ORIGINAL ARTICLE

Fibronectin-binding Protein F1 (*prtF1*) Gene Is Highly Distributed in Tetracycline-resistant, Erythromycin-sensitive *Streptococcus pyogenes* Isolates

Simon Onyema Azi[1,2], Suresh Kumar[1], Leslie Than Thian Lung[1], Malina Osman[1], Rukman Awang Hamat[1]

[1] Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia
[2] Department of Medical Laboratory Science, Faculty of Health Sciences and Technology, Ebonyi State University, Abakaliki, Ebonyi State PMB 053, Nigeria

ABSTRACT

Introduction: The internalization process of group A streptococci (GAS) into human cells is one of the crucial steps in the pathogenesis of GAS infections, which could also affect their susceptibility responses toward several antibiotics. Currently, data on the distribution of internalization-associated genes and susceptibility patterns are still lacking in Malaysia. This study investigated the distribution of fibronectin-binding protein F1 (*prtF1*) and streptococcal pyrogenic exotoxin B (*speB*) genes in GAS isolates with their susceptibility profiles and source of samples. **Methods:** We used 43 GAS isolates from our previous stock culture and performed antibiotic susceptibility testing by Kirby-Bauer disk diffusion method and interpreted the results according to the established guidelines. We detected virulence (*prtF1* and *speB*) and resistance (*ermA, ermB, mefA, tetM* and *lnuA*) genes by PCR method using established primers and protocols. **Results:** High resistance rates were observed against doxycycline (58.1%) and clindamycin (16.3%). In comparison, 100.0% and 46.5% of GAS isolates carried *speB* and *prtF1* genes, respectively. tetM and lnuA genes were detected in all respective resistant isolates (100% for each). No macrolide resistance genes were detected. Interestingly, *prtF1* gene was highly distributed in doxycycline-resistant than doxycycline-sensitive isolates (60.0% versus 27.8%). **Conclusions:** High resistance rate of GAS toward doxycycline in our study may potentially reflect the uncontrol dissemination of tetM gene among our isolates. The presence of *prtF1* gene among this strain would enhance its ability to evade the intracellular action of antibiotics, which may affect the management of GAS diseases. Thus, close monitoring of GAS by molecular methods is required in the future.

Keywords: GAS, Fibronectin-binding proteins, *prtF1*, *speB*, Tetracycline resistance, PCR

Corresponding Author:
Rukman Awang Hamat, MPath
Email: rukman@upm.edu.my
Tel: +603-97692369

INTRODUCTION

Streptococcus pyogenes (Group-A streptococcus) or better known as a "flesh-eating bug" is one of the major human pathogens responsible for over 600 million infections and about 517,000 deaths annually (1). Heightened concerns have been escalating among the scientists and epidemiologists due to its ability to produce a fatal toxin known as streptococcal pyrogenic exotoxins (SPEs) and other deadly virulence factors. This is further complicated by increasing data on serious invasive diseases and its long term sequalae such as rheumatic heart disease (RHD), acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN) that have been reported in resource-poor or limited settings (2).

Nonetheless, the detailed pathophysiological mechanisms of GAS pathogenesis remain elusive. It has been reported that the ability of GAS to be internalized into the epithelial cells by *in vitro* and *in vivo* studies has contributed to the ideas of therapeutic failure and persistent throat carriage among patients with GAS pharyngitis and invasive diseases (3). Streptococcal pyrogenic exotoxin B (SpeB)- a cysteine proteinase that is encoded by *speB* gene has been recognized for not only exhibits broad enzymatic roles that is by degrading or activating a wide spectrum of different human proteins, including fibronectins but also involves in internalization process (4, 5). Surprisingly, there have been conflicting findings regarding the contribution of SpeB to GAS internalization into human cells. Some studies have demonstrated an increased internalization of GAS

isolates into the cells while others reported the reduction of internalization process (6, 7). It has been reported that degradation of Protein F1 by SpeB (an enzyme) would abrogate the entry of GAS into human cells. Thus, it is believed that *speB* is an important regulator for the internalization process in GAS pathogenesis (8).

Meanwhile, the internalization process has also been associated with fibronectin-binding proteins, which are encoded by *prtF1* and other virulence genes (9). Protein F1 that is encoded by *prtF1*, promotes bacterial adherence to and facilitates their entry into human epithelial cells, which could allow them to be intracellularly located. Thus, the bacteria could escape the bactericidal action of antibiotics such as penicillin (10). Many reports have documented that invasive and erythromycin-resistant GAS isolates are equipped with these virulence determinants (11-13). However, recent data reported that these fibronectin-binding proteins (in particular *prtF1*) are also found among non-invasive isolates, suggesting that a peculiar trait may exist among GAS strains and the trait may differ from country to country (9, 14-16). In addition, apart from epithelial cells, GAS isolates have been shown to be internalized into neutrophils and survived intracellularly (17). In Malaysia, GAS isolates have been shown to be highly sensitive to penicillin and erythromycin but demonstrated high resistance rate to tetracyclines (18). While much information has widely accumulated on the internalization process among macrolide-resistant, invasive GAS strains, virtually nothing is known about the association of *speB* and *prtF1* genes with other antibiotic resistance determinants such as tetracycline and clindamycin resistance genes in GAS isolates. We hope that our findings could provide an insight in supporting appropriate antibiotic use and control measure in our hospital setting.

Apart from *speB*, other virulent factors have been found to contribute to GAS pathogenicity such as M protein, streptokinase and fibronectin- binding protein F1 adhesin *prtF1*) and others (19). *prtF1* in particular, has played a pivotal role in the internalization of *speB* into respiratory epithelia which has been linked to antibiotic resistance in GAS due to its ability to block entry of antimicrobial agents (10, 20, 21).This study therefore investigated the potential associations of fibronectin-binding protein F1 (*prtF1*) and streptococcal pyrogenic exotoxin B (*speB*) genes in GAS isolates with their susceptibility profiles and source of samples.

MATERIALS AND METHODS

Sample Collection and Bacterial Identification

Forty-three non-duplicative clinical isolates of GAS were obtained from Hospital Serdang, Hospital Kuala Lumpur and some clinics in Klang Valley, Malaysia. These isolates were kept at -70°C freezer in the Medical Microbiology Laboratory, Faculty of Medicine and Health Sciences, UPM Malaysia during a one-year study period (from January 1, 2015 to January 1, 2016). The isolates were obtained from various clinical samples as follows: blood (n = 8), pus (n = 18), wound (n = 2), tissue (n = 6), throat (n= 1), and others (n = 8). Invasive source of GAS strains were regarded as strains that were isolated from sterile sites such as blood, deep tissues and body fluids as previously described (22). All isolates were grown overnight on 5% defibrinated sheep blood agar plates at 37°C in 5% CO2. The identification of GAS was conducted by using several biochemical tests such as gram staining, bacitracin susceptibility (Oxoid, United Kingdom), pyrrolidonyl arylamidase test (Oxoid, United Kingdom), and confirmed with 16SrRNA sequencing.

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing was performed by using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Isolab Sdn Bhd, Malaysia) supplemented with 5% sheep blood and incubated in 5% CO2 for 24 hours at 37°C. The antibiotics that were tested as follows: erythromycin (15 µg, Oxoid, UK), azithromycin (15 µg, Oxoid, UK), clindamycin (15 µg, Oxoid, UK), and doxycycline (30 µg, Oxoid, UK). Doxycycline was used to represent the tetracycline antibiotic group. Detection of inducible-clindamycin resistance was also performed by the D-test method. The results were interpreted according to CLSI guidelines (23).

Molecular Detection of Antibiotic Resistance and Virulence Genes

Bacterial DNA was extracted by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In view of the silence form of resistance genes that are commonly found among GAS,(18, 24). we subjected all of the isolates to the detection of macrolide resistance genes such as *ermA, ermB*, and *mefA*; clindamycin resistance (*lnuA*) gene and tetracycline resistance (*tetM*) gene and virulence (*prtF1*, *speB*) genes by PCR using specific primers and conditions as previously established (Table I). The flanking region of repeat domain type 2 (RD2) of *prtF1* gene was targeted by the primer sets used for *prtF1* gene detection in the present study which was established by Neeman and colleagues (10). The fibronectin- binding activity involves RD2 region which consist of five repeats in the protein's domain. The number of RD2 repeats was determined based on the size of amplicon detected in gel electrophoresis (25, 26).

EconoTaq® PLUS GREEN master mix (Tiangen Biotech Co. Ltd., Beijing, China) and Bio-Rad Thermocycler (Biorad®, Germany) were used for PCR amplification. The PCR products were analyzed and visualized by gel electrophoresis in 0.8% (w/v) agarose gel and gel documentation system (Alpha ImagerTM 2200, Alpha Innotech, Australia), respectively. Staphylococcus aureus BAA ATCC 977, Streptococcus pneumoniae ATCC 700677, S. pneumoniae ATCC 700676 and S.

Table 1: List of primers used in this study

Gene	Oligonucleotide sequence	Amplicon Size (bp)	References
16sRNA (27f, 1492r)	F 5′ AGAGTTTGATCCTGGCTCAG 3′	1498	(28)
	R 5′ AGAAAGGAGGTGATCCAGCC 3′		
speB	F 5′ CTAGACAATACAACTGGAACAA3′	200	(29)
	R 5′ GTGAACAGATTGGTTGTAGC 3′		
ermA	F 5′ GCA TGA CAT AAA CCT TCA 3′	242	(30)
	R 5′ AGG TTA TAA TGA AAC AGA 3′		
ermB	F 5′ CGA GTG AAA AAG TAC TCA ACC 3′	692	(30)
	R 5′ GGC GTG TTT CAT TGC TTG ATG 3′		
mefA	F 5′ AGT ATC ATT AAT CAC TAG TGC 3′	320	(31)
	R 5′ TTC TTC TGG TAC TAA AAG TGG 3′		
tetM	F 5′ GTTAAATAGTGTTCTTGGAG 3′	517	(28)
	R 5′ CTAAGATATGGCTCTAACAA 3′		
prtF1	F 5′TTTTCAGGAAATATGGTTGAGACA3′	Varies*	(11)
	R 5′TCGCCGTTTCACTGAAACCACTCA3′		
lnuA	F 5′GGTGGCTGGGGGGTAGATGTATTAACT-GG3′	100	(32)
	R 5′GCTTCTTTTGAAATACATGGTATTTTTC-GATC3′		

*The set of primers are complementary to the flanking region of repeat domain type 2 (RD2), a repeat domain of *prtF1* gene; the amplicon size varies due to the number of repeats.

pneumoniae ATCC 6305 were used as positive controls for *ermA, ermB, mefA* and *tetM* genes, respectively. *S. pyogenes* ATCC 19615 was used as a positive control for *speB* gene.

RESULTS

Antibiotic susceptibility patterns

Of 43 GAS isolates, 31 (72.1%) and 12 (27.9%) were isolated from non-invasive and invasive samples, respectively. All GAS isolates (100%) were sensitive to erythromycin and azithromycin. Twenty-five (58.1%) and 7 (16.3%) of them exhibited resistance to doxycycline and clindamycin, respectively and had their resistance genes (Table II). There were no inducible MLSB (iMLSB) or constitute MLSB (cMLSB) or MS phenotypes detected among GAS isolates. Regarding virulence genes, 43 (100.0%) and 20 (46.5%) of GAS isolates carried *speB* and *prtF1* genes, respectively as shown in Table II.

PCR gel electrophoresis of virulence and resistance genes

PCR analysis of a repeat domain of *prtF1* gene, which is repeat domain type 2 (RD2) region produced products approximately ranging from 345 to 680 bp (Fig. 1). Of 20 *prtF1*-positive *S. pyogenes* strains, three (5%), four (70%), five (15%) and six (10%) of RD2 repeats were detected in the present study (Fig. 1). The PCR products of *speB* gene is shown in gel electrophoresis (Fig. 2). In addition, Fig. 3 shows the gel electrophoresis image of *tetM* gene PCR products. *tetM* and *lnuA* genes were detected in all doxycycline and clindamycin-resistant isolates (100% for each). No macrolide resistance genes were detected. PCR products of *lnuA* and macrolide genes were visualized by gel electrophoresis (data not shown).

Table II: Distribution of virulent and antibiotic resistant genes among the 43 *S. pyogenes* in the present study

Genes	Frequency n (%)
prtF1	20 (46.5)
speB	43 (100)
tetM	25 (58.1)
lnuA	7 (16.3)
ermA	0 (0)
ermB	0 (0)
mefA	0 (0)

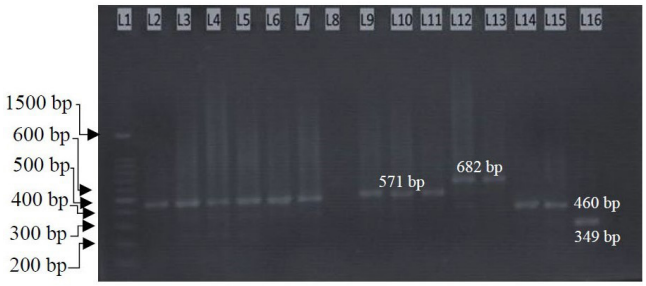

Figure 1: Gel electrophoresis image of *prtF1* gene detected in representative GAS isolates. Lane 1: 100 bp DNA ladder (MyTACG); Lane 2-7 and 14-15: four RD2 repeats of *prtF1* gene (460 bp). Lane 9-11: five RD2 repeats (571 bp). Lane 12-13: six RD2 repeats (682 bp). Lane 16: three RD2 repeats of prtF1 gene (349 bp). Lane 8: negative result of an isolate.

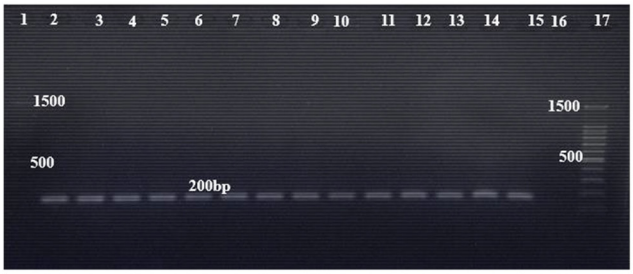

Figure 2: Gel electrophoresis image of *speB* gene detected in representative GAS isolates. Lane 1 and 17: 100bp DNA ladder (MyTACG). Lane 2: *Streptococcus pyogenes* ATCC 19615 as positive control. Lane 3-15: *speB* gene in GAS isolates. Lane 16: DNA free template as negative control.

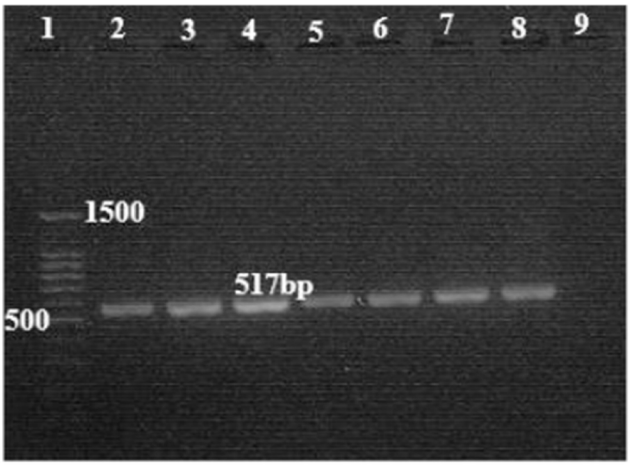

Figure 3: Gel electrophoresis image of *tetM* gene detected in representative GAS isolates. Lane 1: 100bp DNA ladder (MyTACG). Lane 2: *Streptococcus pyogenes* ATCC 700676 as positive control. Lane 3-8: *tetM* gene (517bp) in GAS isolates. Lane 9: negative control.

The *prtF1* gene was highly detected in doxycycline-resistant isolates than in doxycycline-sensitive isolates (60.0% versus 27.8%) (Table III). In addition, *prtF1* gene was also highly detected among GAS isolates obtained from invasive than non-invasive samples (58.3% versus 41.9%). Nonetheless, all associations could not be analyzed due to inadequate number of *S. pyogenes* isolates recruited in the present study.

Table III: The distribution of *prtF1* gene in relation to tetM, lnuA resistance genes and type of samples in 43 GAS isolates

Parameters	*prtF1* gene	
	Positive n %	Negative n %
tetM^{P} (n=25)	15 (60.0)	10 (40.0)
tetM^{N} (n=18)	5 (27.8)	13 (72.2)
lnuA^{P} (n=7)	0 (0)	7 (100)
lnuA^{N} (n=36)	20 (55.6)	16 (44.4)
Invasive (n=12)	7 (58.3)	5 (41.7)
Non-invasive (n=31)	13 (41.9)	18 (58.1)

DISCUSSION

The present study investigated the susceptibility patterns of selected antibiotics and the molecular detection of resistance and virulence genes, which might be involved in the pathogenesis of GAS diseases. It is not surprising that all GAS isolates in our study were sensitive to 14 and 15-membered rings of macrolides. Decreased resistance rates to macrolides have also been increasingly reported in many studies locally and abroad. For instance, previous studies have reported the macrolide resistance rates ranging from 0-9 % in Malaysia, Tunisia and Finland.(27-29). Macrolides and lincosamides are commonly used as alternative drug of choices in patients who are allergic to penicillin. (30) Nonetheless, higher resistance rates of 93.5 % and 70.0% were recently reported in China and the Middle East and North Africa, respectively (31, 32). The differences in the prevalence rates of macrolide resistance are associated with changes in macrolide use (33). Thus, low resistance rate of macrolides could be explained by the judicious use of macrolides in previous studies including Malaysia.

To the best of author's knowledge, the presence study reported the first of clindamycin-resistant (L phenotype) *S. pyogenes* strains (16.3%) and its resistance gene (*lnuA*) in Malaysia. More than 90% of *S. pyogenes* isolates exhibited resistance to clindamycin in China (31). Whereas, only 0.5% of *S. pyogenes* isolates in the United States were resistant to clindamycin (34). This is worrying as clindamycin is usually used to treat necrotizing fasciitis and other invasive diseases for its inhibitory role in reducing the production of *S. pyogenes* superantigens as well as improving patient outcomes (35-37). Though it is rare, *lnuA* gene inactivates lincosamide by chemical modification (38). Therefore, clinicians should not empirically prescribe clindamycin for necrotizing fasciitis and invasive cases. Tetracyclines are not the main therapeutic option for treating invasive GAS diseases, they are commonly used for the treatment of respiratory and skin diseases in humans (39). However, the high resistance rate to doxycycline is of a great concern as all doxycycline-resistant isolates harbored *tetM* gene in our study.

Our finding corresponds with other findings that reported 70 % and 86.4 % of *S. pyogenes* were resistant to tetracycline in Tunisia and China, respectively (27, 29). In China, 95.9% of 121 *S. pyogenes* isolates had *tetM* gene detected (31). *tetM* is the major resistance determinant detected in most *S. pyogenes* isolates as it is easily translocated from chromosome to chromosome (40, 41). *tetM* gene is carried by the Tn916-related transposons which are readily transmissible via mobile genetic elements (27, 42). In contrast to many studies, tetracycline-resistant *S. pyogenes* strains is unique as these strains had no macrolide-resistance genes detected in the present study (24, 43, 44). *ermB* gene is physically associated with *tetM* gene in the mobile genetic elements and most tetracycline-resistant strains would exhibit resistance to macrolides (24). However, we did not perform the whole genome sequencing on these strains to investigate for gene mutations or deletions in the present study. In Malaysia, rampant use of tetracyclines has been reported in poultry and pig farms and it is likely that these mobilomes would be easily transferred among inter and intra species of bacteria (29). In addition, resistance to tetracycline has been considered as a cofactor for the development of erythromycin resistance (45). Nonetheless, we observed that there were no tetracycline-macrolide co-resistant genes detected in all isolates. In contrast, few studies have reported *ermB* and *tetM* genes are closely associated in streptococci as both resistance genes are found on the same transposon (27, 42).

In the present study, we detected *speB* gene in all isolates regardless of its invasiveness. Our finding corroborates with other study which proposed *speB* gene as an important biomarker for the identification of *S. pyogenes* (20). Similarly, all 140 *S. pyogenes* isolates carried *speB* gene regardless of its invasiveness in China (31). Despite a very high susceptibility rate to erythromycin, our GAS isolates exhibited higher proportion of *prtF1* gene compared to few studies conducted in Germany (30.0%) and Israel (41.8%) (10, 46). The molecular basis for this interaction is poorly understood and deserves more studies on functional genomics in future. Nonetheless, it was slightly lower than other studies in Israel (51.4%) and Japan (69.5%) (13, 25). In contrast to our finding, *prtF1*-harbouring strains are commonly detected among erythromycin-resistant GAS isolates in other studies (11-13).

The repeat domain type 2 (RD2) region of *prtF1* gene

has an important role for the entry of *S. pyogenes* into epithelial cells (47). The number of RD2 repeats varies among *S. pyogenes* strains ranging from one to six (10, 48). In the present study the most common repeat domain was four RD2 repeats (70%). Similarly, four RD2 repeats of *prtF1*-positive *S. pyogenes* strains (39%) were commonly reported in Italy (49). Unlike erythromycin-sensitive *S. pyogenes* strains in the present study, strains with more than one RD2 repeat of *prtF1* gene are commonly associated with macrolide resistance (13). Nonetheless, the variability of the RD2 region is not related to the activity of fibronectin-binding proteins (25). With regard to *prtF1* gene among tetracycline-resistant strains, our finding highlights the potential threat of the combined features of *prtF1*/*tetM*-positive GAS strains which could escape both penicillin (the antibiotic of choice) and tetracyclines by means of intracellular location and resistance, respectively. More worryingly, it has been postulated that the resistance genes and *prtF1* gene can be simultaneously transferred on mobile genetic elements, which can be acquired from similar or different species of throat microflora (9). Based on the type of samples obtained in our study, *prtF1*-positive isolates were highly detected in invasive than non-invasive samples (58.3 % versus 41.9%). This is in contrast with other studies that reported more *prtF1*-positive isolates were found in non-invasive GAS strains (39, 50). Nonetheless, few studies reported more *prtF1*-positive isolates were detected in GAS invasive diseases which support our finding (12, 13). Apparently, it has been proposed there is no specific association between virulence traits and clinical manifestations in GAS infections (12).

Our study is limited in few ways. First, the small sample has restricted the statistical analysis of several variables (for example, *lnuA* and *speB* genes) plus we lacked resources to include other genes that are involved in adhesion process for instance, serum opacity factor (sof) gene. Secondly, the presence of *prtF1* gene was not evaluated by western blot or RT-PCR was not investigated to determine the functional assay or gene expression. Nonetheless, although this is only preliminary, the findings in the present study could offer an insight on the potential interaction of virulence and resistance determinants among *S. pyogenes* in Malaysia.

CONCLUSION

Fibronectin-binding protein F1 (*prtF1*) gene is commonly detected in doxycycline resistance isolates. High frequency of tetracycline-resistant gene carried by our GAS isolates could eventually initiate the development of erythromycin-resistant strains via rapid transmissibility of mobile genetic elements among bacteria in the future. Moreover, emergence of clindamycin-resistant strains is of a great concern too. Thus, close monitoring of this peculiar trait by molecular approaches is strongly advisable to detect any subtle changes which could affect the management of GAS diseases in our region.

ACKNOWLEDGMENTS

The authors wish to extend their very special thanks to the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia and their staff for their continuous support. This work was funded by the Universiti Putra Malaysia Research Grant (GP-IPS/2018/9656600) and the Tertiary Education Trust Fund (TetFund), Ebonyi State University, Abakaliki, Nigeria. (TETF/ES/UNI/ESU/ABAKALIKI/EBONYI/ASTD/2017.The ethical approval for this study was obtained from Malaysia Ethics Committee for Research Involving Human Subjects (Reference number: UPM/TNCP/RMC/1.4.18.2/JKEUPM).

REFERENCES

1. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. Lancet Infect Dis. 2005;5(11):685-94.
2. Arias-Constanti V, Trenchs-Sainz de la Maza V, Sanz-Marcos NE, Guitart-Pardellans C, Gene-Giralt A, Luaces-Cubells C. Invasive disease by Streptococcus pyogenes: patients hospitalized for 6 years. Enferm Infecc Microbiol Clin. 2018;36(6):352-6.
3. Hanski E, Caparon M. Protein F, a fibronectin-binding protein, is an adhesin of the group A streptococcus Streptococcus pyogenes. Proc Natl Acad Sci U S A. 1992;89(13):6172-6.
4. Shumba P, Mairpady Shambat S, Siemens N. The Role of Streptococcal and Staphylococcal Exotoxins and Proteases in Human Necrotizing Soft Tissue Infections. Toxins (Basel). 2019;11(6).
5. Kapur V, Topouzis S, Majesky MW, Li LL, Hamrick MR, Hamill RJ. A conserved Streptococcus pyogenes extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb Pathog. 1993;15(5):327-46.
6. Tsai PJ, Kuo CF, Lin KY, Lin YS, Lei HY, Chen FF. Effect of group A streptococcal cysteine protease on invasion of epithelial cells. Infect Immun. 1998;66(4):1460-6.
7. Chaussee MS, Cole RL, van Putten JP. Streptococcal erythrogenic toxin B abrogates fibronectin-dependent internalization of Streptococcus pyogenes by cultured mammalian cells. Infect Immun. 2000;68(6):3226-32.
8. Nyberg P, Rasmussen M, Von Pawel-Rammingen U, Bjorck L. *speB* modulates fibronectin-dependent internalization of Streptococcus pyogenes by efficient proteolysis of cell-wall-anchored protein F1. Microbiology. 2004;150(Pt 5):1559-69.
9. Facinelli B, Spinaci C, Magi G, Giovanetti E, P EV. Association between erythromycin resistance and ability to enter human respiratory cells in group A streptococci. Lancet. 2001;358(9275):30-3.
10. Neeman R, Keller N, Barzilai A, Korenman Z, Sela

S. Prevalence of internalisation-associated gene, *prtF1*, among persisting group-A streptococcus strains isolated from asymptomatic carriers. Lancet. 1998;352(9145):1974-7.
11. Haller M, Fluegge K, Arri SJ, Adams B, Berner R. Association between resistance to erythromycin and the presence of the fibronectin binding protein F1 gene, *prtF1*, in Streptococcus pyogenes isolates from German pediatric patients. Antimicrob Agents Chemother. 2005;49(7):2990-3.
12. Baldassarri L, Creti R, Imperi M, Recchia S, Pataracchia M, Orefici G. Detection of genes encoding internalization-associated proteins in Streptococcus pyogenes isolates from patients with invasive diseases and asymptomatic carriers. J Clin Microbiol. 2007;45(4):1284-7.
13. Hotomi M, Billal DS, Togawa A, Ikeda Y, Takei S, Kono M. Distribution of fibronectin-binding protein genes (*prtF1* and prtF2) and streptococcal pyrogenic exotoxin genes (spe) among Streptococcus pyogenes in Japan. J Infect Chemother. 2009;15(6):367-73.
14. LaPenta D, Rubens C, Chi E, Cleary PP. Group A streptococci efficiently invade human respiratory epithelial cells. Proc Natl Acad Sci U S A. 1994;91(25):12115-9.
15. Hagman MM, Dale JB, Stevens DL. Comparison of adherence to and penetration of a human laryngeal epithelial cell line by group A streptococci of various M protein types. FEMS Immunol Med Microbiol. 1999;23(3):195-204.
16. Molinari G, Chhatwal GS. Streptococcal invasion. Curr Opin Microbiol. 1999;2(1):56-61.
17. Staali L, Morgelin M, Bjorck L, Tapper H. Streptococcus pyogenes expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. Cell Microbiol. 2003;5(4):253-65.
18. Mohammed Kalgo H, Syawani Jasni A, Rohani Abdul Hadi S, Huda Umar N, Nur Adila Hamzah S, Awang Hamat R. Extremely Low Prevalence of Erythromycin-Resistant Streptococcus pyogenes Isolates and Their Molecular Characteristics by M Protein Gene and Multilocus Sequence Typing Methods. Jundishapur J Microbiol. 2018;11(5):e12779.
19. Rohde M, Cleary PP. Adhesion and invasion of Streptococcus pyogenes into host cells and clinical relevance of intracellular streptococci. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. Streptococcus pyogenes : Basic Biology to Clinical Manifestations. Oklahoma City (OK)2016.
20. Chiang-Ni C, Wu JJ. Effects of streptococcal pyrogenic exotoxin B on pathogenesis of Streptococcus pyogenes. J Formos Med Assoc. 2008;107(9):677-85.
21. Strus M, Heczko PB, Golinska E, Tomusiak A, Chmielarczyk A, Dorycka M. The virulence factors of group A streptococcus strains isolated from invasive and non-invasive infections in Polish and German centres, 2009-2011. Eur J Clin Microbiol Infect Dis. 2017;36(9):1643-9.
22. Defining the group A streptococcal toxic shock syndrome. Rationale and consensus definition. The Working Group on Severe Streptococcal Infections. JAMA. 1993;269(3):390-1.
23. C.L.S.I. Performance Standards for Antimicrobial Susceptibility Testing 2016 January 2016. 256 p.
24. Brenciani A, Bacciaglia A, Vecchi M, Vitali LA, Varaldo PE, Giovanetti E. Genetic elements carrying erm(B) in Streptococcus pyogenes and association with tet(M) tetracycline resistance gene. Antimicrob Agents Chemother. 2007;51(4):1209-16.
25. Natanson S, Sela S, Moses AE, Musser JM, Caparon MG, Hanski E. Distribution of fibronectin-binding proteins among group A streptococci of different M types. J Infect Dis. 1995;171(4):871-8.
26. Sela S, Aviv A, Tovi A, Burstein I, Caparon MG, Hanski E. Protein F: an adhesin of Streptococcus pyogenes binds fibronectin via two distinct domains. Mol Microbiol. 1993;10(5):1049-55.
27. Hraoui M, Boutiba-Ben Boubaker I, Doloy A, Ben Redjeb S, Bouvet A. Molecular mechanisms of tetracycline and macrolide resistance and emm characterization of Streptococcus pyogenes isolates in Tunisia. Microb Drug Resist. 2011;17(3):377-82.
28. Huang CY, Lai JF, Huang IW, Chen PC, Wang HY, Shiau YR. Epidemiology and molecular characterization of macrolide-resistant Streptococcus pyogenes in Taiwan. J Clin Microbiol. 2014;52(2):508-16.
29. Antibiotic Use and Antibiotic Resistance in Food Animals in Malaysia: A Threat to Human And Animal Health [press release]. Malaysia: Consumers' Association of Penang, 10 October 2013 2013.
30. Shulman ST, Bisno AL, Clegg HW, Gerber MA, Kaplan EL, Lee G. Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America. Clin Infect Dis. 2012;55(10):e86-102.
31. Lu B, Fang Y, Fan Y, Chen X, Wang J, Zeng J. High Prevalence of Macrolide-resistance and Molecular Characterization of Streptococcus pyogenes Isolates Circulating in China from 2009 to 2016. Front Microbiol. 2017;8(1052).
32. Rafei R, Hawli M, Osman M, Dabboussi F, Hamze M. Distribution of emm types and macrolide resistance determinants among group A streptococci in the Middle East and North Africa. Journal of Global Antimicrobial Resistance. 2020.
33. Silva-Costa C, Frires A, Ramirez M, Melo-Cristino J, Portuguese Group for the Study of Streptococcal I. Differences between macrolide-resistant and -susceptible Streptococcus pyogenes: importance

of clonal properties in addition to antibiotic consumption. Antimicrob Agents Chemother. 2012;56(11):5661-6.
34. Richter SS, Heilmann KP, Beekmann SE, Miller NJ, Miller AL, Rice CL. Macrolide-resistant Streptococcus pyogenes in the United States, 2002-2003. Clin Infect Dis. 2005;41(5):599-608.
35. Sriskandan S, McKee A, Hall L, Cohen J. Comparative effects of clindamycin and ampicillin on superantigenic activity of Streptococcus pyogenes. J Antimicrob Chemother. 1997;40(2):275-7.
36. Norrby-teglund A, Ihendyane N, Darenberg J. Intravenous Immunoglobulin Adjunctive Therapy in Sepsis, with Special Emphasis on Severe Invasive Group A Streptococcal Infections. Scand J Infect Dis. 2003;35(9):683-9.
37. Andreoni F, Zьrcher C, Tarnutzer A, Schilcher K, Neff A, Keller N. Clindamycin Affects Group A Streptococcus Virulence Factors and Improves Clinical Outcome. The Journal of Infectious Diseases. 2016;215(2):269-77.
38. Brisson-Noл A, Arthur M, Courvalin P. Evidence for natural gene transfer from gram-positive cocci to Escherichia coli. J Bacteriol. 1988;170(4):1739-45.
39. Delvecchio A, Currie BJ, McArthur JD, Walker MJ, Sriprakash KS. Streptococcus pyogenes prtFII, but not sfbI, sfbII or fbp54, is represented more frequently among invasive-disease isolates of tropical Australia. Epidemiol Infect. 2002;128(3):391-6.
40. Le Bouguйnec C, de Cesрdиs G, Horaud T. Presence of chromosomal elements resembling the composite structure Tn3701 in streptococci. J Bacteriol. 1990;172(2):727-34.
41. Hammerum AM, Nielsen HUK, Agersш Y, Ekelund K, Frimodt-Moller N. Detection of tet(M), tet(O) and tet(S) in tetracycline/minocycline-resistant Streptococcus pyogenes bacteraemia isolates. J Antimicrob Chemother. 2004;53(1):118-9.
42. Malhotra-Kumar S, Lammens C, Piessens J, Goossens H. Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. Antimicrob Agents Chemother. 2005;49(11):4798-800.
43. Brenciani A, Bacciaglia A, Vignaroli C, Pugnaloni A, Varaldo PE, Giovanetti E. Phim46.1, the main Streptococcus pyogenes element carrying mef(A) and tet(O) genes. Antimicrob Agents Chemother. 2010;54(1):221-9.
44. Giovanetti E, Brenciani A, Tiberi E, Bacciaglia A, Varaldo PE. ICESp2905, the erm(TR)-tet(O) element of Streptococcus pyogenes, is formed by two independent integrative and conjugative elements. Antimicrob Agents Chemother. 2012;56(1):591-4.
45. Nielsen HU, Hammerum AM, Ekelund K, Bang D, Pallesen LV, Frimodt-Moller N. Tetracycline and macrolide co-resistance in Streptococcus pyogenes: co-selection as a reason for increase in macrolide-resistant *S. pyogenes*? Microb Drug Resist. 2004;10(3):231-8.
46. Brandt CM, Allerberger F, Spellerberg B, Holland R, Lutticken R, Haase G. Characterization of consecutive Streptococcus pyogenes isolates from patients with pharyngitis and bacteriological treatment failure: special reference to *prtF1* and sic / drs. J Infect Dis. 2001;183(4):670-4.
47. Jadoun J, Ozeri V, Burstein E, Skutelsky E, Hanski E, Sela S. Protein F1 Is Required for Efficient Entry of Streptococcus pyogenes into Epithelial Cells. The Journal of Infectious Diseases. 1998;178(1):147-58.
48. Natanson S, Sela S, Moses AE, Musser JM, Caparon MG, Hanski E. Distribution of Fibronectin-Binding Proteins among Group A Streptococci of Different M Types. The Journal of Infectious Diseases. 1995;171(4):871-8.
49. Spinaci C, Magi G, Zampaloni C, Vitali LA, Paoletti C, Catania MR. Genetic diversity of cell-invasive erythromycin-resistant and -susceptible group A streptococci determined by analysis of the RD2 region of the *prtF1* gene. J Clin Microbiol. 2004;42(2):639-44.