated the presence of DNA in all strains. The PCR amplification of virulence genes of *S. pyogenes* was performed, which yielded product sizes of 700 bp, 290 bp, 780 bp, 820 bp, 510 bp, 409 bp, 550 bp, and 580 bp for *emm*, *sagA*, *speA*, *speB*, *scpA*, *slo*, *hylA*, and *ska* genes, respectively (Fig. 3).

Table 2. Cultural, microscopic and biochemical characters of *S. pyogenes*.

Sr. No.	Tests	Results
1	Growth on crystal violet blood agar	White grayish colonies
2	Growth on MacConkey agar	No growth
3	Gram Staining	Gram positive purple color cocci shaped bacteria arranged in chains and pair form
3	Catalase test	No formation of bubbles
4	Oxidase test	Negative result, no appearance of color
5	Bacitracin sensitivity	Positive, a clear zone of hemolysis
6	SIM test	No color change in inoculated SIM after addition of Kovac's reagent indicates negative result
6	TSI test	Color change in inoculated TSI Agar indicates sugar fermentation
7.	6.5 % NaCl test	No turbidity, bacteria were unable to grow in 6.5 % salt broth

Table 3. Resence or absence of SOF in *S. pyogenes* strains.

Serotype	SOF (+) 62.5 %	SOF (-) 37.5 %
1		_
2	+	
3	+	
4	+	
5	+	
6		_
7	+	
8		_

Discussion

S. pyogenes is an important human pathogen, characterized by the infections it causes in humans (Chiang-Ni and Wu, 2008). Although its infections are reported in all months of a year but high incidence was reported during October to November (Hoffmann, 1985). *S. pyogenes* is Gram positive, non-motile, facultative anaerobe, round to ovoid-coccus in shape (Patterson, 1996), non-spore forming bacterium and showed β-hemolysis on blood agar (Liang *et al.*, 2008). Its biochemical features were already reported by Al-Kareem *et al.* (2014), which were in agreement with the features of our bacterial strains. According to Pichichero *et al.* (1999), *S. pyogenes* cultures sensitive to bacitracin are grouped as Group a Streptococcus (GAS) which was followed in this study. The β-hemolytic streptococci possessed specific cell wall antigen which can be detected by agglutination of specific antisera during Lancefield serotyping. In our present study, eight species of *S. pyogenes* were confirmed by Lancefield serotyping (Fig. 1) which is in agreement with previous literature (Slotved and Hoffman, 2017). The cell surface of *S. pyogenes* consists of a protein that consists of C-terminal and N-terminal. Its C-terminal binds with fibronectin where interaction of N-domain with serum makes its appearance, as opaque. In this way, the cell surface protein is involved in opaqueness of serum (Courtney *et al.*, 2003; Courtney and Pownell, 2010).

Table 4. Antibiogram of *S. pyogenes*.

Sr. No.	Antibiotics	Susceptibility patterns (in percentage) according to zones in diameter (in mm)		
		Sensitive	Intermediate	Resistant
1	Penicillin G (10 IU)	87.5 %	-	12.5 %
2	Erythromycin (15 mcg)	100 %	-	-
3	Cefotaxime (30 mcg)	62.5 %	-	37.55 %
4	Tetracycline (30 mcg)	-	-	100 %
5	Clindamycin (2 mcg)	87.5 %	-	12.5 %
6	Gentamycin (30 mcg)	37.5 %	12.5 %	50 %

Its role is to induce the antibodies to *S. pyogenes* in human, rats and rabbits. SOF (+) strains are related to impetigo and acute glomerulonephritis where SOF (-) strains are associated with throat inflammation (Courtney *et al.*, 2003). Here in this study, among eight isolates of *S. pyogenes*, SOF was detected in five strains (62.5 %) (Table 2). Our observations are in accordance with the findings of Timmer *et al.* (2006). Out of eight isolates of *S. pyogenes*, all were found 100 % sensitive to erythromycin whereas Cantón *et al.* (2002) reported 90 % sensitivity. In our study, 87.5 % of the strains were found to be sensitive to penicillin and clindamycin.

Fig. 1. Identification of *S. pyogenes* on reaction card by using Rapid ID 32 STREP® Kit.

According to Ray *et al.* (2016), GAS strains were often susceptible to be sensitive to penicillin. Our findings were in contradiction with Tamayo *et al.* (2005) who reported clindamycin resistance but agreed about resistance to tetracycline. All of the isolates were found to be sensitive to cefotaxime (62.5 %), while it was also important to note the increasing resistance to it (37.55 %) along with tetracycline, due to their incessant use among Pakistani population.

In this study, the virulence genes of *S. pyogenes* (*emm*, *sagA*, *speA*, *speB*, *scpA*, *slo*, *hylA*, *ska*) were amplified (Fig. 3). The amplified product of *emm* gene of *S. pyogenes* was seen to be a 914 bp fragment of the target gene, which was reported by Khosravi *et al.* (2016). Borek *et al.* (2012) reported the amplification fragment size of *speA* gene to be 576 bp. Another study reported its amplicon size to be 248 bp (Vlaminckx *et al.*, 2003). Dunne *et al.* (2013) detected the amplification fragment size of *speB* gene in their study to be of 77 nt (nucleotides), respectively, while also reporting the product size of 346 nt of *speB* gene found in the study of McMillan *et al.* (2010). In contradiction, a product of size 952 bp of *speB* was amplified in the study of Borek *et al.* (2012).

According to Vlaminckx *et al.* (2003), the amplicon size of the *speB* gene was seen to be 955 bp in their study. Dmitriev *et al.* (2004) reported the size of the group A streptococci (GAS) amplicon for *scpA* gene to be equal to 306 bp.

In another study, the product size of the PCR amplification of *scpA* gene was found to be 622 bp (Borek *et al.*, 2012). Jing *et al.* (2006) reported the amplicon size of *ska* gene of *S. pyogenes* to be of 409 bp, while another study reported the fragment size of the amplified gene product to be 439 bp (Mayfield *et al.*, 2014).

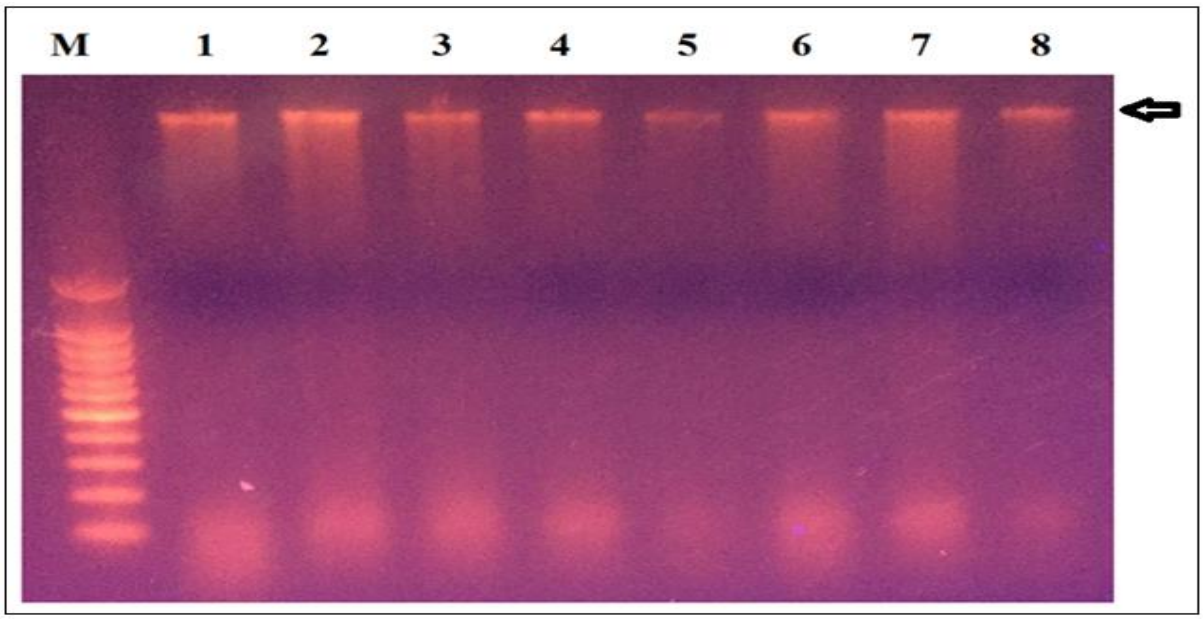

Fig. 2. Isolation of genomic DNA of eight different strains of *S. pyogenes*.

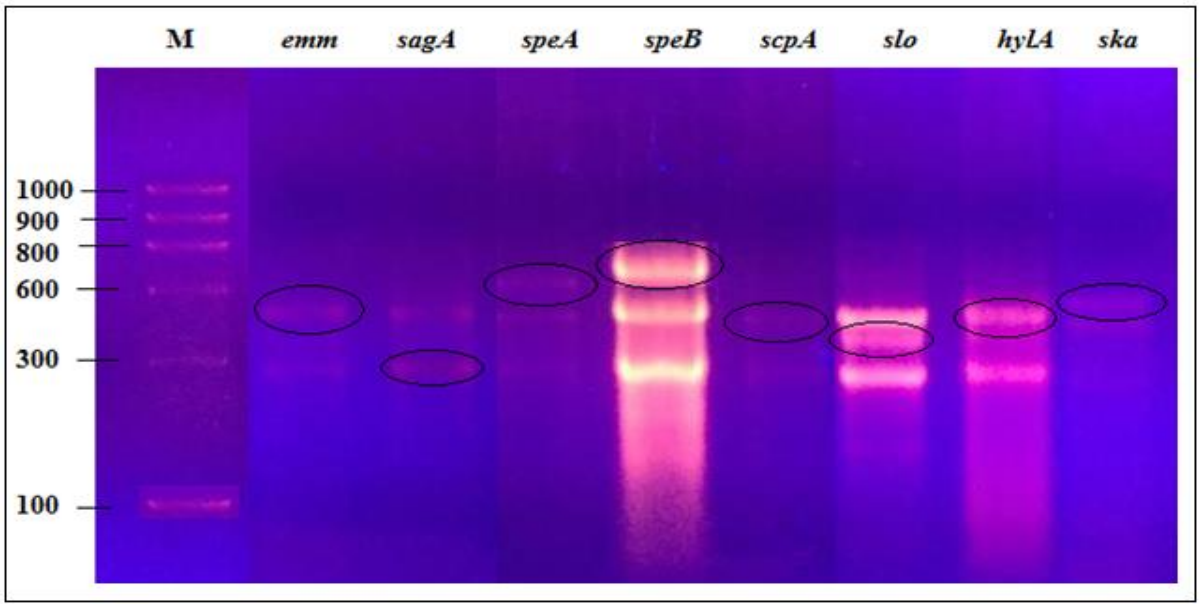

Fig. 3. PCR amplification of virulence genes of *S. pyogenes*. The size of the amplified bands are given below; *emm*= 700 bp, *sagA* =290 bp, *speA* = 780bp, *speB* = 820 bp, *scpA* = 510 bp, *slo* = 409 bp, *hylA* = 550 bp and *ska* = 580 bp.

The amplified product of *hylA* gene of *S. pyogenes* was seen to be of 1296 bp, as reported by Mirjamali *et al.* (2014). Mayfield *et al.* (2014) detected the amplicon size of the *ska* gene to be of 315 bp in their study.

Conclusion

S. pyogenes is an important pathogen which is attributable to the infections of the upper respiratory tract. Proper preventive measures, the use of proper antibiotics as observed from culture sensitivity tests as well as early diagnosis can be helpful in avoiding the complications caused by it.

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