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RESEARCH ARTICLE

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Prevalence and multidrug resistance pattern of β-lactam resistant *Streptococcus pyogenes* isolated from nasopharyngeal infections

ABSTRACT:

Group A Streptococcus (GAS), commonly known as *Streptococcus pyogenes*, is one of the top ten infectious causes of death globally. Increased antibiotic resistance is the main cause of streptococcal infection treatment failure. Therefore, this study was conducted to evaluate the occurrence, antimicrobial resistance, and genetic characterization of *S. pyogenes* isolated from different patients. A total of 60 pharyngitis and tonsillitis throat swabs were obtained. Only 7 isolates (11.6%) were confirmed to be *S. pyogenes*. The highest prevalence of *S. pyogenes* was obtained from children, boys (26.6%) followed by adults (males) (16.6%) while the lowest prevalence was recovered from girls (11.7%). On the other hand, no infection was recorded in the case of females. All *S. pyogenes* isolates were susceptible to ampicillin-sulbactam, ciprofloxacin, chloramphenicol, doxycycline, meropenem, and tetracycline. While 100% showed resistance to amoxicillin-clavulanic acid, cefotaxime, and cephradine followed by ceftriaxone (71%) and cefuroxime (71%). Based on the multidrug-resistance (MDR) profile, a total of 6 out of 7(85.7%) *S. pyogenes* isolates were resistant to 3 or more of β-lactam antibiotics. The PCR assay revealed that the bla_{TEM}, bla_{Z}, bla_{IMP}, and bla_{CTX} genes were detected in 57.1%, 28.5%, 57.1%, 42.8%, 15%, 11.3%, and 5.6% of the isolates. To the best of our knowledge, this is the global study about these beta lactamase genes in *Streptococcus pyogenes*.

KEY WORDS:

Streptococcus pyogenes, β-lactam resistance, bla_{TEM}, bla_{Z}, bla_{IMP}, bla_{CTX} genes, pharyngitis

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INTRODUCTION:

Acute sinusitis, acute otitis media, pharyngitis, community-acquired pneumonia, and acute bronchitis are widespread respiratory tract infections and represent a major health concern, especially in low-resource settings. One of the most common causes of acute respiratory tract infections is *Streptococcus pyogenes* (*S. pyogenes*). *S. pyogenes* is a Gram-positive that belongs to the Streptococcaceae, extracellular, spherical shape and β-hemolytic bacterium that can grow on enriched culture media (Walker *et al.*, 2014). Several clinical conditions such as scarlet fever, acute rheumatic fever, glomerulonephritis, sepsis, necrotizing fasciitis, meningitis, streptococcal toxic shock syndrome, impetigo, and acute pharyngitis

were caused by *S. pyogenes* (Sanyahumbi *et al.*, 2016). Sore throat, abrupt onset fever, red pharynx, swollen tonsils, yellow or blood-tinged exudates, petechiae on the soft palate and posterior pharynx are some of the clinical signs of acute pharyngitis (Choby, 2009). Every year, over a hundred million people become infected with *S. pyogenes.* It was reported that from 2009 to 2014, *S. pyogenes* generated approximately 660,000 invasive infections and 616 million instances of pharyngitis, resulting in 163,000 deaths (Imöhl *et al*., 2017).

Streptococcus pyogenes was isolated from children with acute pharyngitis in African countries, with a prevalence rate of 66.7, 28, 2.3, and 11.3% in Nigeria (Uzodimma *et al*., 2017), Egypt (Sultan and Seliem, 2018), Kenya (Osowicki *et al*., 2019; Kebede *et al*., 2021) and Jimma, Ethiopia (Tesfaw *et al*., 2015), respectively. *S. pyogenes* can be transmitted through direct contact, contaminated fomites, or food-borne contamination or droplets from those with pharyngeal infection or colonization (Do *et al*., 2019). Even though untreated *S. pyogenes* acute pharyngitis causes post-infection complications such as acute rheumatic fever (ARF) and rheumatic heart disease (RHD) and glomerulonephritis (Khandekar, 2019).

Streptococcus pyogenes was considered susceptible to β-lactam antibiotics, such as penicillins and cephalosporins. As a result, penicillin is used as a first-line antibiotic, and macrolides are a different possibility (Camara *et al*., 2013). The emergence of *S. pyogenes* isolates with resistance to β-lactam antibiotics or reduced susceptibility to penicillin had been reported in several studies. Therefore, this work was performed to evaluate the prevalence and β-lactam resistance of *S. pyogenes* obtained from different patients in Benha Teaching Hospital, Qalyubia Governorate, Egypt.

MATERIAL AND METHODS:

Ethical Aspects:

The Ethics Committee of Benha University Hospital gave its approval to the study protocol. All procedures were performed following the Declaration of Helsinki.

Sampling:

A total of 60 samples were taken from Benha Teaching Hospital, Qalyubia Governorate, Egypt. Out of all samples, fifty-five were recovered from the throat, 4 were collected from Ear discharges, and only one was obtained from sputum. All samples were collected during the period between July 2018 and November 2020. Samples were collected under hygienic conditions via sterile cotton swabs preserved in an Amie's Transport Medium. A code number was assigned to each sample and transported immediately to the laboratory for microbiological investigation.

Isolation and identification:

Streptococcus pyogenes was isolated using the method described by the Clinical Laboratory of Standard Institute (CLSI, 2019). Samples were cultivated for 24 to 48 hours at 37°C on Tryptic-soya agar (TSA) supplemented with 5% sheep blood and incubated in 5% CO_2. Bacteriological features were used to phenotypically identify *S. pyogenes* isolates (including blood haemolysis, Gram stain, catalase, and growth inhibition around a disc containing 0.04 units of bacitracin).

Antibiotic sensitivity test (AST):

Antimicrobial susceptibility was investigated on *S. pyogenes* isolates using antibiotic disk diffusion technique in compliance with the clinical and laboratory standard institute (CLSI, 2018) guidelines. The isolates were tested against 21 antibiotics belonging to β-lactam, Cyclines, Aminoglycosides, Macrolides, Quinolones, Carbapenems, Lincosamides, Glycopeptides, Phenicols and Sulfonamide classes represented by penicillin G (P, 10 µg), cefotaxime (CTX, 30 µg) , ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), cephradine (CE, 30 µg), cefuroxime (CXM, 30 µg), amoxicillin-clavulanic acid (AMC, 30 µg), ampicillin-sulbactam (SAM, 20 µg), piperacillin (PRL,100 µg), erythromycin (E, 15 µg,), clindamycin (DA, 2 µg), vancomycin (VA, 30 µg), chloramphenicol (C, 30 µg) and tetracycline (TE, 30 µg), doxycycline (DO 30 µg), gentamycin (CN, 10 µg), amikacin (AK, 30 µg), novobiocin (NV, 30 µg), meropenem (MEM, 30 µg), ciprofloxacin (CIP, 5 µg), and sulfamethoxazole/trimethoprim (SXT, 25 µg). At 37°C, plates were incubated for 16 – 24 hours. Based on the inhibitory zone, the outcome was classified as resistant, intermediate, or susceptible. Multidrug-resistant strains were those that showed resistance to at least three antibiotic classes (MDR) (Magiorakos *et al*., 2012).

DNA, plasmid extraction and PCR amplification:

The Qiaamp DNA Mini Kit was used to extract DNA from samples (Qiagen, Germany, GmbH). For 10 min at 56ºC, 200 µL of the culture suspension were treated with 10 µL of proteinase K and 200 µL of lysis buffer. After incubation, 200 µL of 100% ethanol was added to the lysate. Following the manufacturer's instructions, the sample was washed and centrifuged. The nucleic acid was eluted with 100 µL of elution buffer provided in the kit. The isolates were confirmed as *S. pyogenes* using 16S rRNA primer (Iwasaki *et al*., 1993) then Plasmid DNAs were extracted from bacterial isolates using Plasmid DNA Miniprep Kits (Thermo Fisher Scientific, Waltham, MA, USA)

following the manufacturer's instructions screened for the presence of the beta-lactamase genes including *bla$_{TEM}$*, *bla$_{Z}$*, *bla$_{IMP}$*, and *bla$_{CTX}$*. The characteristics of all used primers, as well as amplicons size and PCR conditions, are summarized in table 1 as reviewed by Colom *et al*. (2003), Pitkälä *et al*. (2007), Xia *et al*. (2012), and Mohamudha Parveen *et al*. (2012).

Table 1. Primer sequences and cycling conditions during PCR

Primer	Sequence	Amplified product	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16Sr RNA S. pyogenes	CTA CTT GGA TCA AGA CGG GT TTA GGG TTT CCA GTC CAT CC	419 bp	95˚C 2 min.	95˚C 30 sec.	53˚C 30 sec	72˚C 30 sec	35	72˚C 12 min.
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec	72˚C 40 sec	35	72˚C 7 min.
blaZ	CAAAGATGATATAGTTGCTTATTCTCC TGCTTGACCACTTTTATCAGC	610 bp	95˚C 10 min.	95˚C 15 sec.	56˚C 20 sec.	72˚C 18 sec.	35	72˚C 10 min.
blaIMP	CATGGTTTGGTGGTTCTTGT ATAATTTGGCGGACTTTGGC	488 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 40 sec	35	72˚C 10 min.
blaCTX	CGC TTT GCC ATG TGC AGC ACC GCT CAG TAC GAT CGA GCC	307 bp	95˚C 10 min.	95˚C 15 sec.	60˚C 1 min.	72˚C 30 sec.	35	72˚C 10 min.

PCR products visualization and analysis:

The products of PCR were separated by electrophoresis on 1% agarose gel (AppliChem, Germany, GmbH) by running 20 µl of the PCR products. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analysed by computer software.

RESULTS:

Colonial appearance and biochemical identification of *S. pyogenes* isolates:

Streptococcus pyogenes produces beta-haemolytic colonies on blood agar. The colonies were encircled by a zone of full haemolysis and haemoglobin decolonization. They were small, colourless, dry, shiny (sometimes mucoid), and produced an inhibition around a disk containing 0.04 units of bacitracin). *S. pyogenes* isolates were confirmed to be Gram-positive by gram staining, and negative for catalase production test.

Prevalence of *S. pyogenes* among different patients:

A total of 60 samples were isolated from the Department of Otolaryngology from Benha Teaching Hospital, 32 paediatric patients (2–15 years old) and 28 from adults (18–60 years old). Among all isolates, 7 (11.6%) were positive beta-haemolytic *S. pyogenes*. It was observed that the highest prevalence of *S. pyogenes* was recorded in children (boys) (26.6%) and adults (males) (16.6%). While the lowest colonization of *S. pyogenes* was found in girls (11.7%). On the other hand, no infection was detected in females (Table 2).

Table 2. Distribution of *S. pyogenes* among different patients with respiratory tract infection (n = 60).

Patients		No. of tested samples	No. of Samples positive for *S. pyogenes* (%)
Adult	Male	6	1 (16.6)
	Female	22	0.0
Children	Boys	15	4 (26.6)
	Girls	17	2 (11.7)
Total		60	7 (11.6)

Antibiotic Susceptibility Testing:

The antibiotic sensitivity and resistance rates for whole isolates are represented in table 3. Of the 7 isolates, 100% showed resistance to amoxicillin-clavulanic acid, cefotaxime, and cephradine followed by ceftriaxone, cefuroxime, clindamycin, novobiocin, vancomycin (71 % for each). All isolates were susceptible to ampicillin-sulbactam, ciprofloxacin, chloramphenicol, doxycycline, meropenem, and tetracycline. Interestingly, 6 out of 7 (85.7%) of the tested *S. pyogenes* were multidrug-resistant (resistant to three or more antibiotics).

Table 3. Antibiotic susceptibility patterns of *S. pyogenes* isolated from different patients (n = 7)

Antibiotics		Sensitive		Resistant	
		No.	%	No.	%
β-lactam	Penicillin-G	3	43	4	57
	Cefotaxime	0	0	7	100
	Ceftriaxone	2	29	5	71
	Ceftazidime	5	71	2	29
	Cephradine	0	0	7	100
	Cefuroxime	2	29	5	71
	Amoxicillin-clavulanate	0	0	7	100
	Ampacillin-sulbactam	7	100	0	0
	Piperacillin	4	57	3	43
Cyclines	Doxycycline	7	100	0	0
	Tetracycline	7	100	0	0
Aminoglycosides	Amikacin	5	71	2	29
	Novobiocin	2	29	5	71
	Gentamycin	6	86	1	14
Macrolides	Erythromycin	5	71	2	29
Quinolones	Ciprofloxacin	7	100	0	0
Carbapenems	Meropenem	7	100	0	0
Lincosamides	Clindamycin	2	29	5	71
Glycopeptides	Vancomycin	2	29	5	71
Phenicols	Chloramphenicol	7	100	0	0
Sulfonamide	Sulfamethoxazole/Trimethoprim	5	71	2	29

Molecular characterization by 16Sr RNA gene:

All tested isolates gave characteristic bands at 419 bp and confirmed as *S. pyogenes* (Fig. 1).

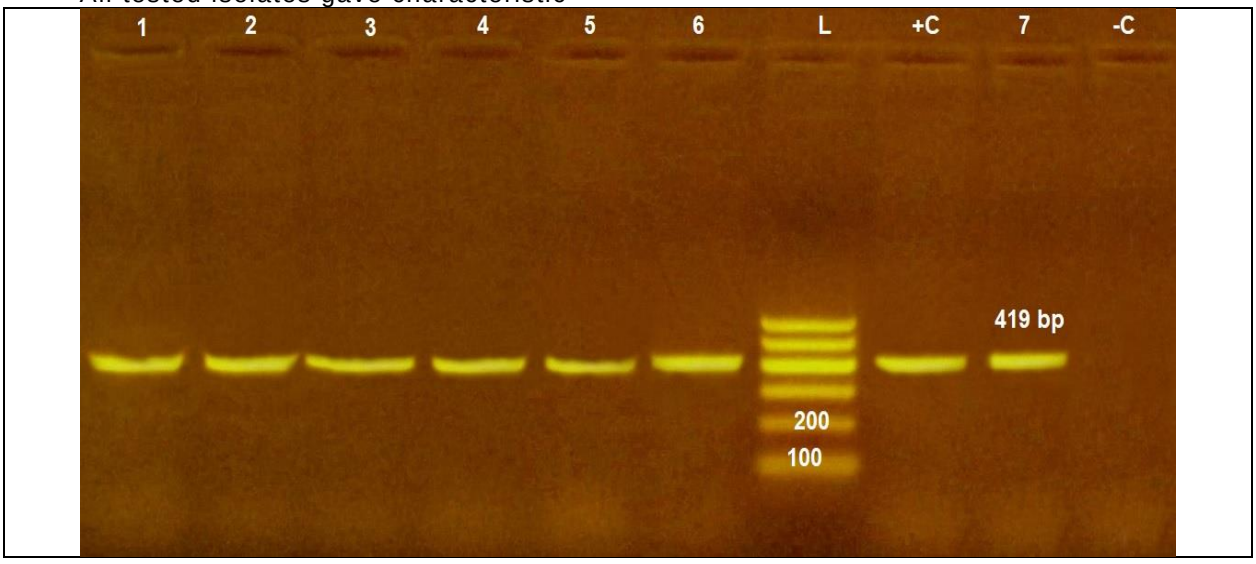

Fig. 1. Agarose gel electrophoresis of PCR- for amplification products of 16S RNA; Lane +C: Control positive (reference strain of code no. ATCC 12344), Lane L: 100-bp ladder (marker), Lanes 1-7: Positive samples; Lane -C: control negative.

Detection of β-lactamase genes in *S. Pyogenes* isolates:

A total of 6 out of 7 (85.7%) of the obtained *S. pyogenes* were harboured the β-lactamase genes. The dominant *bla* gene responsible for resistance to beta-lactam antimicrobials of *S. Pyogenes* isolates was found to be variants of *bla* genes. The bla_{TEM}, bla_{Z}, bla_{IMP}, and bla_{CTX} genes were detected in 57.1%, 28.5%, 57.1%, and 42.8% of the isolates (Figs 2 - 5).

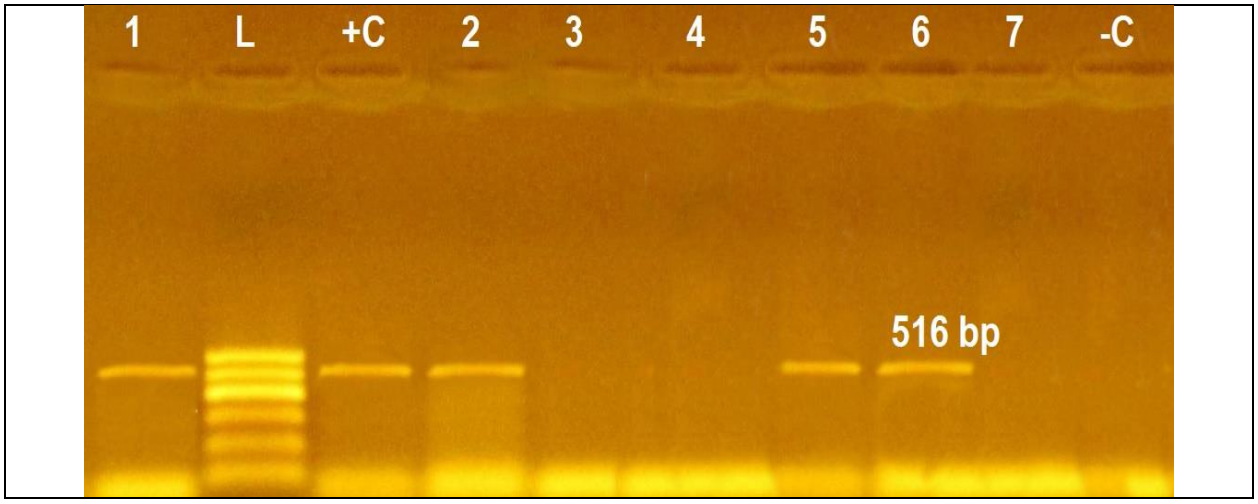

Fig. 2. Agarose gel electrophoresis of PCR- for amplification products of bla TEM gene; Lane +C: Control positive (reference strain of code no. ATCC 12344), Lane L: 100-bp ladder (marker), Lanes 1,2,5, 6: Positive samples for bla TEM gene; Lane -C: control negative.

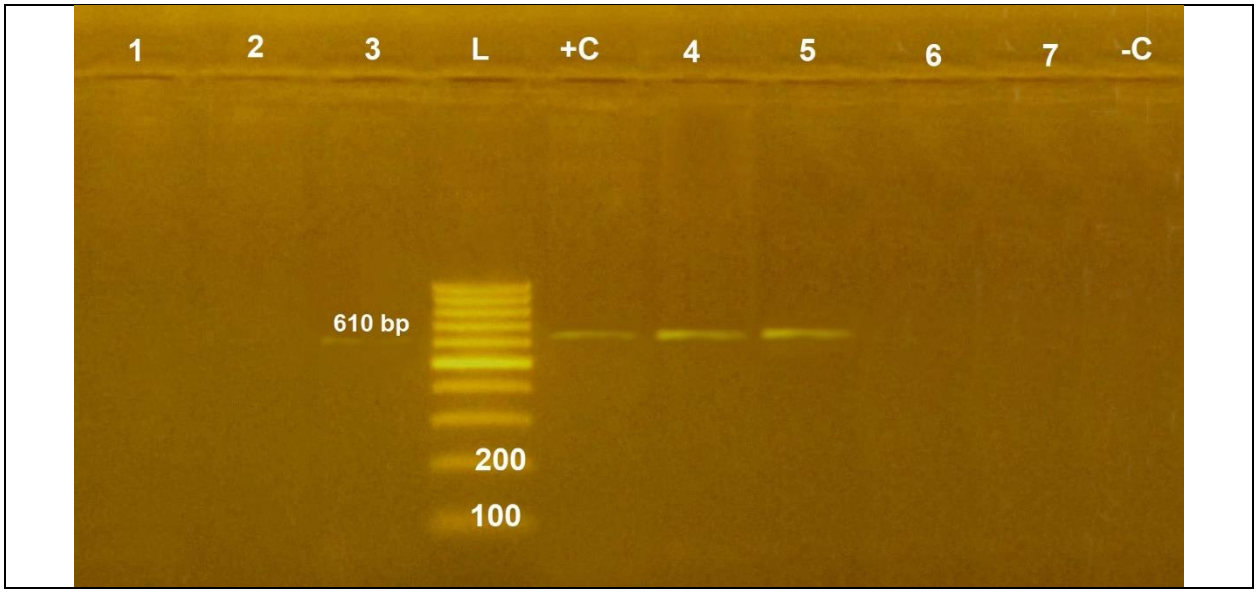

Fig. 3. Agarose gel electrophoresis of PCR- for amplification products of *bla Z* gene; Lane +C: Control positive (reference strain of code no. ATCC 12344*)*, Lane L: 100-bp ladder (marker), Lanes 3-5: Positive samples for *bla Z* gene; Lane -C: control negative*.*

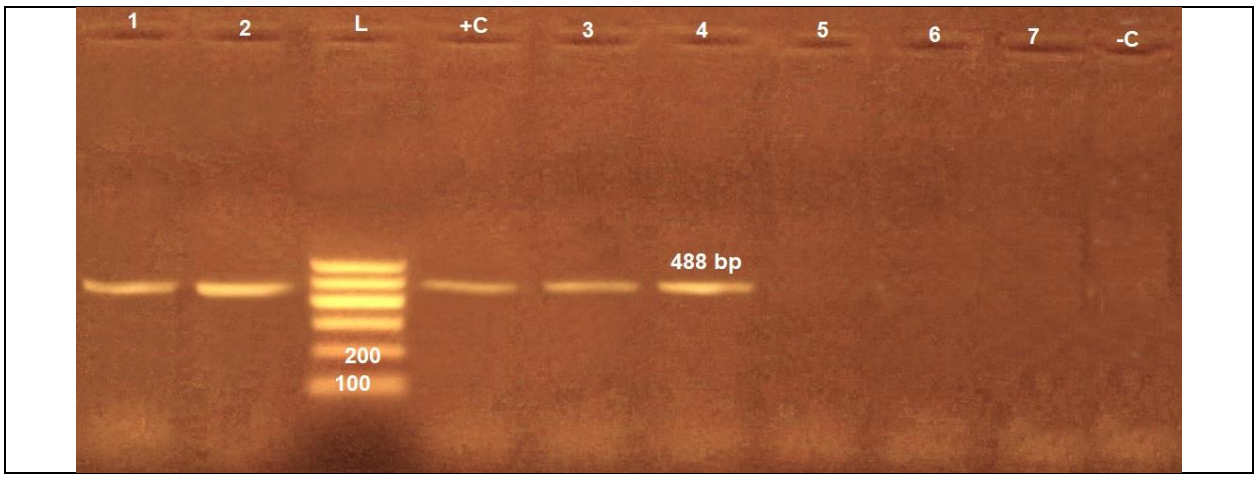

Fig. 4. Agarose gel electrophoresis of PCR- for amplification products of *bla IMP* gene; Lanes +C: Control positive (reference strain of code no. ATCC 12344*)*, Lane L: 100-bp ladder (marker), Lanes 1-4: Positive samples for *bla IMP* gene; Lane -C: control negative*.*

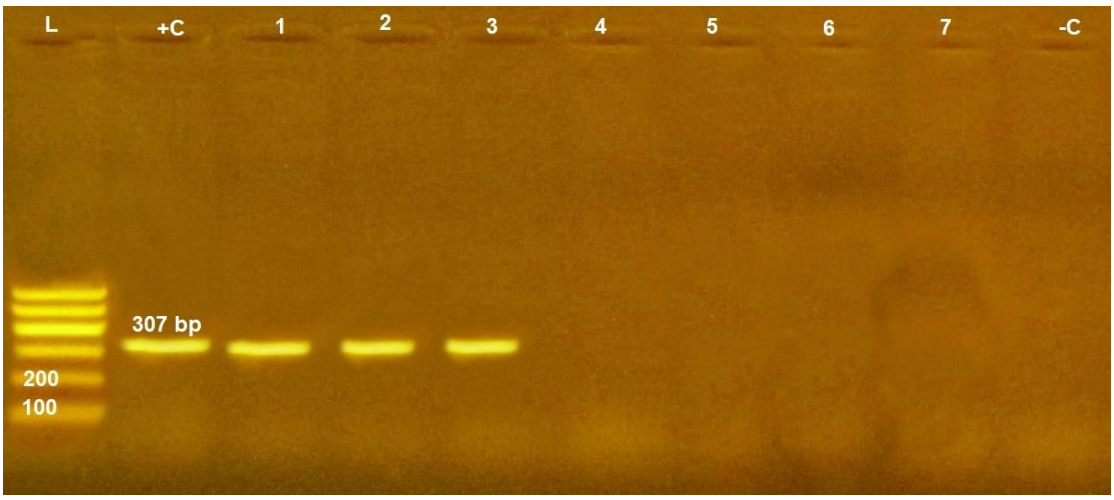

Fig. 5. Agarose gel electrophoresis of PCR- for amplification products of *bla CTX* gene; Lane +C: Control positive (reference strain of code no. ATCC 12344*)*, Lane L: 100-bp ladder (marker), Lanes 1-3: Positive samples for *bla CTX* gene; Lane -C: control negative.

DISCUSSION:

Streptococcus pyogenes is a bacterium that causes a wide range of human infections and is a major cause of morbidity and mortality around the world, from non-invasive diseases like acute pharyngitis to life-threatening invasive infections like sepsis and toxic shock syndrome (Gherardi *et al.*, 2015).

The present study investigated the occurrence and the antimicrobial resistance patterns of *S. pyogenes* collected from pharyngitis. The obtained data revealed an overall prevalence rate of 11.6% (7 of 60 isolates). The colonization of *S. pyogenes* in throat swabs of children was 85.7%. This prevalence is an indication that the organism is active in the area with the potential of causing widespread disease.

The prevalence observed in this study was lower than that obtained in Benin City (14%), 30% in Iran (Sayyahfar *et al.*, 2015), 29.2 % in Iraq (Ali *et al.*, 2015). In contrast, our results were higher than the Jimma, Ethiopia 11.3% (Tesfaw *et al.*, 2015), Japan 5.8% (Igarashi *et al.*, 2017), India 5.5% (Khandekar, 2019), Romania 4% (Bobia *et al.*, 2019) , Brazil 3.9% (Alexandre *et al.*, 2017), Saudi Arabia 1.5% (Ashgar *et al.*, 2015) and Mexico 0.04 – 0.42% (Gutiérrez-Jiménez *et al.*, 2018). These differences may be attributed to different geography, method, socio-economic conditions, and sample size, seasonal variations.

All *S. pyogenes* isolates were susceptible to ampicillin-sulbactam, ciprofloxacin, chloramphenicol, doxycycline, meropenem and tetracycline. and absolute resistance (100%) was obtained among the isolates against amoxicillin-clavulanic acid, cefotaxime and cephradine followed by ceftriaxone, cefuroxime, clindamycin, novobiocin, vancomycin (71 %), penicillin-G (57 %), piperacillin (43 %), ceftazidime, amikacin, erythromycin, sulfamethoxazole-trimethoprim (29%), and gentamycin (14%).

In the present study, the highest antibiotic resistance was determined to be against β-lactam with the rate of (85.7%). Several reports had evaluated the emergence of *S. pyogenes* isolates that are non-susceptible or even resistant to β-lactam antibiotics, the majority of which were published in Chinese journals between 2002 and 2018. Most of these reports were from the large Antimicrobial Surveillance Network in China and were published in Chinese Journals. A study in Mexico (Amábile-Cuevas *et al.*, 2001) reported diminished susceptibility to penicillin in 10 isolates (5%). In India, 7 of 34 strains (20.6%) were discovered to be non-susceptible to penicillin (Capoor *et al.*, 2006), while in Japan 2 of 93 strains were found to be “resistant” to penicillin (Ogawa *et al.*, 2011a). *S. pyogenes* may develop penicillin resistance by evading therapy by infiltrating epithelial cells that are poorly penetrated by penicillin (Kaplan *et al.*, 2006), developing a biofilm (Ogawa *et al.*, 2011b), the production of B-lactamases genes that are known to hydrolyse b-lactams (Murray, 1992), the overproduction of penicillin-binding proteins (PBPs) that bind to antimicrobial agents rendering them inactive (Fontana *et al.*, 1996) and protection of *S. pyogenes* by other β-lactamase-producing bacterial species (Brook and Gober, 2008 ; Brook, 2013). In the present study, a high rate of beta- lactam antimicrobial resistance was observed in 6 out of 7(85.7%) of isolates.

Although it has been stated that streptococci are unable to acquire foreign *bla* genes (Haenni *et al.*, 2018), at least two studies have reported the presence of these genes in *Streptococcus pneumonia* (Ding *et al.*, 2004; Chang *et al.*, 2016). Also, a recent study based on whole-genome sequencing revealed the presence of β-lactamases

determinants of *S. uberis* and SDSD isolates bovine mastitis (Vélez *et al.*, 2017). In our study, the dominant beta-lactamase genes discovered were variants of *bla*$_{TEM}$, *bla*$_{Z}$, *bla*$_{IMP}$, and *bla*$_{CTX}$.

bla$_{TEM}$ has been reported worldwide and *bla*$_{CTX}$ is currently the most widespread and threatening mechanism of antibiotic resistance, particularly in community-acquired infections (Lachmayr *et al.*, 2009). Resistance to benzylpenicillin is mainly caused by the *blaZ* gene encoding production of beta-lactamases, which hydrolytically destroy beta-lactams. The *blaZ* gene can be located chromosomally or on plasmids. This type of penicillin resistance may thus emerge via two mechanisms: spread of resistant clones or through horizontal dissemination of mobile elements containing the *blaZ* gene (Malachowa and DeLeo, 2010). Regarding the different types of detected beta-lactamase genes, *bla*$_{TEM}$ and *bla*$_{IMP}$ were the most common followed by *bla*$_{CTX}$ *and bla*$_{Z}$. These higher rates of *bla*$_{TEM}$ and *bla*$_{IMP}$ among our isolates may be associated with studies performed in Italia; 45.4% (Carattoli *et al.*, 2008) and Portugal; 40.9% (Fernandes *et al.*, 2014).

CONCLUSION:

In the current research, we noted that the highest prevalence of *S. pyogenes* was recorded in boys and males. Moreover, *S. pyogenes* isolates showed resistance to β-lactam antibiotics. Also, our study is the first to highlight the presence of *bla* genes (*bla*$_{TEM}$, *bla*$_{Z}$, *bla*$_{IMP}$, and *bla*$_{CTX}$) in β-lactam resistant *S. pyogenes* isolates. Although β-lactams may still be effective, their future might be hindered by the presence of β-lactam-resistant bacteria. To maintain the required efficacy, limited use of β-lactam is recommended.

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دراسة مدى انتشار ونمط مقاومة الأدوية المتعددة لبيتالاكتام في البكتريا العقدية المقيحة والمعزولة من عدوى البلعوم الأنفي

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تعد البكتريا العقدية المقيحة والمعروفة باسم (GAS) أحد أكبر أسباب الوفاة حول العالم. إن السبب الرئيسي لفشل علاج عدوى البكتريا العقدية المقيحة هو زيادة مقاومتها للمضادات الحيوية. ولذا تم إجراء هذه الدراسة لتقييم مدى انتشار هذه البكتريا ومقاومتها للمضادات الحيوية المختلفة وأيضا التوصيف الجيني للبكتريا العقدية المقيحة والتي تم جمعها من مرضى مختلفين. تم جمع 60 مسحه من الحلق والبلعوم لمرضى التهابات اللوزتين من فئات عمريه مختلفة. من بين هذه العينات حملت 7 عينات فقط (بمعدل 11,6٪) البكتريا في منطقة الحلق. وقد أظهرت معدل انتشار أعلى في الاولاد بمعدل 6,26٪ يليهم البالغين الذكور بمعدل 6,16٪ بينما كانت أقل انتشارا في البنات بمعدل 7,11٪. من ناحية أخرى لم تسجل أي إصابة في البالغين الإناث. أظهرت نتائج اختبار الحساسية للمضادات الحيوية المختلفة أن جميع العزلات كانت حساسة للأمبيسيلين، سولباكتام، سيبروفلوكساسين، الكلورامفينيكول، الدوكسيسيكلين، الميروبينيم والتتراسيكلين. بينما أظهرت 100٪ مقاومة للأموكسيسيلين - كلافولانيك، سيفوتاكسيم، وسيفرادين يليه سيفترياكسون وسيفوروكسيم بمعدل (71٪). أظهرت 6 من أصل 7 عينات مقاومة لـ 3 أو أكثر من المضادات الحيوية لمجموعة البيتا لاكتام اعتمادا على المقاومة المتعددة للدواء. أظهر اختبار PCR أن جينات blaTEM و blaZ و bla IMP و blaCTX تم اكتشافها في 57.1٪، 28.5٪، 57.1٪، 42.8٪، 15٪، 11.3٪ و 5.6٪ من العزلات على الترتيب. وعلى حد علمنا، عالميا هذه هي الدراسة التي ذكرت حول مجموعة البيتا لاكتاماز في البكتريا العقدية المقيحة.