Isolation and antibiotic sensitivity pattern of drug resistant bacteria in ulcerative foot of type 2 diabetic patients

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Abstract: The present study aimed to decipher the bacterial infections in diabetic foot human patients in Pakistan and the anti-microbial susceptibility for clinical relevance. A total of 30 samples were collected from hospitalized type 2 diabetic patients (men and women) having foot ulcers. The collected samples were cultured on mannitol salt agar, Blood agar and MacConkey's agar and cetrimide agar. Gram staining and specific biochemical tests were performed to identify the invading bacteria. Antibiotic sensitivity and resistance pattern was performed for isolated bacteria by Kirby-Bauer disc diffusion method. In diabetic foot ulcers, most prevalent bacteria were *S. aureus* with percent positivity of 83% followed by *E. coli* (66%), *K. pneumoniae* (40%) and *P. aeruginosa* (16%). The infected ulcer with poly-microorganisms was 83.4% and the infected ulcer with single isolates was 16.6%. Imipenem was found to be most sensitive antibiotic against Gram positive as well as Gram negative bacterial isolates from diabetic foot ulcer human patients. Gram negative isolates from diabetic foot showed resistance to ampicillin, sulfamethoxazole/trimethoprim, cefotaxime/clavulanate, metronidazole. The diabetic foot ulcers of human patients revealed high prevalence of *S. aureus* followed by *E. coli, K. pneumoniae* and *P. aeruginosa* respectively. Imipenem was found to be the most sensitive antibiotic for all the bacterial isolates from foot ulcers of type 2 diabetic patients. This study suggests imipenem as effective antibiotic for treatment of diabetic foot ulcers against bacteria.

Keywords: Diabetic foot, antibiogram, drug resistance, imipenem.

INTRODUCTION

Diabetes mellitus is a chronic disease developing world wide and it is continuously expanding due to more obesity and less physical activities among population (Shaw *et al*., 2010). Beside many advances in diagnosis and treatment, incidence of diabetes is increasing every day (Tarkun and Özgoksu, 2017). The most feared complexity is diabetic foot that occurs due to interlinked risk factors in diabetic patients (Al-Shabaki, 2014). The prevalence of foot ulcer ranges from 4-10% in diabetic patients worldwide. Each year the incidence of population for diabetic foot is 1.0-4.1% and the lifetime prevalence of foot ulcer may be up to 25%. During the course of treatment, almost half of wounds on foot become infected (White and Mclntosh, 2008). The bacterial diabetic foot infections and prevalence of drug resistant bacteria in diabetic foot have been increased during the recent years in Asia (Karmaker *et al*., 2016).

The secondary bacterial infections results from foot ulceration that appear after trauma to neuropathic foot manifesting the diabetic foot complications (Rao and Lipsky, 2007). The bacterial infections are enhanced due to immunological deficiencies particularly neutrophils deficiency: provides first line of defence during bacterial infections (Richard *et al.*, 2012). The secondary infectious origin complications include sepsis, osteomyelitis, amputation and death in severe cases (Aamir *et al*., 2011). There is 25% chances of establishing foot ulcer in diabetic patients (Jain *et al*., 2017) and the hazards of lower limb amputation extends by 15-46 times greater than non-diabetic patients (Vijayakumar *et al*., 2012).

Diabetic foot infections may be categorized into mild, moderate, or severe infection. In untreated mild to moderate foot infection, Gram-positive bacteria *i.e*., *Staphylococcus aureus* (*S. aureus*) was majorly isolated pathogen and previously treated severe or chronic infections were due to poly microorganisms (Bader, 2008). Mostly, the mixed micro-flora is present in infectious foot ulcers *i.e*., *S. aureus, E. coli, K. pneumoniae* and *P. aeruginosa* (Hefni *et al*., 2013; Champoux *et al*., 2004). *Enterobacteriaceae* are commonly found in chronic diabetic foot infections with previous treatment history (Vijayakumar and Gundela, 2012). In Pakistan, previous data reported polymicrobial infection of diabetic foot in human with 65% antibiotic resistance (Alavi *et al*., 2007). For antibiotic selection in diabetic foot treatment, factors of previous antibiotics used, hospital stay and antibiotic susceptibility should be kept in mind (Lipsky, 1999). Recently, the biofilm

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forming aerobic bacterial pathogens from patients with chronic wound infections were reported, among them the diabetic foot ulcers were the most common (Rahim *et al*., 2016; Xie *et al*., 2017). A little is known about the bacterial infections and their antimicrobial susceptibility in diabetic foot ulcer patients in Pakistan, therefore, the present study was designed to know the occurrence of bacterial infections and antimicrobial profile in diabetic foot of human patients

MATERIALS AND METHODS

Ethical approval

The study was conducted in compliance (Approval No. CE 1657, dated 20-08-2015) with local Institutional Bioethics Committee (IBC), University of Agriculture, Faisalabad, Pakistan and Faisalabad Medical University, Faisalabad, Pakistan.

Sample size and area of sample collection

The present study was conducted on a total 30 samples from both male and female individuals suffering with type 2 diabetes (t2d) regardless of their age. All patients were subjected to full assessment of personal and clinical history, clinical, general, and local examination of the affected foot lesion. The patients were admitted to hospital for management of infected diabetic foot ulcer (DFU). Before collection of samples, an informed consent of patients was also taken and they were told about the purpose of sample collection and research study.

Sample collection

Aseptic sampling protocol was adopted for collection of samples. Briefly, ulcerative foot wounds were washed vigorously with normal saline to avoid chances of colonizing (rather than pathogenic) bacterial flora (Banashankari *et al*., 2012). Sterile swabs were used for sample collection from foot ulcers of t2d patients. The DFU was classified according to Wagner's classification (Hefni *et al*., 2013; Hayat *et al*., 2011) as shown in table 1, table 2. table 3 defines the detailed description of diabetic patients with ulcerative foot wounds.

Samples processing and identification of bacterial isolates

A microbial evaluation of infected foot wounds in diabetic patients was conducted to determine the possible pathogens present in wound. Study was done on the samples collected from patients suffering with DFU and their infections. Aerobic samples were cultured on to MacConkey agar, Mannitol salt agar and Blood agar and chocolate agar (Jaber and Saeed, 2013) (Ahmed *et al*., 2013). After overnight incubation at 37°C, the morphology of the pathogens was recorded. The culture plates with no growth at 48 hours were incubated for at least 5 days before discarding and sterile culture plates were considered as non-infected foot wound as described previously (Ahmed *et al*., 2013). Isolation and identification of the species of pathogen was done by standard microbiological methods. All the isolates were first identified by morphological features (culture characteristics and colony appearance) followed by microscopic examination. Further confirmation of the isolates was done by various biochemical and enzymatic tests such as indole, catalase, coagulase, mannitol fermentation, glucose fermentation and lactose fermentation tests (Jasmine *et al*., 2013).

Antibiotic susceptibility testing of isolates recovered from diabetic foot ulcers

All the isolates were subjected to antibiotic sensitivity testing by the Kirby–Bauer disc diffusion method using various commercially available antibiotic discs such as imipenem (10µm), gentamycin (10µm), meropenem (10µm), cefotaxime (30µm), ciprofloxacin (5µm), trimethoprim/sulphamethoxazole (25µm), ampicillin (10µm), amoxicillin (10µm), clavulanate/cefotaxime (40µm), oxacillin (1µm) and metronidazole. The standard overnight fresh bacterial suspension (0.5 McFarland) of each isolate was spread on Mueller Hinton Agar. Antibiotic discs were dispensed and plates were incubated at 37^{o}C for 24 hours. Zones of bacterial growth inhibtion were measured and results were interpreted according to Clinical and Laboratory Standard recommendations (Ahmed *et al*., 2013).

STATISTICAL ANALYSIS

The results were presented as percent positivity of bacterial isolates prevalent in diabetic foot human patients.

RESULTS

We first classified and calculated the percentage of diabetic foot condition according to well known Wagner's classification which indicated highest percentage (36%) of patients in grade 2, while it was up to 46% according to severity of disease in the same grade (table 4). The lowest percentage (6%) of diabetic foot patients were observed in grade 0 or grade 5 (table 4). Out of total collected samples, the bacterial positive cultures were 96%. The negative cultures were considered as sterile cultures. On the basis of Gram staining and shape, the isolated bacteria were grouped into two types, *i.e.* Gram positive cocci (*S. aureus*) and Gram negative bacilli (*E. coli, K. peumoniae, P. aeruginosa*) and their average percentage was 40% and 60% (fig. 1). The infectious ulcer with poly-microorganisms was 83.4% and the infectious ulcer with single isolate was 16.6%. fig. 2 (a, b, c & d) represents the culture characteristics of all bacteria (*S. aureus*, *E. coli, K. pneumoniae,* and *P. aeruginosa*) isolated from ulcerative foot of t2d patients.

The antibiotic susceptibility test was performed against isolated bacteria; it was found that all the bacterial

Table 2: Classification of diabetic foot according to Wagner's classification

Grades	Ulcer characteristics
Grade 0	No ulcer with intact skin
Grade 1	Superficial ulcer in which only skin is involved without tissue damage
Grade 2	Deep ulcer, penetrating to tissues (tendons/ligaments/muscles), without bone damage
Grade 3	Deep ulcer with cellulitis or abscess formation, often with osteomyelitis
Grade 4	Localized gangrene
Grade	Extensive gangrene involving the whole foot

Table 2: Classification according to severity of infection in diabetic foot ulcer

Grade	Severity of infection
Grade 1	No infection
Grade 2	Mild infection; No evidence of systemic infection and systemic illness including pus or inflammation. Skin or subcutaneous tissues involved. Cellulitis <2 cm around the wound
Grade 3	Moderate infection; Deep tissue infection involving subcutaneous tissue, bone, tendon. Abscesses involved. Cellulitis >2 cm
Grade 4	Severe and systemic infection

isolates were sensitive to imipenem indicating 100% sensitivity (fig. 3a). The antibiotic sensitivity of *E. coli* isolates was found to be 100% for imipenem/meropenem and cefotaxime (50%) (fig. 3a). However, the *E-coli* isolates from diabetic foot were resistant to gentamycin (100%), ciprofloxacin (100%), ampicillin (100%), sulfamethoxazole/trimethoprim (100%), clavulanate/ cefotaxime (100%), and metronidazole (100%) (fig. 3b). The *K. peumoniae* bacterial isolates were 100% sensitive to imipenem/meropenem and for gentamycin (75%), ciprofloxacin (50%) and cefotaxime (25%), respectively (fig. 3a). The *K. peumoniae* isolates from diabetic foot were resistant to ampicillin (100%), cefotaxime (75%), sulfamethoxazole/trimethoprim (100%), clavulanate/ cefotaxime (100%), metronidazole (100%) (fig. 3b). The *S. aureus* isolates sensitivity was recorded as imipenem (100%), gentamycin (35%), ciprofloxacin (100%), ampicillin (50%), cefotaxime (75%), SXT (75%), and oxacillin (75%) (fig. 3a). The *S. aureus* bacterial isolates were resistant to gentamycin (65%), ampicillin (50%), sulfamethoxazole/trimethoprim (25%), clavulanate/ cefotaxime (100%), metronidazole (100%) and oxacillin (25%) (fig. 3b). The *P. aeruginosa* isolated from diabetic foot ulcers was sensitive to imipenem (100%), gentamycin (40%), ciprofloxacin (60%) and cefotaxime (60%) but it was resistant to gentamycin (60%), ampicillin (100%), sulfamethoxazole/trimethoprim (100%), clavulanate/cefotaxime (100%), metronidazole (100%) and cefotaxime (40%) as shown in (fig. 3 a & b).

DISCUSSION

The present study was investigated for the occurrence of drug resistant bacteria in diabetic foot ulcers of human patients and the samples were collected by swabbing. The parameters of family history, examination details, age, sex, foot trauma, type of diabetes, duration of diabetes, status of glycemic control, type of treatment received, exact location of ulcer (plantar/non plantar), duration of ulcer, duration of hospital stay and co-morbidities in human patients were recorded as previously reported by Oliveira and Fihlo, 2014. It was found that 96% cultures were positive for *Escherischia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* having 66%, 40%, 16% and 83%, respectively. Similar bacterial isolates from diabetic foot ulcers have also been reported by Jain *et al*., (2017).

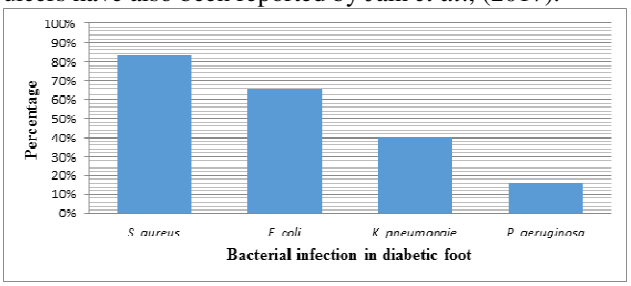

Fig. 1: Percentage prevalence of secondary bacterial isolates from diabetic foot of human patients.

In addition to the above mentioned bacterial isolates, some other studies have reported the presence of *Alcaligenes and Stenotrophomonas* spp. in diabetic foot ulcers (Shahi *et al.,* 2016). *S. aureus* was the major isolate in our study. In a study conducted, Bozkurt compared the superficial swab cultures with deep tissue biopsy cultures from diabetic foot human patients and reported *S. aureus* (81%) in swab cultures and deep tissue culture (Bozkurt *et al*., 2011). High prevalence of *S. aureus* (47%) in samples collected from diabetic foot ulcers have also been reported by Jain *et al*. (2017). Our results were somewhat contrasting to findings of Shahi *et al.,* 2016), who reported a high prevalence of *Enterococcus* spp. (47.61%) in infected DFU followed by *E. coli* (35.71%) and *S. aureus* (33.33%). Considering the occurrence of bacterial infection in DFU, the most common isolated pathogens were Gram negative *i.e.* 60% followed by Gram positive

(40%). In another study it was found that Gram negative are more commonly present in diabetic foot patients *i.e.* 51% than Gram positive were 36.9% (Wu *et al*., 2018). Xie *et al.,* (2017) have also reported a higher proportion of Gram negative bacteria (54.1%) in DFUs. Our results demonstrated similar trend in percentage of bacterial isolates from diabetic foot patients in human. Based on questionnaire, we observed that the male patients were 2.3 times more prone than female patients to develop the diabetic foot ulcer. Also the patients with age more than 50 years had higher chances of getting foot ulcers. Diabetes longer than 5 years or random blood sugar level above 200mg/dL was also among the major factors for developing foot ulcers.

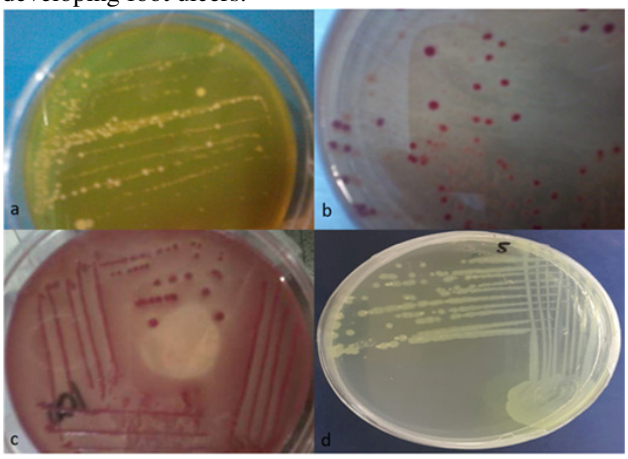

Fig. 2: a) Small yellow coloured colonies of *S. aureus* on mannitol salt agar isolated from diabetic foot patient b) Pink lactose fermenting colonies of *E. coli* on MacConkey agar medium isolated from diabetic foot c) Pure culture of *K. pneumoniae* exhibiting lactose fermenting colonies on MacConkey agar medium d) Growth of *P. aeruginosa* isolate on Cetrimide agar medium

The aerobic bacterial pathogens invaded the diabetic foot isolated in this study were *S. aureus* (83%), *E. coli* (66%), *K. pneumoniae* (40%) and *P. aeruginosa* (16%). The aerobic bacterial isolates such as *S. aureus, Streptococcus agalactiae* and *P. aeruginosa* were commonly found in diabetic foot wounds and antibiotic resistance was reported (Hayat *et al*., 2011; Perim *et al*., 2015). Esmat and Islam in 2012 conducted a study to determine the common aerobic bacterial causes of diabetic foot infections and there *in vitro* antibiotic susceptibility pattern (Esmat and Islam, 2012). This study found *E. coli* (20.3%), *K. pneumoniae* (17.4%), *S. aureus* (16.2%) and *P. aeruginosa* (12.6%) as major pathogens of infection in diabetic patients. We found 96% samples as positive cultures and 4 % samples as negative cultures that may be termed as sterile cultures. Sugandhi and Parasanth conducted a study on 60 human patients, out of which 40 were positive cultures and 10 were negative cultures yielding no bacterial isolates (Sugandhi and Parasanth 2014). In our study the infectious ulcer with poly microorganisms were 83.4% and the infectious ulcer with single isolates were 16.6%. Citron in his study found that out of 427 positive specimens the ratio of poly-microbial infection was 83.8% (Citron *et al*., 2007). We isolated 2.06 bacteria per culture in average, while Citron found an average of 2.7 organisms per sample.

Table 3: Summary of data and information taken from human patients by filling the questionnaire

Sr. No.	Factors	Percentage
1	Age ≤50 years >50 years	 20% 80%
2	Sex Male Female	 70% 30%
3	Family history Yes No	 60% 40%
4	Status of glycemic control ≤200 >200	 26.6% 73.4%
5	Duration of diabetes ≤5 years >5 years	 14% 86%
6	Foot trauma Plantar Non plantar	 33.3% 66.7%
7	Duration of ulcer ≤1 month >1 month	 33.4% 66.6%
8	Duration of hospital stay ≤2 days >2 days	 6.6% 93.4%
9	Co-morbidities Hypertension Retinopathy Neuropathy Nephropathy	 66.6% 50% 36.6% 16.6%

Table 4: Percentage of diabetic foot infections according to Wagner's classification and severity of infection

Grades	Percentage according to Wagner's classification	Percentage according to severity of infection
Grade 0	0%	___
Grade 1	23%	0%
Grade 2	36%	46%
Grade 3	23%	36%
Grade 4	10%	16%
Grade 5	6%	--

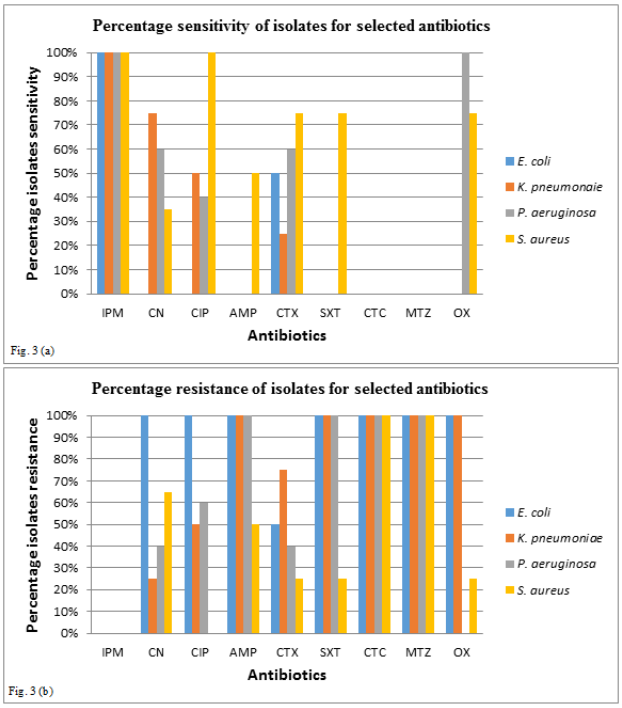

Fig. 3: Comparison of sensitivity (fig. a) and resistance (fig. b) of selected antibiotics against isolated *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* from diabetic foot of human patients

The aim to find the antimicrobial profile of isolated bacteria in our study was to provide the knowledge of most prevalent bacteria in infectious foot ulcer and their antimicrobial profile which may provide a guideline for clinicians to treat and manage the infectious foot ulcer. In our study the 100% isolated bacteria (Gram positive as well as Gram negative) were found to be sensitive for imipenem. Vijayakumar in his study found that imipenem was active against Gram positive orgainsms and cefepime and amikacin was found to be active against Gram negative bacteria (Vijayakumar and Gundela, 2012). Ahmed found that for most of the bacterial pathogens in diabetic foot, imipenem was active antibiotic (Ahmed *et al*., 2013). Jasmine and colleagues in his study found that 95 % isolates were sensitive to imipenem (Jasmine *et al.*, 2013). Among the combinations, cefipime-tazobactum and cefoperazone-sulbactum were the most effective. It was reported that the Non Steroidal Anti-inflammatory Drugs (NSAIDS) such as aspirin and tylenol also showed anti-bacterial effect against human clinical bacterial isolates from diabetic foot in Pakistan (Akhter *et al*., 2010). However, recent data evidenced that the methicillin and extended spectrum β-lactamase (ESBL) resistance has been increased in the diabetic foot human patients in Pakistan (Chaudhry *et al*., 2016). In a study conducted by Jain (2017), 53% of the Gram-negative bacilli were extended spectrum beta-lactamase producers, while 41% were methicillin-resistant S. aureus and 19% were vancomycin-resistant Enterococcus. In our study the Gram negative isolates (100%) were resistant for ampicillin, sulfamethoxazole/trimethoprim, cefotaxime/ clavulanate, metronidazole. The *E. coli* (50%) were resistant for cefotaxime, while 50% were sensitive. The members of *Enterobacteriacae* were resistant to oxacillin.

CONCLUSION

The incidence of antibiotic resistant bacteria are increasing in diabetic foot patients, therefore, bacterial isolation and culture sensitivity of the infected diabetic foot patients is recommended for rational use of antibiotics.

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