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Research Article

Prevalence and Characterization of *Streptococcus pyogenes* Clinical Isolates from Different Hospitals and Clinics in Mansoura

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Streptococcus pyogenes are associated with many bacterial diseases in both humans and animals and are capable of causing a multitude of human diseases. *S. pyogenes* isolates were identified by their bacitracin sensitivity, positive *spy1258* detection, and positive GAS latex agglutination. Different isolates were typed serotypically and genotypically by BOX-PCR. Different virulence factors were identified in *S. pyogenes* isolates. In addition, antimicrobial resistance was tested to eleven different antibiotics. Furthermore, the resistance mechanisms were determined phenotypically by the disc diffusion method. Finally, the correlation between both molecular and serotypes identified and the profile of virulence factors and clinical and geographical sources was determined for all isolates. Thirty-eight *S. pyogenes* isolates were collected from different clinical sources. Resistance testing indicated high resistance to mostly used antibiotics except amoxicillin/clavulanic acid, amoxicillin, and ampicillin. Serotyping results indicated five different serotypes, M1, M2, M3, M4, and M6, in *S. pyogenes* isolates, while six isolates were identified as untypeable. In addition, positive PCR results identified most of the tested SAgs genes in which *speJ* gene was mostly identified followed by *speI, speC,* and *ssa* genes being identified in 81.6%, 63.3%, 60.5%, and 60.5%, respectively. However, *speH* was the least detected. In contrast, *speL, speM*, and *smeZ* genes could not be detected in all tested isolates. Finally, BOX-PCR molecular typing was a more effective clustering method when compared to the serotyping method in all *S. pyogenes.* In conclusion, the isolates in this study were highly resistant to mostly used antibiotics. M1 was the most identified serotype. No significant association was found between serotypes, BOX-PCR cluster groups, and SAgs genes profiles. However, by the application of BOX-PCR, effective molecular typing was obtained.

1. Introduction

Streptococcus species are associated with many bacterial diseases in both humans and animals. Arthritis, neonatal sepsis, meningitis, and pneumonia are some examples of diseases in humans, while in animals they mainly cause mastitis [1]. *S. pyogenes* is differentiated as group A streptococcus (GAS) as it contains N- acetyl glucosamine linked to rhamnose polymer [2]. *S. pyogenes* is capable of infecting humans, mainly through adhesion and colonization of the host mucosal surface epithelial cells of the upper respiratory tract [3]. Furthermore, the pathogenicity of *S. pyogenes* is apparent in some different mild *infectious* diseases, such as mild pharyngitis and skin infections, in addition to severe ones, such as streptococcus toxic shock and necrotizing fasciitis. The infection rate is more than 600 million infections annually [4]. A variety of virulence factors are associated with the severity of GAS infection including streptolysin O and S (hemolysin), streptokinase, streptodornase, M protein and its related protein, hyaluronic acid capsule, hyaluronidase, the cysteine protease SpeB, superantigen proteins (SAgs), and several phage-encoded exotoxins. M-like protein is a term applied to the surface protein that resembles the M protein in its structure [5]. Virulence factors are equally distributed within *S. pyogenes*; some are encoded by chromosomes, while others depend on the presence of mobile genetic elements. Confirmation of their presence or absence is considered as a simple clinical diagnosis method [6]. M protein is the most analyzed virulence factor which can be used in the serotype classification of *S.*

pyogenes. SAgs contribute to GAS pathogenicity based on their immune stimulatory activity. SAgs gene distribution has been used as a method for the detection of genomic heterogeneity, the correlation between gene contents, and the determination of clinical manifestation [7].

This study aimed to identify *S. pyogenes* in different clinical sources and detect the antibiotic susceptibility and virulence factors associated with this organism. Finally, this study aimed to classify the isolates phenotypically by serotyping and genotypically by BOX-PCR.

2. Materials and Methods

2.1. Bacterial Strains [8]. During the period from April 2017 to July 2018, a total of 342 isolates were collected from different hospitals and clinics in Mansoura city in the Dakahlya governorate in Egypt. These specimens were collected from different sources (burn, sore throat, urine, blood, and vaginal smears) at Burn and Cosmetics Center (BCC), Mansoura International Hospital (MIH), Mansoura University Hospital (MUH), Pediatric University Hospitals (PUH), and different clinics in Mansoura city, Dakahlya governorate, Egypt. As a result, seventy isolates were identified as beta-hemolytic streptococci by Gram stain, blood hemolysis, and catalase production test. After the detection of isolates, they were cultured on brain heart infusion broth and stored in 50% v/v glycerol stock in −80 till use.

A reference strain ATCC19615, obtained from the MIRCENS center (Faculty of Agriculture, Ain Shams University, Egypt), was included as a positive control.

2.1.1. Ethics Statement. The experimental protocol conducted in this study complies with the ethical guidelines adopted by the Ethics Committee in the Faculty of Pharmacy, Mansoura University, which accepted this study. This study was conducted according to the Helsinki Declaration.

2.2. Phenotypic Identification of S. pyogenes Isolates. The bacitracin sensitivity test was conducted according to the method of Abraham and Sistla [9]. In brief, after adjusting the optical density (OD) of the overnight culture to about 10^6 CFU/ml, it was spread by sterile swab on a blood agar plate, followed by placing a bacitracin disc in the center of each plate. After overnight incubation, the diameter of the inhibition zone was measured by a suitable tool.

2.3. Determination of the Antimicrobial Sensitivity of the Isolates. All the identified GAS in addition to the standard strains ATTCC19615 were subjected to antibiotic sensitivity test, by using Kirby-Bauer antibiotic disc diffusion method [10] according to the standard values provided by CLSI 2015 [11]. Eleven different antibiotics were used in the study, namely, amoxicillin, amoxicillin/clavulanic acid, ampicillin, ceftriaxone, cefepime, erythromycin, azithromycin, tetracycline, gentamicin, levofloxacin, and vancomycin.

2.4. Identification of Resistance Phenotype. According to the methods mentioned previously [12, 13] after adjusting the OD to about 10^6 CFU/ml, all strains were inoculated on Mueller–Hinton agar plates followed by the addition of erythromycin and clindamycin discs 1 cm apart. Isolates in this study were classified either possessing cMLS (constitutive macrolide-lincosamide streptogramin B resistance phenotype), iMLS (inducible macrolide-lincosamide streptogramin B resistance phenotype), or M phenotype (resistant to macrolide but susceptible to lincosamide and streptogramin B antibiotic) as previously reported by Wu et al. [12] and Björnsdóttir et al. [13].

2.5. Serotyping Reactions. In glass capillary tubes, different antisera specific to M-type were used for the determination of strain serotype according to Johnson et al. [14].

2.6. Phenotypic Identification of Some Virulence Factors of β-Hemolytic Streptococci Isolates. All GAS and ATTC 19615 were subjected to phenotypic identification of virulence factors.

2.6.1. Streptokinase Production (3rd Edition of European Pharmacopeia). *S. pyogenes* isolates in addition to the standard strain were cultured in 10 ml BHI broth followed by incubation overnight at 37°C with shaking at 150 rpm. The bacterial enzyme was collected from the supernatant by centrifuging at 3000 rpm for 15 min. Streptokinase precipitate was obtained by mixing the supernatant with an equal volume of 40% (v/v) cold alcohol (prepared) and freezing at −20 for 48 hrs. The precipitated streptokinase was collected by centrifugation at 10,000 rpm for 15 min at 4°C followed by drying in the air for half an hour and dissolution in one-tenth of its original volume PH (7.2) phosphate-buffered saline. The purified enzyme was stored by freezing at −20 until utilization. Clot formation was induced by mixing 200 μl of thromboplastin with 100 μl diluted plasma solution (1 ml of sterile plasma to 5 ml phosphate-buffered saline PH 7.2) and both are prewarmed for 30 min at 37°C. Enzyme activity was tested by the addition of 1 ml of each enzyme solution. The lysis of the clot was observed for 30 min depending on the enzyme activity [15].

The assay of streptokinase by clot lysis was performed in a sterile Eppendorf with known weight ($W0$) containing nearly 500 μl human venous blood. Clotting of the blood was enhanced by incubation for 45 min. After the aspiration of the total fluid produced, the weight of each Eppendorf was measured ($W1$). The clot weight was determined by calculating the Eppendorf's weight difference in the presence and absence of the clot in each tube. Clot lysis activities of different isolates were tested using 200 μl of the enzyme extract. All the Eppendorf tubes were then incubated for 2 hrs followed by fluid aspiration. For the quantitative determination of the enzyme activity, the difference in the weight before and after lysis ($W2$) was measured. Negative control was prepared using 200 μl distilled water instead of enzyme extract [16].

The results were interpreted as

$$\%\ \text{of clot lysis} = \frac{\text{difference in the clot weighrt before and after lysis}}{\text{weight of the clot before lysis}} \times 100. \quad (1)$$

2.6.2. Streptodornase Production [17]. The method of identification is based on its DNase activity that can be detected in agarose gel electrophoresis. All isolates were cultured separately in 5 ml BHI broth. After overnight incubation at 37°C, each fresh culture was then centrifuged at 3000 for 15 min at 4°C. The supernatant of each isolate was used for the identification of the streptodornase activity. For the detection of streptodornase activity, 15 μl of each solution to be tested for streptodornase activity was mixed with an equal volume of DNA (from the tested isolates) and incubated at 37°C for 2 hrs (determined experimentally). The reaction mixture was subjected to 1.5% agarose gel electrophoresis at 80 mV for 30 min and was examined under the UV lamp. DNA in phosphate buffer was used as a negative control. Isolates that result in disappearance or fading of the DNA bands were considered as streptodornase producers [17].

2.6.3. Quantitative Detection of Biofilm Production [18]. Biofilm production was tested in microtiter plates by the adsorption method. One pure colony of the isolates was cultured in 5 ml TSB and incubated overnight at 37°C. After adjusting the fresh culture OD to 0.23–0.27, 200 μl aliquots were inoculated into a 96-well sterile microtiter plate with a flat bottom and incubated overnight at 37°C without shaking. Each well was aspirated to remove the content followed by washing with 200 μl sterile physiological saline three times. After washing the wells, the plate was vigorously shaken to remove nonbiofilm producing cells. Biofilm producing cells were fixed by the addition of 150 μl absolute methanol in each well followed by incubation for 20 min. Staining was performed using 150 μl of 1% crystal violet for 15 min. After the washing step to remove the excess dye, 150 μl of 33% (v/v) glacial acetic acid was added to solubilize the dye taken by the attached biofilm. Results were recorded by measuring the plate absorbance at 490 nm.

2.7. Molecular Detection of S. pyogenes and Some Virulence Factors. Extraction of the chromosomal DNA and detection of the virulence genes by polymerase chain reaction (PCR): DNA was extracted using a bacterial specific DNA isolation kit (Gene JET Genomic DNA Purification kit k0721) according to the manufacturer's instructions. *S. pyogenes* detection was performed using *Spy1258* listed in Table 1.

The virulence factors of *S. pyogenes* SAgs genes (*speA, speC, speG, speH, speI, speJ, speK, speL, speM, ssa*, and *smeZ*) were screened by PCR using the primers listed in Table 1. In a total of 25 μl, the reaction volume was prepared by mixing 12.5 μl master mix (My Red TaqMix, Bioline, United Kingdom), 1 μl (0.5 μM) each of both forward and reverse primers of the tested gene, and 3 μl of the template DNA and nuclease-free water. Negative control was prepared by using nuclease-free water. All PCR reactions were performed using Cycler 003 PCR Machine (A & E Lab (UK)) under the following conditions: 2 min of initial denaturation at 94°C, 35 cycles of denaturation (30 s at 94°C), annealing (30 s as indicated in Table 1), extension (60 s at 72°C), and a final extension at 72°C for 10 min. Finally, PCR products were analyzed by electrophoresis through 1% agarose gel and then stained with ethidium bromide and visualized under UV light.

2.8. BOX-PCR. The 22-mer BOXA1R oligonucleotide (CTA CGG CAA GGC GAC GCT GAC G) was used to generate BOX-PCR profiles [20]. Amplification reactions were performed in volumes of 25 μl reaction volume prepared by mixing 12.5 μl master mix (My Red TaqMix, Bioline, United Kingdom), 2 μl (0.5 μM) of Air BOX-PCR primer, and 3 μl of the template DNA and nuclease-free water. Negative control was prepared by using nuclease-free water. BOX-PCR was performed using Cycler 003 PCR Machine (A & E Lab (UK)) starting with an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 2 min at 53°C, and 2 min at 72°C with a final elongation step of 5 min at 72°C. Finally, PCR products were analyzed by electrophoresis through 1% agarose gel and then stained with ethidium bromide and visualized under UV light. DNA fingerprints of strains were first compared for similarity by visual inspection of band patterns. Variations in intensity were not taken as differences. Different migration bands were considered identical when all scored bands in each pattern had the same migration distance. Finally, the patterns of all of the isolates were analyzed more rigorously using the UPGMA (unweighted pair group method with arithmetic mean) software following the indicated software recommendations.

2.9. Statistical Analysis. A Chi-square test was used for statistical analysis where the significance of the obtained results was judged at the 0.05 level.

3. Results

3.1. Collection, Isolation, and Identification of the Isolates. In this study, in a total of three hundred forty-two clinical isolates, seventy clinical isolates were identified as β-hemolytic *Streptococcus* species. The isolated colonies were small, grey, β-hemolytic, and negative for catalase production test indicating no effervescence produced by both slide methods in addition to the tube method. After examination under the microscope, all isolated colonies produce violet ovoid and coccid shaped bacteria in pair or chain arrangement. Out of 342 clinical specimens, thirty-eight

Table 1: Different oligonucleotides used in this work to detect different *S. pyogenes* genes.

Gene name	Type	Nucleotide sequences (5′ to 3′)	Amplicon size (bp)	Tm (°C)	Ref.
spy1258	Fw	AAAGACCGCCTTAACCACCT	407	56	[19]
	Rv	TGGCAAGGTAAACTTCTAAAGCA			
speA	Fw	AGGTAGACTTCAATTTGGCTTGT	576	59	
	Rv	GGGTGACCCTGTTACTCAC			
speC	Fw	GCCAATTTCGATTCTGCCG	405	59	
	Rv	TGCAGGGTAAATTTTTCAACGAC			
speG	Fw	TGGAAGTCAATTAGCTTATGCA	384	59	
	Rv	GCGAACAACCTCAGAGGGCAAA			
speH	Fw	TGAGATATAATTGTCGCTACTCAC	480	59	
	Rv	CCTGAGCGGTTACTTTCGG			
speI	Fw	TTCATAGACGGCGTTCAACAA	176	59	
	Rv	TGAAATCTAGAGGAGCGGCCA			
speJ	Fw	TCCTTGTACTAGATGAGGTTGCA	286	59	[6]
	Rv	GGTGGGGTTACACCATCAG			
speK	Fw	CCTTGTGTGTGTATCGCTTG	568	59	
	Rv	TTGCTGTCCCCCATCAAA			
speL	Fw	CCTGAGCCGTGAAATTCCC	657	59	
	Rv	ACACCAGAATTGTCGTTTGGT			
speM	Fw	ATCGCTCATCAAACTTTTC	496	59	
	Rv	CCTTGTGTGTGTATCGCTTG			
ssa	Fw	AAGAATACTCGTTGTAGCATGT	678	59	
	Rv	AATATTGCTCCAGGTGCGG			
smeZ	Fw	TTTCTCGTCCTGTGTTTGG	246	59	
	Rv	TTCCAATCAAATGGGACGGAGAAC			

(11.1%) isolates were identified by bacitracin sensitivity, positive GAS kit latex agglutination, and positive *spy1258* gene. Concerning clinical sources, *S. pyogenes* isolates were 22 (57.9%) isolates from burn and 16 (42.1%) isolates from sore throat.

3.1.1. Serological M Protein Typing. Thirty-two isolates (84.2%) were classified into five serotypes; however, the remaining six isolates (15.8%) could be identified as untypeable. In addition, M1 was the most commonly identified serotype (39.5%) followed by M3 (18.4%) and M2 (13.15%) serotypes. In contrast, M4 (5.25%) and M6 (7.9%) were the least identified serotypes (Figure 1). The results obtained indicated that no significant difference was detected in M serotype distribution between isolates from burn and sore throat sources ($P > 0.05$).

3.2. Antibiotic Susceptibility Testing. Antimicrobial sensitivity was detected by antibiotic disc diffusion method according to the standard values provided by CLSI 2015. As a result, different isolates in this study were classified as sensitive, intermediate, or resistant (Figure 2). The sensitivity pattern of *S. pyogenes* revealed that all the *S. pyogenes* isolates were sensitive to amoxicillin/clavulanic acid, 81% to amoxicillin, 43% to ampicillin, and 7% to vancomycin. In contrast, all *S. pyogenes* isolates were resistant to ceftriaxone, cefepime, erythromycin, azithromycin, clindamycin, tetracycline, gentamicin, and levofloxacin antibiotics. Concerning the isolation source, no significant difference was found between burn and sore throat isolates regarding resistance except for ampicillin resistance ($P = 0.02$).

3.3. Phenotypic Identification of the Resistance Mechanism. The results of the present study concluded that 100% of *S. pyogenes* were resistant to both clindamycin and erythromycin which indicate the cMLS phenotype.

3.4. Phenotypic Characterization of Some Virulence Factors

3.4.1. Detection and Assay of Streptokinase Production. Twenty-eight isolates (73.6%) and *S. pyogenes* ATTC19615 reference isolate were identified as streptokinase producers. In addition, different isolates in this study exhibited different degrees of clot lysis ranging from 8% to 97.7% including 30.6% produced by ATTC19615. Moreover, depending on the enzyme activity, most of the isolates (68.4%) produced clot lysis less than 50%. However, a high degree of clot lysis (≥97%) was exhibited by only two isolates (Figure 3) Concerning the isolation source, no significant difference was found between burn and sore throat isolates ($P > 0.05$).

3.4.2. Quantitative Detection of the Biofilm Formation Using Microtiter Plates. According to biofilm formation, isolates in this study were classified into eight (21%) strongly adherent isolates, three (8%) moderately adherent isolates, and 13 (34.2%) weakly adherent isolates. The remaining 14 (36.8%) isolates in addition to the *S. pyogenes* ATTC19615 were identified as nonadherent strains. Concerning the

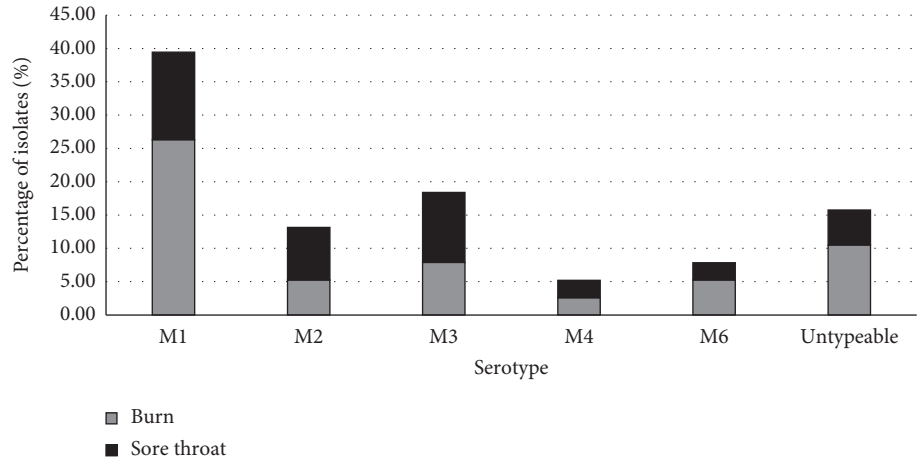


Figure 1: Prevalence of different serotypes among S. pyogenes isolates.

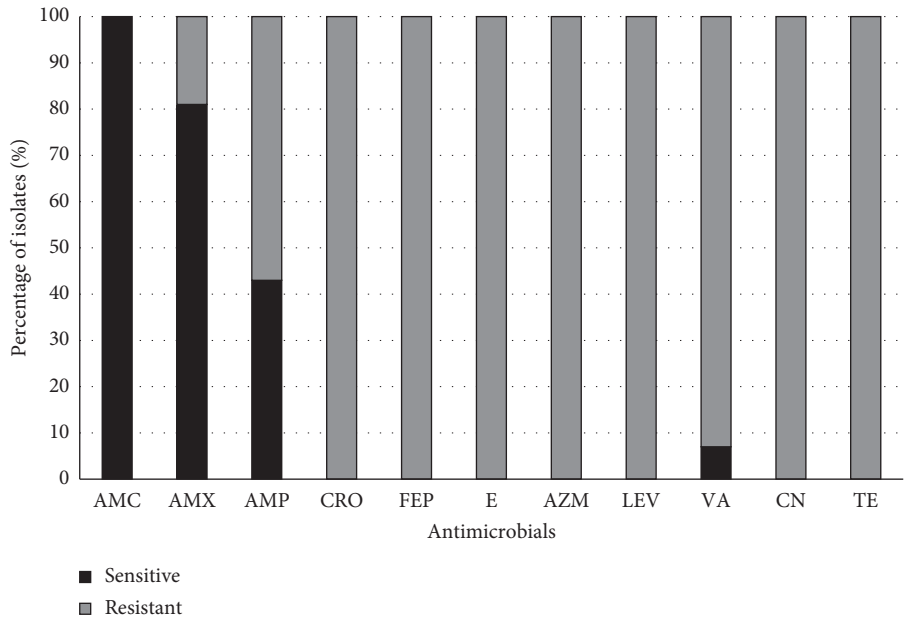


Figure 2: Antimicrobial sensitivity results of *S. pyogenes* isolates. AMC : amoxicillin/clavulanic acid, AMX : amoxicillin, AMP : ampicillin, CRO : ceftriaxone, FEP : cefepime, E : erythromycin, AZM : azithromycin, LEV : levofloxacin, VA : vancomycin, CN : gentamicin, TE : tetracycline.

clinical sources of the isolates, no significant difference was found between burn and sore throat isolates ($P > 0.05$).

3.4.3. Streptodornase Production. In this study, it was found that 28 (73.6%) of all isolates were identified as streptodornase producers in which 17 (44.7%) isolates showed complete disappearance of DNA bands, while 11 (28.9%) isolates showed incomplete disappearance or fading of DNA bands. Regarding *S. pyogenes* ATCC19615, a negative result was detected. Concerning the clinical sources of the isolates, no significant difference was found between burn and sore throat isolates ($P > 0.05$).

3.5. PCR Detection of Superantigens. The prevalence of SAgs genes was investigated by PCR testing. In this study, *SpeJ* was the most prevalent gene, detected in 31 (81.58%) isolates followed by *speI, speC,* and *ssa* genes which were identified in 24 (63.25%), 23 (60.5%), and 23 (60.5%) isolates, respectively. However, *SpeH* gene was identified at a lower level as it was harbored by only six (15.8%) isolates (Figure 4(a)). The other three SAgs genes, *speM*, speL, and *smeZ*, were not detected in any tested isolate. These results indicated that *speJ* was the mostly identified superantigen in M1, M3, and the untypeable strains; however, *speI* was most common in M6 serotype. Regarding the number of SAgs genes per each isolate, all isolates contained at least two SAgs genes, while

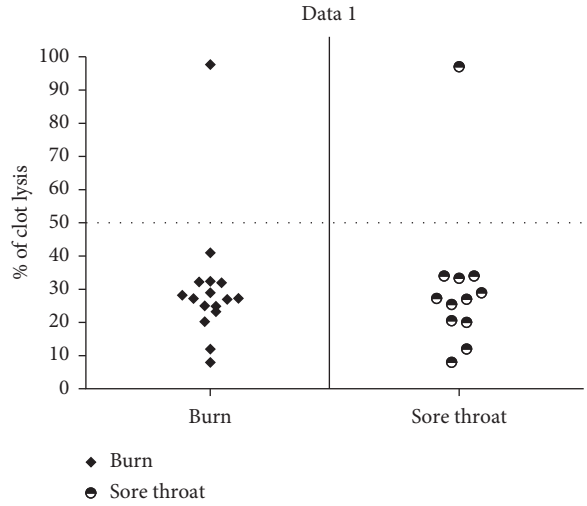


Figure 3: Percentage of clot lysis produced by *S. pyogenes* from different clinical sources.

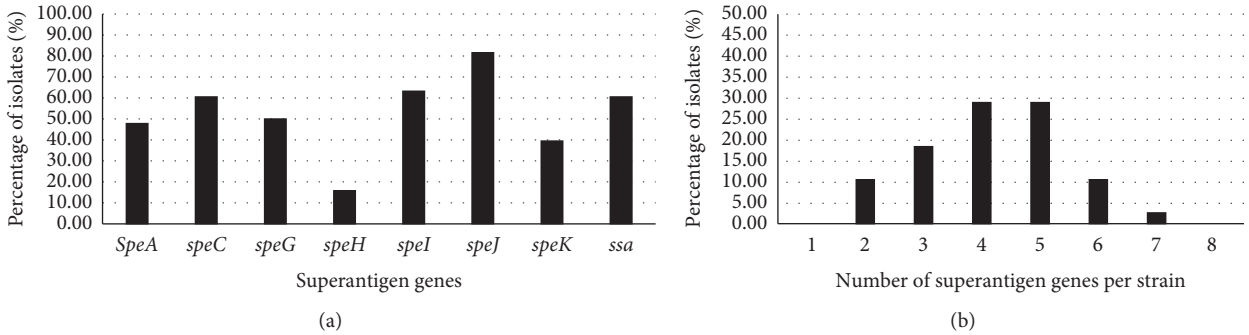


Figure 4: Prevalence of eight SAgs genes in 38 *S. pyogenes* isolates: (a) the occurrence of each SAgs gene in isolates and (b) the percentage of isolates containing different numbers from 1 to 8 SAgs genes.

34 (89.5%) isolates carried three or more SAgs genes. Although the average SAgs per isolate were 4, four isolates carried six genes and only one carried seven genes (Figure 4(b)).

3.6. Identification of Different SAgs Gene Profiles. Thirty-three different SAgs gene profiles were obtained in 38 *S. pyogenes* isolates (Table 2) including 29 unique profiles demonstrated by only one isolate, while only four profiles were exhibited by two or more isolates. The *speC-speJ* and *speI-speJ* combinations were the most commonly detected in 20 (52.6%) and 18 (47.4%) isolates, respectively. However, the coexistence of *speA-speJ* combination was found in 14 (36.84%) isolates. Other combinations, *speA-speC, speC-speG,* and *speA-speG* genes, were presented in 11 (29%), 10 (26.32%), and 7 (18.4%) isolates, respectively.

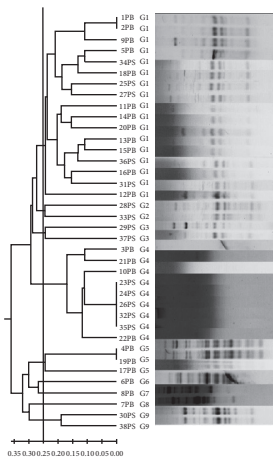
3.7. SAgs Gene Association with Different Serotypes. In this study, the correlation between SAgs genes and different serotypes indicated that no significant difference was found ($P > 0.05$); however, *speI* and *speJ* SAgs genes were the most identified in the M1 serotype, being identified in 11/15 (73.3%). In addition, *SpeA-speI* combination was the most identified in M1 serotype exactly in 8/15 (53.3%) isolates, followed by *speI-speJ* combination in 7/15 (46.67%) isolates. Moreover, each of *speC-speJ*, *speI-ssa,* and *speK- ssa* combination was identified in 6/15 (40%) isolates. In serotype M2, *speG* and *speJ* were the most identified genes in 4/5 (80%) isolates, while *speG-speJ* combination was the most identified combination in 3/5 (60%) isolates.

Furthermore, *SpeJ* gene was detected in all (7/7) M3 serotype isolates. The coexistence of *speJ* and *ssa* genes was identified in 6/7 (85.7%) isolates of M3 serotype. *speJ-speK-ssa* combination was identified only in M1, M3, and untypeable serotypes, being identified in 4/15 (26.6%), 4/7 (57.1%), and 2/6 (33.3%) isolates, respectively. *speI-speK-ssa* combination was identified in the M1, M3, and untypeable serotypes in 4/15 (26.6%), 3/7 (42.8%), and 1/6 (16.67%) isolates, respectively. *speI-speJ-speK-ssa* combination was identified mainly in M3 serotype 3/7 (42.8%), followed by M1 and untypeable serotypes in 2/15 (13.3%) and 1/6 (16.6%) isolates, respectively.

3.8. BOX-PCR Typing. Molecular typing of *S. pyogenes* isolates using BOX-PCR produced 9 different BOX-PCR clusters indicating the classification of all isolates into 9

Table 2: SAgs gene profiles in different *S. pyogenes* isolates.

Profile no.	*Superantigen profile*	No. of isolates	Serotype
P1	*SpeC, speJ*	1	M1
P2	*SpeG, speJ*	1	M2
P3	*SpeI, speJ*	1	M1
P4	*SpeJ, ssa*	1	M3
P5	*SpeA, speC, speJ*	1	N. T
P6	*SpeA, speI, speJ*	1	M1
P7	*SpeC, speG, speJ*	1	M1
P8	*SpeC, speI, speJ*	1	M2
P9	*SpeC, speK, ssa*	1	M4
P10	*SpeG, speI, ssa*	1	M2
P11	*SpeG, speJ, speK*	1	M2
P12	*SpeA, speC, speG, speI*	1	M1
P13	*SpeA, speC, speI, speJ*	3	M1, M1, M6
P14	*SpeA, speG, speI, ssa*	1	M6
P15	*SpeA, speI, speJ, ssa*	1	M1
P16	*SpeA, speJ, speK, ssa*	1	M3
P17	*SpeC, speG, speI, speJ*	1	M3
P18	*SpeC, speJ, speK, ssa*	1	M1
P19	*SpeG, speH, speJ, ssa*	1	M2
P20	*SpeG, speJ, speK, ssa*	1	M1
P21	*SpeA, speC, speG, speJ, ssa*	2	M4, N. T
P22	*SpeA, speC, speI, speJ, speK*	1	N. T
P23	*SpeA, speC, speI, speJ, ssa*	2	M3, N. T
P24	*SpeA, speG, speI, speK, ssa*	1	M1
P25	*SpeA, speH, speI, speK, ssa*	1	M1
P26	*SpeC, speG, speH, speI, speJ*	1	M6
P27	*SpeC, speG, speH, speI, ssa*	1	M1
P28	*SpeC, speI, speJ, speK, ssa*	1	M1
P29	*SpeG, speI, speJ, speK, ssa*	1	N. T
P30	*speA, speG, speI, speJ, speK, ssa*	1	M1
P31	*SpeC, speG, speI, speJ, speK, ssa*	2	M3, M3
P32	*SpeC, speH, speI, speJ, speK, ssa*	1	M3
P33	*SpeA, speC, speG, speH, speJ, speK, ssa*	1	N. T



Isolate	Serotype	AMC	AMX	AMP	CRO	FEP	E	AZM	CN	VA	TE	LEV	Resistance phenotype	*speA*	*speC*	*speG*	*speH*	*speI*	*speJ*	*speK*	*ssa*	Streptokinase	Streptodornase	Biofilm
1PB	M6	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	+	+	+	+	+	−	−	+	−	S
2PB	M3	S	S	S	R	R	R	R	R	S	R	R	cMLS	−	+	+	−	+	+	−	−	+	+	M
9PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	+	−	−	+	+	−	−	+	−	W
5PB	M1	S	S	S	R	R	R	R	R	R	R	R	cMLS	+	−	−	−	+	+	−	+	+	−	W
34PS	M4	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	+	+	−	−	+	−	+	+	+	S
18PB	N.T	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	+	−	−	+	+	+	−	+	+	S
25PS	M6	S	S	S	R	R	R	R	R	R	R	R	cMLS	+	+	−	−	+	+	−	−	+	+	W
27PS	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	+	−	−	+	+	−	−	+	+	S
11PB	N.T	S	R	R	R	R	R	R	R	R	R	R	cMLS	+	+	−	−	+	+	−	+	−	+	NA
14PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	+	+	+	+	−	−	+	+	+	M
20PB	N.T	S	R	R	R	R	R	R	R	S	R	R	cMLS	−	−	−	−	+	+	+	+	+	+	W
13PB	M4	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	+	−	−	−	−	+	+	−	+	NA
15PB	M3	S	R	R	R	R	R	R	R	R	R	R	cMLS	−	+	−	+	+	+	+	+	−	−	W
36PS	N.T	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	+	+	−	−	+	−	+	−	+	S
16PB	N.T	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	+	+	+	−	+	+	+	−	+	NA
31PS	M2	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	−	+	+	−	+	−	+	+	+	NA
12PB	M1	S	S	S	R	R	R	R	R	R	R	R	cMLS	+	+	+	−	+	−	+	+	+	+	W
28PS	N.T	S	R	R	R	R	R	R	R	R	R	R	cMLS	+	−	−	−	−	+	−	−	−	+	NA
33PS	M1	S	S	S	R	R	R	R	R	R	R	R	cMLS	+	−	+	−	+	+	+	+	+	−	S
29PS	M2	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	+	+	−	−	+	+	−	+	+	NA
37PS	M1	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	−	−	−	+	+	+	+	+	+	W
3PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	−	−	+	+	−	+	+	+	+	W
21PB	M3	S	R	S	R	R	R	R	R	R	R	R	cMLS	−	+	−	−	−	+	−	+	+	+	S
10PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	+	−	+	−	+	−	−	−	+	−	NA
23PS	M2	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	+	−	−	+	+	−	−	−	+	W
24PS	M3	S	S	S	R	R	R	R	R	R	R	R	cMLS	+	−	−	−	−	+	+	+	+	+	NA
26PS	M1	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	+	−	−	−	+	−	−	+	+	NA
32PS	M1	S	S	S	R	R	R	R	R	R	R	R	cMLS	−	+	−	−	−	+	+	+	+	−	W
35PS	M3	S	S	S	R	R	R	R	R	R	R	R	cMLS	+	+	−	−	+	+	−	+	+	+	W
22PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	−	+	−	−	+	+	+	+	+	W
4PB	M1	S	R	R	R	R	R	R	R	R	R	R	cMLS	+	−	−	−	+	+	−	−	+	−	M
19PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	−	−	−	+	+	−	−	+	+	NA
17PB	M2	S	S	R	R	R	R	R	S	R	R	R	cMLS	−	−	+	−	−	+	−	−	−	+	W
6PB	M1	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	+	+	−	−	+	−	−	−	+	S
8PB	M6	S	R	R	R	R	R	R	R	R	R	R	cMLS	+	−	+	−	+	−	−	+	+	+	NA
7PB	M2	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	−	+	−	+	−	−	+	+	+	NA
30PS	M3	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	+	+	−	+	+	+	+	+	−	NA
38PS	M3	S	S	R	R	R	R	R	R	R	R	R	cMLS	−	+	+	−	+	+	+	+	−	−	NA

Figure 5: Dendrogram of *S. pyogenes* BOX-PCR results showing the clustering of isolates into 9 different groups, illustrating different serotypes, SAgsgenes, sensitivity patterns, and some virulence factors.

groups. These patterns were designated as G1-G9. Group G1 included most of the isolates, followed by G4 and G5 with 17, 9, and 3 isolates, respectively. Isolate numbers 23PS, 24PS, 26PS, 32PS, and 35PS could be identified as untypeable (Figure 5).

3.9. Correlation between the Two Typing Methods and Clinical Sources. Regarding clinical sources of the isolates, the majority of the isolates, 22 isolates (57.8%), were isolated from burns, while the remaining 16 (42.2%) were obtained from sore throat samples. Serotyping methods concluded

that the M1 serotype was the most identified serotype, compromising 15 isolates including 10 burn isolated strains. Regarding the molecular typing method, G1 included the majority of the isolates (17 isolates), where 12 isolates were burn isolated strains, followed by G4 with 9 isolates, in which four isolates were obtained from burn source, while the remaining 5 isolates were sore throat isolates.

4. Discussion

S. pyogenes is a β-hemolytic GAS associated with the human respiratory tract, skin, and soft tissue infections [21–23]. *S. pyogenes* isolates can be identified according to bacitracin sensitivity [24]. In addition, other more sensitive identification methods have been introduced including GAS latex agglutination and PCR test for *spy 1258* gene [9, 19, 25]. In this study, by the application of mostly applied techniques, 38 isolates out of 342 (11.1%) clinical specimens were identified as *S. pyogenes*. Our results showed a higher incidence of *S. pyogenes* compared to the level previously detected in India as 160 *S. pyogenes* isolates were detected in a total of 34065 clinical samples (0.0047%) [9]. However, the annual incidence of *S. pyogenes* infections varied greatly and ranged between 0.029 and 2.84% per 100,000 population [26].

In this study, antimicrobial sensitivity testing results indicated high sensitivity (100%) of all *S. pyogenes* isolates to amoxicillin/clavulanic acid. A similar observation has been previously reported in Pakistan [27]; however, a lower sensitivity level (81%) was reported in Egypt [28]. In addition, in this study, a lower amoxicillin sensitivity (81%) was compared to 100% mentioned previously in Pakistan and Senegal, respectively [27, 29].

Regarding ampicillin sensitivity, 43% detected was higher than 11% found by Mahdil et al. [30] and lower than that previously reported in one study in Egypt [28] and another study of Khosravi et al. [31] where 88% and 100% were identified, respectively.

In this study, ceftriaxone resistance was identified in all the tested isolates. In contrast, Camara et al. [29] and Villasenor et al. [32] reported 100% ceftriaxone sensitivity. However, variable results were obtained in Egypt and Iran where lower resistance percentages were obtained (50% and 12%), respectively [28, 31]. This increased level of resistance to B-lactam reflects the modification of sensitivity patterns of the bacteria to the mostly used antibiotic [33].

In our study, the double disc diffusion method indicated a high level of resistance (100%) to both erythromycin and clindamycin (cMLS phenotype). However, lower percentages were detected in Taiwan where cMLS phenotype was identified in 50% and 25% of the tested isolates, respectively [12, 34]. This increased level of antimicrobial resistance against erythromycin antibiotic may be attributed to the excessive use of macrolide in the treatment of most upper respiratory tract infections which is usually caused by a viral infection [34].

In our study, the M protein serotyping test indicated a higher prevalence of M1 serotype identified in 39.5% of all the *S. pyogenes* isolates. A similar observation has been documented in a previous study in the UK [35]. Interestingly, the M1 serotype was mainly identified in burn isolates compared to sore throat ones, while the M3 serotype was more common in sore throat isolates. However, there is no direct relation between severe GAS infections or any M-serotype [36].

In our study, multiple virulence factors have been tested in *S. pyogenes* isolates. In the case of streptokinase, an enzyme is produced by most β-hemolytic streptococci strains, which acts as a spreading factor by the activation of plasminogen [37]. In *S. pyogenes* tested isolates, 73.7% of all isolates were positive streptokinase producers which is in agreement with the results previously documented in France [38]. However, other different results were obtained in some previous studies in Egypt and the USA, 55% and 97.5%, respectively [28, 39]. In addition, by testing another spreading factor, streptodornase, produced by group A, C, and G streptococci which acts to liquefy the viscous components of the host cells such as DNA [40], our results indicated 73.7% of positive streptodornase producers. This result was higher compared to 55% previously documented in Egypt [28].

Biofilm is another tested virulence factor that is associated with antimicrobial resistance and medical device mediated infections [41]. In the present study, 63.2% of the tested isolates exhibited positive biofilm formation abilities. Different biofilm production abilities were identified among the tested isolates. Twenty-one percent were strongly adherent, 8% were moderately adherent, and 34.2% were weakly adherent. This variation in different studies may be attributed to divergent sample sizes, different isolation sources, and nutritional content of the medium [42]. By performing SAgs PCR test, the chromosomally encoded SAgs gene *speJ* was the most identified gene being identified in 81.58% followed by the three phage associated genes *speI, speC,* and *ssa* in 63.25%, 60.5%, and 60.5% of total isolates, respectively. In contrast, *speM, speL,* and *smeZ* genes could not be identified in any of the tested isolates. However, in some previous studies [36, 43], *speA* and *speC* genes were the most identified in Canada and Serbia where 35.3% and 17.2% could be detected, respectively. In contrast to our results, the chromosomally encoded gene *smeZ* was the most identified in Serbia, China, Portugal, and Norway where it was identified in 100%, 100%, 98%, and 98.3%, respectively [44–47].

By performing SAgs PCR test, 15 (39.5%) of the isolates expressed from five to eight genes; however, 23(60.5%) expressed less than five genes. In contrast, in a previous study in Poland [6] and in another study in China [7], 17.4% and 82.5% of *S. pyogenes* isolates expressed more than 5 SAgs genes, respectively. In addition, as previously documented, SAgs are usually present in a bacteriophage which confirms the direct or indirect function of the M protein acting as barriers for horizontally transferred genes [48]. Moreover, the genome of the bacteriophage can reach 12% of the total GAS genome which indicates the horizontal transfer modification which is the main process affecting the appearance and evolution of pathogenic strains [49, 50].

The assessment of the combination between virulence gene profiles, serotypes, and clinical sources of *S. pyogenes*

could increase the investigation ability of genetic studies of *S. pyogenes* from different geographical areas [51]. Analyzing the identified SAgs genes in the tested isolates revealed high diversity in the SAgs gene profiles, where 33 profiles were identified in 38 isolates. However, other results were previously reported in some previous studies. In a total 191 *S. pyogenes* isolates, 57 SAgs gene profiles were detected in Portugal [46]. In addition, in another study in China, eight profiles were identified among a total 74 *S. pyogenes* isolates [45].

In this study, by analyzing the SAgs gene profile, *speC-speJ* and *speI-speJ* combinations were mostly presented in 52.6% and 47.4% of total isolates, respectively. However, no significant association between the coexistence of different SAgs genes and specific M serotypes was found. In other previous studies, the coexistence of the two chromosomally encoded genes *smeZ* and *speG* was identified in Portugal and Norway in 91.7% and 91.6% of all isolates, respectively [46, 47]. Additionally, the coexistence of *smeZ* and *speC* was identified in all the tested isolates in one study in China [45].

Interestingly, by the application of BOX-PCR method, in this study, effective molecular typing covering highly virulent *S. pyogenes* strains was obtained in this study compared to the traditional serological one. Regarding the two typing methods, serological and molecular typing methods, BOX-PCR was demonstrated to be the most effective typing method to group the most virulent isolates into similar genetic relatedness (G1 and G4) among isolates of different serotypes and clinical sources. Where isolates 3PB, 12PB, 14PB, 18BP, 20PB, 34PS, and 35PS contained five different SAgs genes, positive streptodornase, streptokinase production, and biofilm formation are clustered under two groups while they are clustered under M1, M3, M4, and untypeable serotypes (Figure 5). It is worth mentioning that no previous studies were found for typing of *S. pyogenes* by BOX-PCR; other studies were conducted on *Streptococcus pneumonia* [20] and *Salmonella enteric* [52].

5. Conclusion

S. pyogenes pathogens are not only associated with upper respiratory tract infections but also commonly identified among burns. In our study, *S. pyogenes* isolates were highly resistant to mostly used antibiotics, which is considered a serious issue affecting public health. M1 serotype was the most identified serotype among *S. pyogenes* isolates. All the isolates were cMLS phenotype. The results showed high genetic variation in SAgs gene profiles where 33 different profiles were produced. By the application of BOX-PCR, effective molecular typing covering highly virulent *S. pyogenes* strains was obtained.

Data Availability

All datasets generated or analyzed during this study are available upon reasonable request from the corresponding author.

Disclosure

This work was performed at the Microbiology Department, Faculty of Pharmacy, Mansoura University, Egypt.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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