In vitro activity of antimicrobial agents against *streptococcus pyogenes* isolated from different regions of Khyber Pakhtun Khwa Pakistan

Muhammad Rizwan[1], Jehan Bakht[2], Nafees Bacha[1]* and Bashir Ahmad[1]
[1]Centre of Biotechnology and Microbiology, University of Peshawar, KPK, Pakistan
[2]Institute of Biotechnology and Genetic Engineering, University of Agriculture, Peshawar, KPK, Pakistan

Abstract: The present study investigates the antibiotic resistance of S. *pyogenes* of 600 isolates collected from different body parts including throat and sputum were analyzed for their antimicrobial susceptibility to 5 antibiotics using the Kirby Bauer disc diffusion method. Based on different identification tests including, gram staining, beta hemolysis, catalase test and bacitracin sensitivity test, a total of 138 isolates were confirmed as S. *pyogenes*. The prevalence of *S. pyogenes* was 80% in sore throat and 29% in sputum samples. These isolates were further tested for antibiotics resistance using disk diffusion method. Out of 138 isolates, 49.27% isolates showed resistance towards cefixime, 28.98% towards cefotaxime and 17.39% towards ciprofloxacin, 17.39% towards ampicillin, 17.39% towards erythromycin, 15.94% towards streptomycin, 0.724% isolates towards chloromphenicol and 0% towards penicillin. Among the resistant isolates of *S. pyogenes*, 60.71% showed resistance towards cefixime, 57.14% towards ciprofloxacin, 57.14% towards streptomycin, 50% towards erythromycin and 25% towards cefotaxime.

Keywords: *S. pyogenes*. Hemolysis, GAS, rheumatic fever, pharyngitis, Pyrrolidonyl arylmidase (PYR)

INTRODUCTION

Streptococcus pyogenes is an important species of gram-positive, spherical and non-spore forming extra cellular bacterial pathogen. *S. pyogenes* is a facultative anaerobic bacterium, which is present in chain form. This bacterium is a conditional and opportunistic pathogen, which is always in search for suitable environment to cause a disease (Todar, 2002). *S. pyogenes* shows its unique nature which does not cause just one disease, but have capability to cause a number of different diseases. This bacterium built colonies in the skin or throat and causes a wide range of complexes. The diseases range from simple pharyngitis (strep throat) and skin infections (cellulitis, impetigo, and erysipelas) to sever like necrotizing fasciitis, pneumonia, Streptococcal Toxic Shock Syndrome (STSS), bacteremia, acute rheumatic fever, reactive arthritis and glomerulonephritis (Cunningham, 2000; Efstratiou, 2000; Pfoh *et al*., 2008). Non suppurative autoimmune sequelae, such as glomerulonephritis and Rheumatic Fever (RF) can also develop infections with *S. pyogenes* and are of major concern in developing countries (Stollerman, 1997; Cunningham, 2000).

The respiratory tract infections are common in children as well as in adults in Pakistan. The estimated incidence of respiratory infections in children and adults are 1192 visits to the physicians per 2000 population per annum. According to the National Health Survey, upper respiratory tract infection including pharyngitis and sore throat are responsible for 800 visits to a physician per 2000 population annually in Pakistan. In Pakistani big cities air pollution due to industrial areas and a great number of vehicles has increase the risk of viral and/or bacterial sore throat and pharyngitis as well (Somro *et al*., 2010). It has been reported that rheumatic heart disease is also in high ratio in both urban (22 per 1,000 persons) and rural (5.7 per 1,000 persons) areas of Pakistan (Rizvi *et al*., 2004; Rehman *et al*., 2007).

A global trend of increasing antimicrobial resistance is well-documented in the literature (Livermore, 2003). Strong evidence supports an association between antibiotic use and resistance in hospitals (McGowan, 1983: Harbarth *et al*., 2001). By contrast, the relationship between antibiotic consumption and resistance has been more difficult to establish for the outpatient setting, although some data suggest a direct correlation for streptococcal infections (Seppälä *et al*., 1997; Harbarth *et al*., 2001; Pihlajamaki *et al*., 2001). Antimicrobial resistance among GAS is an emerging concern. Penicillin is still the antibiotic of choice, for all streptococcal infections, and surprisingly up to now no resistant strains have been reported (Horn *et al*., 1998; Macris *et al*., 1998). However, after penicillin-treatment failure to eradicate infection has been reported in up to 25% of GAS pharyngitis (Pichichero, 1991; Kaplan and Johnson, 1988), suggesting that additional mechanisms to circumvent its effects by this pathogen have developed. Erythromycin is the second option for penicillin hyper sensitive patient (Holmstrom *et al*., 1990; Gerber, 1995). However, resistant to erythromycin increases with passage of time. It has been reported that resistance of *S. pyogenes* to erythromycin is 11.1% in Pakistan compared with previous study where 4% isolates were resistance to erythromycin (Rehman *et al*., 1996; Malik *et al*., 2005).

**Corresponding author:* e-mail: nafees.bacha@gmail.com

MATERIALS AND METHODS

The research work was conducted in the Microbiology Research Laboratory, Centre of Biotechnology and Microbiology, University of Peshawar.

Sample collection

A total of 600 samples were collected from three major districts (Peshawar, Charsadda and Mardan) of Khyber Pakhtunkhwa province Pakistan. Samples were collected from different sources including sore throat, sputum and pus. Out of 600 isolates 264 samples were positive for *S. pyogenes*.

Culture media used

Five different types (Blood Agar Media; Tryptic Soy Agar Media; Tryptic Soy Broth; Muller Hinton Agar Media and Muller Hinton Broth) were used during the present study.

Identification

The collected isolates were identified using different morphological and biochemical tests. The collected samples were screened on blood agar media with 5% sheep blood to observe the growth and beta hemolytic pattern of *S. pyogenes*. The bacterial isolates were subjected to microscopic studies and four type of identification tests i.e. hemolysis, gram staining, catalase test and bacitracin sensitivity test. Pyrrolidonyl arylmidase (PYR) test were positive for all 264 isolates.

Disk susceptibility test

Disk diffusion method was used for determination of Antibiotic resistance of *S. pyogenes* specified by *et al.* (1966). For this purpose first 0.5 McFarland's turbidity standard was made by mixing barium chloride and HCl and the O.D was adjusted using spectrophotometer. The fresh bacterial culture incubated at 37^{0}C over night was compared with 0.5 McFarland's solution used for turbidity standard. McFarland's solution provides turbidity as comparable to bacterial suspension containing 1.5 x 10^{8} C.F.U/ml. Normal saline will be used for culture dilution. Muller Hinton agar media with 5% sheep blood were used for disk diffusion technique. Results were interpreted according to CLSI guideline (Clinical and Laboratory Standards Institute, 2005).

Determination of MICs

For determination of minimum inhibitory concentration Agar dilution method was used (NCCLS, 2009). The antibiotics used for the antibiotics resistance activity included Chloramphenicol (30 mcg), Streptomycin (10mcg), Ciprofloxacin (5mcg), Cefixime (5mcg), Cefotaxime (30mcg), Ampicillin (25 mcg), Penicillin (10mcg), and Erythromycin (15 mcg). The isolates that showed resistance against different antibiotics were then subjected to the determination of minimum inhibitory concentration (MICs) by agar dilution method on Muller Hinton agar with 5% sheep blood in the environment of 5% CO_2. *Staphylcoccus aureus* ATCC 25923 were used as control. CLSI break points were used for all antimicrobial agents except for ciprofloxacin, for which the German Institute of Antimicrobial Susceptibility Testing (DIN) was used.

RESULTS

Prevalence

The results revealed that the overall prevalence of *S. pyogenes* in the targeted three districts including Mardan, Charsadda and Peshawar was found to be 46% (table 1). The result showed that out of 300 samples, 138 samples (46%) were found positive for *S. pyogenes* as indicated by complete hemolysis of blood on blood agar plates. The samples detail of our study showed that 58 samples (29%) of sputum were positive for *S. pyogenes*. Eighty samples (80%) of throat swabs were positive for *S. pyogenes*. Furthermore, 30 samples (52%) of sputum were isolated from females while 28 samples (48%) were from males. Similarly, the prevalence rate in males and females were equal in case of throat swabs (table 1).

Identification of the isolates

The complete hemolysis showed the presence of *S. pyogenes* (fig. 1). It was observed that out of 300 samples, 138 showed complete hemolysis. Gram staining test indicated the gram-positive cocci chains when the slides were observed under the microscope. The hemolytic pattern and the microscopic observation confirmed that these isolates could be of *S. pyogenes*. Samples were further subjected to catalase activity test. Those isolates with catalase negative activity were confirmed as *S. pyogenes*. Finally, the bacitracin sensitivity test was performed against the supposed *S. pyogenes* culture. The isolates showed ≤14 mm zone of inhibition against the bacitracin disc indicating the confirmation of *S. pyogenes* (fig. 2).

Antibiotic resistance and minimum inhibition concentration (MIC)

The current study showed that among total of 138 isolates, 132 isolates (95.675%) were susceptible to chloramphenicol with susceptibility range of ≥21mm (ZI) (fig. 3) and one isolate was found resistant with the resistant range of ≤17mm (ZI) (fig. 5). These result further revealed that only 5 isolates (3.6%) showed intermediate resistance (fig. 4). Gur *et al.* (2002) reported that resistance to chloramphenicol by *S. pyogenes* was 98%.

The results showed that 19 isolates (13.76%) out of 138 isolates were found to be intermediately resistant to streptomycin measuring 12-14mm zone of inhibition**.** The susceptibility rate of bacterial isolates against the streptomycin was found to be 62.31% (fig. 3). The susceptibility range for streptomycin was ≥15mm. Among the 138 isolates of the *S. pyogenes,* 33 isolates (23.91%) were resistant to the streptomycin antibiotic with ≤11mm zone of inhibition (fig. 4). Similarly, MICs of streptomycin indicated that out of 28 isolates, 9 isolates (32.14%) were susceptible (fig. 6), 3 isolates (10.71%) showed intermediate resistance and 16 isolates (57.14%) were resistant against streptomycin antibiotics.

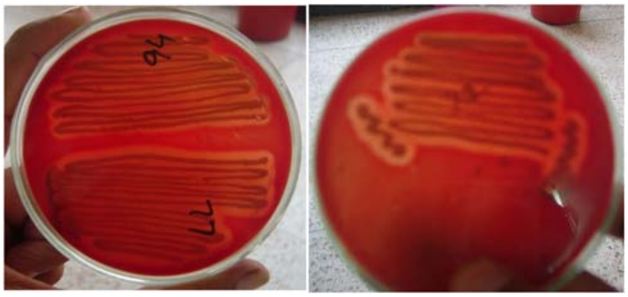

Fig. 1: Figiure showing the positive beta hemolytic pattern of the isolated bacterial species.

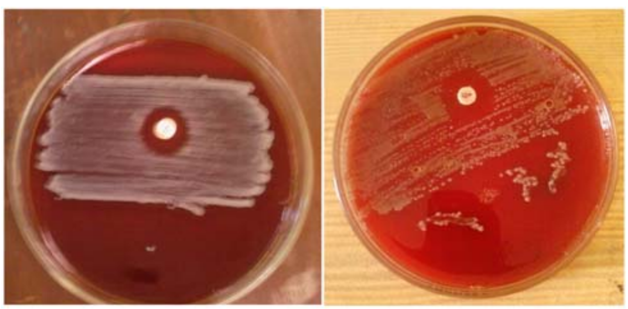

Fig. 2: Figure showing the bacitracin sensitivity test for *S. pyogenes.*

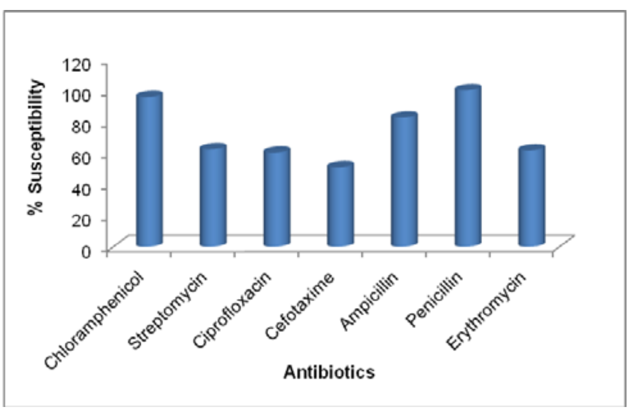

Fig. 3: Percent susceptibility of *S. pyogenes* against all antibiotics used

The results indicated that out of 138 isolates, 31 isolates (22.46%) were intermediately resistant to ciprofloxacin antibiotic measuring 16-20 mm zone of inhibition (fig. 4). Among 138 isolates, 83 isolates (60.14%) were susceptible to ciprofloxacin (fig. 3). The remaining 24 isolates (17.39%) were resistant to the antibiotic revealing ≤15mm zone of inhibition (fig. 4). The data further suggested that out of 28 selected isolates, 12 isolates (42.85%) were susceptible (fig. 7) with MICs of≤ 1µg/ml and 16 isolates (57.14%) were resistant to ciprofloxacin antibiotic with MICs of ≥4µg/ml. Our results also showed that out of 138 isolates, 70 isolates (50.72%) were susceptible to cefixime with susceptibility range of ≥24mm (fig. 3). Similarly, 68 isolates (49.27%) were non-susceptible measuring <24 mm zone of inhibition (fig. 5). Results regarding MIC revealed that out of 28 isolates, 11 isolates (39.28%) were susceptible with MICs rang of ≤0.5µg/mL and 17 isolates (60.71%) were non-susceptible with the MICs range >0.5µg/mL.

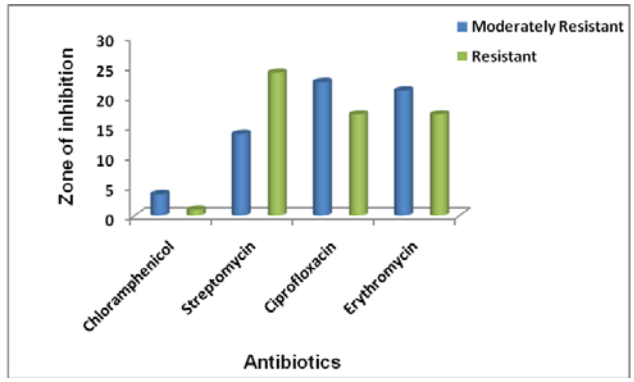

Fig. 4: Percent moderate and total resistance of *S. pyogenes* against four antibiotics used

The data also suggested that out of 138 isolates, 40 isolates (28.98%) were non susceptible with <24mm zone of inhibition (fig. 5). The remaining 98 isolates (71.01%) were susceptible to cefotaxime with susceptibility range of ≥24mm (fig. 3). MICs results showed that 11 isolates (9.28%) were susceptible (susceptibility range= ≤0.5µg/mL) and 7 isolates (25%) were resistant (resistance range=≥ 2µg/mL).

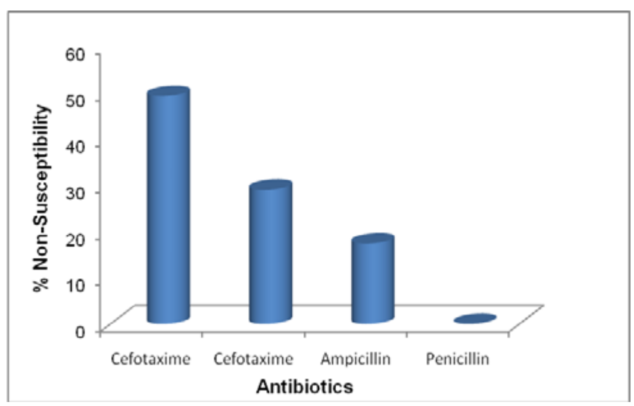

Fig. 5: Percent non-susceptible *S. pyogenes* against four antibiotics used

The susceptibility range noted for ampicillin was ≥24 mm and non-susceptibility range >24mm. Out of 138 isolates, 24 isolates (17.39%) were non-susceptible (fig. 6) and 114 isolates (82.60%) were susceptible to ampicillin (fig. 3). The results further suggested that all of the 138

Table 1: Percentage occurrences of *S. pyogenes* from different sources

Sources	Sample collected	Positive sample	Percentage	Males	Females
Sputum	200	58	29	28	40
Throat	100	80	80	40	40

isolates were susceptible to penicillin (100%). However, among 138 isolates, few isolates were nearer to intermediate resistance value i.e. 24mm zone of inhibition.

Among 138 isolates, 29 isolates (21.01%) showed intermediate resistance towards erythromycin (fig. 4). Out of the remaining 109 isolates, 24 isolates (17.39%) showed resistance towards erythromycin (fig. 5). The remaining 85 isolates (61.59%) were susceptible to erythromycin antibiotic (fig. 3). The result obtained from MICs, showed that out of 28 isolates, 8 isolates (28.57%) displayed intermediate resistance (MIC =0.5μg/mL), 14 isolates (50%) were resistant (MIC≥1μg/mL) and 6 isolates (21.42%) were susceptible (MIC ≤0.25μg/mL) (fig. 6).

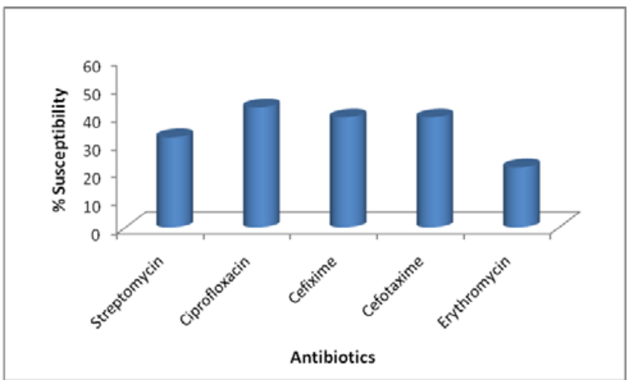

Fig. 6: Percent MICs values of susceptible strains against different antibiotics.

DISCUSSION

The data indicated that more than half of the samples were found positive for *S. pyogenes*. Similar results were also reported by Yan *et al*. (2000) and Gazi *et al*. (2004). Our results revealed that 29% and 80% of sputum and throat swabs were positive for *S. pyogenes* respectively. Similarly, 52% of sputum was isolated from females while 48% were from males. The data also indicated that the prevalence level in males and females were equal in case of throat swabs. It was observed that out of 300 samples, 138 showed complete hemolysis. The hemolytic pattern and the microscopic observation confirmed that these isolates could be of *S. pyogenes*. The data suggested that isolates with catalase negative activity were confirmed as *S. pyogenes*. The isolates showed ≤14 mm zone of inhibition against the bacitracin disc revealing the confirmation of *S. pyogenes*. After identification and confirmation of bacterial isolates as *S. pyogenes*, the antibiotics resistance and minimum inhibitory concentration of the said bacterium was determined. The present study indicated that 95.675% of the isolates were susceptible to chloramphenicol except one isolate which was found resistant The data further showed that only 3.6% measured intermediate resistance. Gur *et al.* (2002) reported that resistance to chloramphenicol by *S. pyogenes* was 98%. The reason of this high susceptibility rate may be due to the less frequent use of this antibiotic against *S. pyogenes.* It can be seen from the results that 13.76% of the isolates were found to be intermediately resistant to streptomycin with the susceptibility rate 62.31%. Among the 138 isolates of the *S. pyogenes,* 23.91% were resistant to the streptomycin antibiotic. Similarly, MICs of streptomycin indicated that 32.14% of the isolates were susceptible, 10.71% showed intermediate resistance 57.14% were resistant against streptomycin antibiotics. This antibiotic is usually used against the gram negative bacterium, however, in our study it was observed it can also inhibit the growth of gram positive bacteria like *S. pyogenes*. The reason of this susceptibility may be due to the less frequent use of this antibiotic against the gram positive bacteria.

It has been reported that 42.85% isolates of *S. pyogenes* were found susceptible and 57.14% resistant to ciprofloxacin in Japan (Sugita *et al*., 1985; Mechthild *et al*., 2001; Sabestian *et al*., 2005). The reason of this resistance may be due to the indiscriminate use of this antibiotic for both gram positive and gram-negative bacteria resulting in increased resistance with the passage of time. Our results also indicated that 50.72% of the isolates were susceptible to cefixime and 49.27% were found non-susceptible. Results regarding MIC showed that 39.28% of the isolates were susceptible and 60.71% were scored as non- susceptible. Similar results are also reported by Brittain *et al.* (1985) and Brook and Gober (1999). The increased variations in these results are due to the massive use of cefixime in Khyber Pakhtunkhwa province of Pakistan that leads *S. pyogenes* to develop resistance against cefixime.

The results also indicated that 22.46% of the total isolates were intermediately resistant to ciprofloxacin and 60.14% were susceptible to ciprofloxacin 17.39% were found resistant to the same antibiotic. The data further suggested that 28.98% of the isolates were non susceptible and the remaining 71.01% were susceptible to cefotaxime MICs results showed that 9.28% of the isolates were susceptible and 25% were found resistant. It has been reported that cefotaxime was 100% active against *S. pyogenes*

(Wendell *et al*., 1980; Emilia *et al*., 2000; Gustafsson *et al*., 2001; Jones *et al*., 2003; Ralf *et al*., 2004). Again, these variations in results may be due to the massive use of cefixime in Khyber Pakhtunkhwa of Pakistan resulting in the development of resistance against cefotaxime by *S. pyogenes*. A variety of factors could be responsible for this, however, the selective pressure exerted by inappropriately used antibiotics is likely to be the most important (Lipsitch and Samore, 2002). The increasing emergence of cefotaxime-resistant *S. pyogenes* is a major concern, for patients allergic to cefotaxime, therapeutic alternatives are required. Out of 138 isolates, 17.39% were non-susceptible 82.60% were susceptible to ampicillin. It has been reported that all selected isolates of *S. pyogenes* were susceptible to ampicillin (Kallings *et al*., 1983; Barbro *et al*., 1992). The high susceptibility rate of the ampicillin antibiotic in the present study may be due its less frequent use in Khyber Pakhtunkhwa province of Pakistan. The data further suggested that all of the 138 isolates were susceptible to penicillin. However, few isolates were nearer to intermediate resistance value. Up till now, no resistant strains have been reported against penicillin (Brown and Rybak, 2004; Doren and Brown, 2004). These results shows that it is possible that the bacterium, *S. pyogenes* may develop resistance against penicillin antibiotic with the passage of time.

It can be seen from the results that among 138 isolates, 29 isolates showed intermediate resistance towards erythromycin and 24 isolates measured resistance towards erythromycin. The remaining 85 isolates were susceptible to erythromycin antibiotic. The result obtained from MICs, showed that 28.57% of the total isolates displayed intermediate resistance, 50% were resistant and 21.42%) were susceptible. Erythromycin resistance was reported in different ratios in different geographical regions. Highest rate of resistance was noted in Italy (Giuseppe *et al*., 1998). In Pakistan the resistance shown by *S. pyogenes* against erythromycin was 4% in 2000 and 11.1% in 2002 respectively. This antibiotic is the second option for the physicians next to penicillin. However, due to the frequent allergic reaction of the maximum human population towards the penicillin antibiotic, erythromycin is the drug of choice for the doctors. This frequent use of the erythromycin antibiotic make it resistant and with the passage of time, the resistance towards this antibiotic may increase to such an extent that we may no more rely on this antibiotic use for the treatment of GAS infections.

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