# Distribution of some virulence factors related to Streptococcus pyogenes isolated from tonsilitis in AL –Diwaniyah provence

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## Abstract

Streptococcus pyogenes(group A streptococcus ,GAS) is a human pathogen that causes a wide spectrum of clinical diseases .The pathogenic properties of GAS strains are often linked to the production of virulence factors such as toxins, proteases or DNases. Detection of virulence factors produced by GAS strains can be used to either determine pathogenic potential of the strain or as a rapid screening method. We recently developed a method to detect simultaneously 7 GAS virulence factors (spe C, I, A, H, G, smeZ and ssa). In this study we found a high rate and degree of resistance to penicillins group and tetracyclin, wetherase were strains very susceptible to meropenem and chloramphenicol The results of molecular screening showed SpeA gene were 46.6% , speC gene was 80 %,smez gene was 53.3%, speI gene was 10%, ssa gene was 73.3,speH gene was 56.6% and speG gene was 60% of total isolates.

## INTRODUCTION

Streptococcus pyogenes(group A streptococcus ,GAS) are spherical bacteria, gram positive , usually containing a capsule composed of hyaluronic acid. It has the ability to ferment lactose, and produce acid without releasing gas, decomposing arginine and non-hydrolyzing sodium hiprate or esculin and not having the ability to grow in media containing bile salts at a concentration of 40% or NaCl at a concentration of 6.5%, resistant to optokine, sensitive to bacitracin, and does not grow at a temperature of 10 or 45 °C (Macfaddin, 2000).

(Okamoto et al. 2004) pointed out the important role played by the capsule in the invasion of host cells by being able to bind to some types of filtrates such as IAV Influenza A virus that causes respiratory infection Anaerobic It needs rich growth media because it is highly sensitive, but in general it grows on a medium that can detect complete hemolysis of beta cells, and its diameter ranges from 0.5 - 1.0 μm (Shulman et al., 2012) .

It is the first to do so, and it is the main cause of many diseases that affect humans of different ages and are broad-spectrum of infections and disease (Brook et al., 2014). In 1928, Rebecca Lancefield published a method used to determine serotypes based on the M protein and was able to describe the serological classification of antigen T, as four out of twenty T-antigens were detected (Ryan and Ray, 2004). It is estimated that 5-15% of health individuals can have S. pyogenes present in their respiratory tract and occur without showing symptoms as normal flora (Chang, 2011).

Streptococcal Superantigens S. pyogenes

Streptococcal Superantigens are proteins with prominent peptides that divide at secretion with a molecular weight 22–28 k Dalton (McCormick et al., 2006). They share amino acid sequences between 17% and 48%, and are part of a large family of toxins that include SAgs (Streptococcal Superantigens) structurally bonded together (Proft et al., 2003). To date, approximately 13 toxins have been labeled in S. pyogenes: SpeA, SpeB, SpeC, SpeF, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, and the mitotoxin Z (SmeZ) streptococcal mitogenic exotoxin Z (Tortora et al., 2013). streptococcal Super antigens (SAgs) bind to the molecules of the main histocompatibility complex II MHCII (MHCII Major histocompatibility complex II) on host cells and thus provide antigen and T cell receptors, releasing large amounts of cytokines. These cytokines have been shown to play major roles in fever, tissue destruction, shock, hypotension, and organ failure associated with bacterial infections (Lappin and Ferguson, 2009), however, these antigens are located on mobile genetic elements (Tortora et al., 2013), SpeA binds to the MHCII α subunit, and binds other superantigens to the MHCII β subunit, while SpeC has binding sites for each of the alpha subunits. and beta MHCII (Tortora et al., 2013). Erybodococcus febrile exotoxins, or red toxins, appear to be responsible for many manifestations of scarlet fever and toxic streptococcal syndrome (Buonpane et al., 2005).

Exotoxins causing red (Spes A, B, and C) (Streptococcal pyrogenic exotoxins), single-origin toxins are known to damage plasma membranes in subcutaneous capillaries and produce a red rash (Tortora et al., 2013). Exotoxin A (SpeA) is a potent antigen and cytoxin in causing necrotizing soft tissue infections and toxic shock syndrome caused by S. pyogenes (Arad et al., 2011). Exotoxins that causing Fever (streptococcal pyrogenic exotoxins), or erythrogenic toxins, appear to be responsible for many manifestations of scarlet fever and toxic streptococcal syndrome. (Buonpane et al., 2005)

The genes for SpeA, SpeC, and SpeH-M are encoded by phages (Boyd et al.,2012), while the gene for SpeB and SpeJ are chromosomes (Llewelyn and Cohen, 2002). In contrast, the SpeG gene, to which both attribute both the primary chromosome and non-isogenic bacteriophages (Brosnahan and Schlievert,2011).

SpeA is a virulent factor that can invade and cause soft-tissue infections or bacteremia caused by S. pyogenes, and the role of this toxin can be different in hosts with a superior antigen response (Llewelyn and Cohen, 2002). The SpeA gene has previously been identified as a virulence factor associated with a disease that invades the body and invades its tissues. SpeA production by S. pyogenes has therefore been linked to streptococcal toxic shock syndrome (Beres et al., 2006).

Streptococcal proteins or B protoxins (cysteine protease) are one of the most important virulence factors in S. pyogenes cystosis secreted as a 40 kDa zemogen which is broken down into a mature active 28 kDalone protease enzyme under reducing conditions (Chuan and Jiunn, 2008).

SpeB is rapidly expressed in the host's primary immune response and acts on localized skin infections as well as diffusion in saliva (Shelburne et al., 2005). It was found that the proteolytic effectiveness of SpeB in decomposition allows S. pyogenes to invade deeper into the tissues. Furthermore, SpeB is also the main antigen involved in the pathogenesis of acute post-streptococcal glomerulonephritis (APSGN) (Batsford et al., 2005).

The SpeC is an antigen produced from several S. pyogenes strains that are closely related to pathotoxic traumatic syndrome (STSS) and other rosary diseases. This gene encodes a mature protein made up of 208 amino acids, with a calculated molecular weight of 24,354. The mature amino acid sequences of SpeC are homogeneous with amino acid sequences of exogenous toxins type A (Spaulding et al., 2013).

SpeA and SpeC stimulate the activation of nonspecialized T-lymphocyte lymphocytes, inhibit antibody synthesis, stimulate fever, promote the release of precursor cytokines and may contribute to multiple organ failure (Cunningham, 2008).

SpeA and SpeC are more common in S. pyogenes infection, but SpeA are important virulent factors in septic infection and have four alleles (SpeA1-SpeA4). The SpeA2 and SpeA3 alleles are the most common alleles in infections (Alba et al., 2006).

SpeA and SpeC are responsible for scarlet fever rash, which stimulates the formation of specific anti-toxin antibodies that provide immunity to scarlet rash when infected again in the future (Hraoui et al., 2010).

## Materials and Methods

### Collection of samples

This study was proceeded on three hundred (300)throat samples collected from patients with tonsillitis aged from 12 to 45 years , who refered to AL-Diwaniyah Teaching Hospital , A Hospital for women and children ,Afak General Hospital during the peroid from January 2022 to August 2022 . Patients who presented with fever and phyrngitis were encluded into the study. Throat swab was taken from the pharynx of each patient , and disposable tongue depressor was used to depress the tongue so that the pharynx was apparent , and immediately placed in a trypton soya broth with 5% blood and transferred to the microbiology laboratory , where the broth was incubated at 37℃ for 24 hours .

### Antimicrobial susceptibility Testing

Antimicrobial resistance is popular problem worldwide and the consuming of antimicrobials has been identified as a driving force for the development and transmission of antimicrobial resistance in bacteria. The antimicrobial susceptibility profile of ten antibiotics belonging to 7 classes including Beta-lactams (Ampicillin 25mg, Ceftriaxon 10mg), Lincosamins (Clidamycin 2mg),(Macrolids) (Erythromycin 15mg and Azithromycin 15mg), fluoroquinlones (Levofloxacin 5mg) , Glycopeptides (Vancomycin 30mg), phenicols (chloramphenicol 30mg),Carbapenem (Meropenem 10mg),Tetracyclins (Tetracyclin 30mg) Tested for the sensitivity of bacterial isolates under current study according to CLSI (2021) was performed using standard disc diffusion method against 30 isolates of Streptococcus pyogenes.

### Bacterial DNA extraction

Bacterial genomic DNA was extracted bacterial isolates by using (Presto™ Mini gDNA Bacteria Kit) as and done according to company instructions.

### Detection of superantigens

### Primers

The PCR primers that used in This study primers were provided by Scientific Reserchер.Co.Ltd in Iraq as following table (1)

**Table 1**

| Primer | Sequence (5'-3') | | Product Size |
|---|---|---|---|
| *SpeC* | F | GCCAATTTCGATTCTGCCGC | 405bp |
| | R | TGCAGGGTAAATTTTTCAACGACA | |
| *SmeZ* | F | TTTCTCGTCCTGTGTTTGGA | 246bp |
| | R | TTCCAATCAAATGGGACGGAGAACA | |
| *SpeI* | F | TTCATAGACGGCGTTCAACAA | 176bp |
| | R | TGAAATCTAGAGGAGCGGCCA | |
| *Ssa* | F | AAGAATACTCGTTGTAGCATGTGT | 678bp |
| | R | AATATTGCTCCAGGTGCGGG | |
| *SpeA* | F | AGGTAGACTTCAATTTGGCTTGTGT | 576bp |
| | R | GGGTGACCCTGTTACTCACGA | |
| *SpeH* | F | TGAGATATAATTGTCGCTACTCACAT | 480bp |
| | R | CCTGAGCGGTTACTTTCGGT | |
| *SpeG* | F | TGGAAGTCAATTAGCTTATGCAG | 384bp |
| | R | GCGAACAACCTCAGAGGGCAAA | |
| | R | GGTGGGGTTACACCATCAGT | |

## Results and Discussion

Antibiotic susceptibility Testing

In this study, isolated 30 isolates of streptococcus pyogenes by using vitek 2 compact and molecular identification the susceptibility test of Streptococcus pyogenes strains showed that high activity with chloramphenicol 100% of the strain were susceptible. This result is in agreement with the finding of Wu. et al., (2014) and khalaf (2020). While the result was disagreement with percentage 82.1obtained by Camara et al., (2013). also this result disagreement with study by Willims (2021) was gave resistance 50% to this antibiotic . The reason is due to the presence of flow enzymes, which encode their inheritance carried on plasmids. Is shown in Table (2).

All isolates showed complete resistance to Ampicillin antibiotic and these results agreement with the findings of Vannice et al., (2020). also these results were agreed with study by Hero (2021) that gave resistance to Ampicillin 100%. While results of study by khalaf (2020) was observed the activity of β-lactams antibiotic against the Streptococcus pyogenes, were the Ampicillin showed high activity with 84%.

The persist susceptibility of GAS to Penicillin group is noticeable and probable due to a limited power to a quire foreign DNA and a lower fitness that may be correlating with Beta- lactam resistance Zabriskie et al.,

(1998). This result agreed with that reported in a local study Ali et al., (2015). This finding is in contrast to a work done by OKonko et al.,(2009) whose work reported resistance of Streptococcus pyogenes to Ampicillin .

As for Ceftriaxone , it showed greater activity were 70% of strain sensitive to Ceftriaxone , because of its unusually long half-life. it has high antibacterial activity against group A Streptococci Patel et al.,(1981) . Ceftriaxone may be potentially advantageous antibiotic in the treatment of streptococcal disease Stevens et al., (1992). This result is in agreement with a previous reported by Beskid et al.,(1981).also agreement with results of study by Kalaf (2020) that showed sensitive to Ceftriaxone 76 %. in this current study , carbapenem antibiotic represented by Meropenem all isolates showed a complete sensitivity of 100% to antibiotic and these results were consistent with the findings of Al-Masoudi (2016), what explains the lack of resistance to carbapenem antibiotic is their high ability to inhibit most betalactamase enzymes (Mirsalehian et al., 2017). The emergence of resistance to these antibiotics is very worrying because they are the last resort .

In this study activity of clindamycin for group A streptococci was good activity 76% of strains were susceptible with 7% resistance. This result is in agreement with study of Ali et al.,(2015) and also low rates of resistance to Clindamycin have been observed in Japan Ikebe et al (2005) , and in Germany Sauermann et al.,(2003)

In this recently study, Levofloxacin showed a good activity 50% and 50% resistance this is disagreement with a study reported by Van Heirsteraetem et al., (2012). Also disagreement with study by Khalaf (2020) that showed sensitive 69% and 23% resistance . while these results were in agreement with local study by Hero (2021) that showed sensitive 60 % to Levofloxacin. Also isin agreement with results other study by Deshwal et al.,(2020) However, based on results, Levofloxacin may play an effective alternative choice for treatment of patients infected with Streptococcus pyogenes infections in case of Penicillin allergy and resistance to macrolides antibiotic . In a study carried out by Berwal et al ., (2019), the isolates of S. pyogenes isolated from respiratory infections in the care units showed resistance of (5.3%) to the antidote Levofloxacin, and Said et al., (2020) were able to obtain susceptible isolates of the Levofloxacin antibiotic through his study conducted in the city of Duhok on the production of pelvic inflammatory disease, which explains the lethal action of this group of antibiotics and their efficacy. High anti-Streptoccus bacteria begins by inhibiting the enzyme DNAgyrase, and the accumulation of cut double strands in the genome of the bacterial cell, which causes obstruction of the basic movements of the DNA molecule and the enzyme RNApoymerase along the DNA template. (Khan et al.,2018 ).

However, the point mutation within the quinolone resistance – determining region situated within the topoisomerase is the main sources for low level Levofloxacin resistance Montes et al.,(2010).

Vancomycin was also good and active against Streptococcus pyogenes with susceptibility rate 50% to these antibiotic . These results are disagreement with findings reported in Iraq by Kalaf (2020) that reported sensitivity 76 % and in other countries (D'oliveira et al; 2003 , Loza et.al 2008) .while other study showed percent of resistant was in this study activity of clindamycin for group A streptococci was activity 40% of strains were susceptible with 60% resistance. This result is disagreement with study of Ali et al.,(2015) and also low rates of resistance to Clindamycin have been observed in Iraq Khalaf (2020) were 76% of strains were susceptible with 7% resistance. and also low rates of resistance to Clindamycin have been observed in Japan

Ikebe et al., (2005) , and in Germany Sauermann et al.,(2003). whereas 94.2, % was resistant to clindamycin Lu et al.,(2017) .Also was the resistance rate to clindamycin 96.6 % in other study by Hongxin Li et al .,(2020).

In this present study we observed high resistance to macrolid antibiotic, the percentage of resistance of Erythromycin and Azithromycin for Streptococcus pyogenes were 65% and 50% respectively. This result is in agreement with a local study by Saleem et al., (2011), Ali et al., (2015) and in agreement with the findings of Yan et al., 2003 who reported high macrolide resistance (40-70%) in Taiwan . other study by Khalaf (2020) showed resistance of Erythromycin and Azithromycin for Streptococcus pyogenes were 69% and 38% respectively . This result is different from that has been According to Khademi et al., (2021), they obtained resistant isolates due to S. pyogenes (12%) in a study he conducted in a children's hospital in Iran. while the resistance rate to erythromycin is 98.3% in study by Hongxin Li et al .,(2020). whereas 93.5% was resistant to erythromycin Lu B, et al.,(2017).This result is dramatically different from that has been reported in Saudi Arabia showed that the resistant was only 6.3% Shibl (2005), and similar percentage have been reported in Germany, UK and Canada. . Macrolide resistance in group A streptococci happen via mechanisms: Target site modification or target drug efflux Seppala et al., (1998). Macrolide resistance was mainly due to the presence of erm (B) between emm 28 and emm 11 types Silva- costa et al., (2008) .(Table 2) .

In this study showed 50% of isolates susceptibility to tetracyclin antibiotic. while the resistance rate to tetracycline is 90.23% in study by Hongxin Li et al .,(2020) . whereas 86.4% was resistant to tetracycline in study by Lu B, et al., (2017).

**Table (2): susceptibility rates of Streptococcus pyogenes (disc diffusion).**

| Antibiotic | R% | I% | S% |
|---|---|---|---|
| Ampicillin | 100 | 0 | 0 |
| Cefitrixone | 70 | 5 | 25 |
| Erythromycin | 50 | | 35 |
| Azithromycin | 50 | 5 | 45 |
| Clindamycin | 60 | 0 | 40 |
| Chloramphenicol | 0 | 0 | 100 |
| Vancomycine | 50 | 0 | 50 |
| Levofloxacin | 50 | 0 | 50 |
| Meropenem | 0 | 0 | 100 |
| Tetracyclin | 50 | 0 | 50 |

Molecular Detection of virulence factors

From exotoxins secreted by many strains of streptococci, this exotoxin that generates fever is the main responsible for the rash of scarlet fever .

The results in this study showed that the bacterial isolates have SpeA gene were 46.6%, with a size of 576pb (Figure 1), when compared with previous studies Be inconsistent with Masoudi (2016) he got 100% in his study that isolated from tonsils and blood, and was contrary to the study of Wang (2012) which scored 12.7%, and also incompatible with Beres and his group (2006) and the percentage was 18.4% and also not identical with the study conducted by Nabat in the province of Babylon (2018) and got 10.8% and was also inconsistent with the results of the study of Tyler (1992) and got 81%, and the results of The current study was not compatible with the findings of the study of Hero (2021), where it obtained 100%. Previous studies have shown that the presence of the gene (speA) in bacterial strains that have a high pathogenic ability to infect the skin and tonsil infection, and also that SpeA is expressed at four times higher levels when strains are grown at 37 ° C than at 26 ° C. ( Wang, Xiaohu, et al. 2012) . Many other studies have suggested that SpeA is typically associated with group A strains of isolated rosaries from patients with highly invasive disease. because this pyrogenian exotoxin is

always present in isolates from patients with acute invasive tissue disease (Wang, Xiaohu et al., 2012). Besides, the genetic codes of SpeA for the toxin that contributes to the virulence of S. pyogenes have been observed in more isolates (46.6%) in people with S. pyogenes infection and these isolates are likely to be very virulent.

Biofilm, capsule, and adhesion genes can probably be indicative of the possible presence of exotoxins because they provide them with protection and cover from antibiotics (Gogos 2020).

From the above, we can say that the pathogenicity of S. pyogenes has evolved through many virulence factors expressed genetically, and the virulence of this bacteria increases our understanding of how the normal flora is able to invade and infect the host's tissues because they are sometimes normal flora.

GAS superantigens, except speG, speJ, and smeZ encoded by chromosome, speA, speC, speH, speI, and ssa are encoded by phage, which is the main driving force for pathogenic strains to obtain pathogenic factors through transfer also the results in this study showed that the bacterial isolates contain , speC gene was 80 % with a size of 405pb (Figure 2),smez gene was 53.3% with a size of 246pb (Figure 3), speI gene was 10% with a size of 176pb (Figure 4), ssa gene was 73.3% with a size of 687pb (Figure 5), speH gene was 56.6% with a size of 480pb (Figure 6) and speG gene was 60% with a size of 384pb (Figure 7). The transfer and mutation of genes can produce highly pathogenic GAS strains, which affect the epidemic situation of the GAS disease, resulting in different distributions of the S. pyogenes superantigen gene spectrum in different periods and geographical areas.

A study from Portugal showed that smeZ (96.0%) and speG (86.9%) were common in GAS, followed by speC, ssa, speJ, speA, speK, and speI Friaes et al.,(2012) .

in other study in china by Hongxin Li et al., (2020) the most common superantigen genes identified from S. pyogenes were smeZ (96.97%), speC (92.59%), speG (91.58%), and ssa (85.52%), while the expression rate of other superantigens was slightly lower: speI (54.55%), speH (52.19%) and speA (34.34%). In this study, 7 superantigens were detected in GAS isolates, and speC, ssa and speH were the most common superantigens, but the content of speI, speG, and smeZ was less. A study from Germany showed that the most common superantigen genes in GAS were speG (92.1%), and speC (42.0%). Simultaneously Imohl M et al.,(2017). the most prevalent types of GAS isolates from pediatric patients during 1993–1994 and 2005–2006. Isolates carrying six or more superantigen genes increased from 46.53% in 1993–1994 to 78.39% in 2005–2006. The level of ssa, speH, and speJ genes increased, while that of speA decreased. The gene profiles of superantigen were associated with the emm type, but strains of the same emm type occasionally carry different superantigen genes in the two periods(Ma Y et al.,2009) .

**Figure (1): Agarose gel electrophoresis image that showed PCR product analysis for detection of SpeA gene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive SpeA gene Streptococcus pyogenes isolates at 576bp product size.**

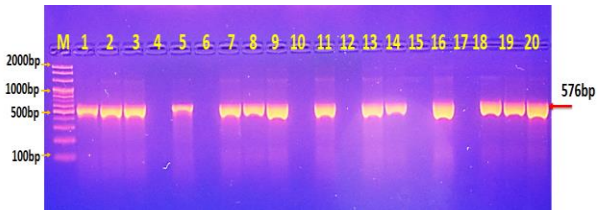


**Figure (2): Agarose gel electrophoresis image that showed PCR product analysis for detection of speC gene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive speC gene Streptococcus pyogenes isolates at 405bp product size.**

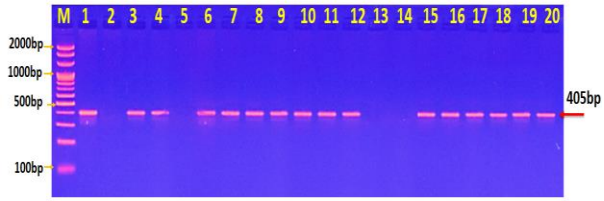



**Figure (3): Agarose gel electrophoresis image that showed PCR product analysis for detection of smeZ gene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive SmeZ gene Streptococcus pyogenes isolates at 246bp product size.**

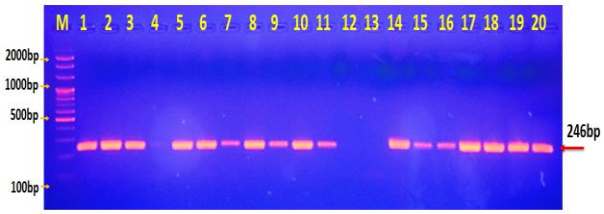



**Figure (4): Agarose gel electrophoresis image that showed PCR product analysis for detection of speI gene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive speI gene Streptococcus pyogenes isolates at 176bp product size.**

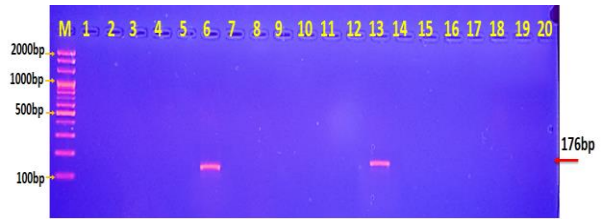



**Figure (5): Agarose gel electrophoresis image that showed PCR product analysis for detection of ssa gene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive ssa gene Streptococcus pyogenes isolates at 678bp product size.**

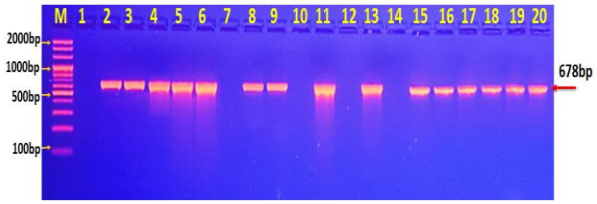



**Figure (6): Agarose gel electrophoresis image that showed PCR product analysis for detection of speH gene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive speH gene Streptococcus pyogenes isolates at 480bp product size.**

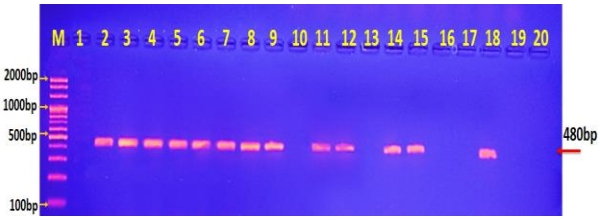
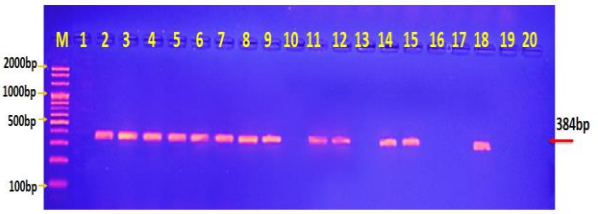



**Figure (7): Agarose gel electrophoresis image that showed PCR product analysis for detection of speGgene Streptococcus pyogenes from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive Mac gene Streptococcus pyogenes isolates at 384bp product size.**




## Conclusions

We conclude streptococcus pyogenes increasing resistant to antibiotics because using random to these antibiotics such as

macrolides and tetracyclins also that have many of virulence factors that help this bacteria to occur infection for human.

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