Bacterial ecology and antibiotic resistance mechanisms of isolated resistant strains from diabetic foot infections in the north west of Algeria

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Abstract

Background In front of the polymorphic bacterial ecology and antibiotic resistance in diabetic patients with foot infections and good patient care, collaboration between clinicians and microbiologists is needed to improve assessment and management of patients with this pathology.

Objective This study was designed to characterize the bacterial ecology of diabetic foot infection (DFIs) and to determine the different mechanisms of resistance involved.

Methods In this study bacterial strains and antibiotic resistance profiles were determined from diabetic foot infections patients ($n = 117$). The identification of resistance mechanisms, such as penicillinase and/or extended-spectrum β -lactamase production (ESBL), methicillin-resistant *Staphylococcus aureus* (MRSA) and efflux pump over-expression were performed.

Results A high prevalence of Gram-negative bacteria (61%) with *Escherichia coli*, and other Enterobacteriaceae and *Pseudomonas aeruginosa* being the predominant isolates. Gram positive bacteria mainly represented by *Staphylococcus aureus* accounted for 39% of the isolates. 93.5% of the Enterobacteriaceae were resistant to, at least, one molecule in the β -lactam family, while the majority of the *Staphylococci* were resistant to penicillin G and tetracycline (93.3% and 71.7%). The majority of non-fermenting Gram negative bacteria were also resistant to fluoroquinolones. β -lactamase detection tests revealed the presence of extended-spectrum β -lactamase in 43.5% of the Enterobacteriaceae, while methicillin-resistant *Staphylococcus aureus* represented 18.2% of the isolates. Additionally, 50.9% of non-fermenting Gram negative bacteria were overproducing efflux pumps.

Conclusion All *Acinetobacter Baumannii* were Multidrug-Resistant (MDR), as the majority of *Staphylococci*, and Enterobacteriaceae. These results should be taken into account by the clinician in the prescription of probabilistic antibiotic therapy in this context.

Keywords Diabetes Complications · Foot infection · Bacteria · Antimicrobial agents

Introduction

Diabetes mellitus (DM) is a clinical syndrome associated with a deficiency in insulin secretion or action [1]. It is a chronic metabolic syndrome with a high risk of developing complications [2], like cardiovascular diseases, kidney failure and eye problems, but foot complications are among the most common and most feared [3]. One of these complications is chronic foot ulcers with severe infection [4]. It is defined by an invasion of tissue with multiplication of microorganisms causing tissue damage with or without inflammatory response of the organism [5]. This is the most common cause of hospitalization among patients with diabetes and often requires long term hospital admissions [6].

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Diabetic foot infection (DFI) must be diagnosed clinically rather than bacteriologically because all skin ulcers harbour micro-organisms [7]. Mostly, DFIs are mixed bacterial infections [8]. They can be caused by pathogenic bacteria from the external environment as well as by bacteria belonging to the physiological microflora of the skin [9]. In patients with diabetes, superficial skin infections are mainly caused by the same organisms as those in healthy hosts, namely group A *Streptococcus* and *Staphylococcus aureus* [10]. Gram negative bacilli (mainly *Enterobacteriaceae*, sometimes *P. aeruginosa* or other non-fermenter species) are usually isolated in conjunction with Gram positive cocci from patients with chronic or previously treated infections. In the case of DFIs, antibacterial therapy using broad-spectrum antibiotics is often used. However, indiscriminate use of common antibiotics will eventually lead to the emergence of multiresistant strains [11].

For this, our study was conducted in order to characterize the bacterial ecology of diabetic foot infection (DFIs) in selected Algerian patients, investigate the bacterial resistance profile of identified strains and determine the different mechanisms of resistance involved (extended-spectrum β -lactamase (ESBL) production, methicillin-resistant *Staphylococcus aureus* (MRSA), penicillinase and efflux pump over-expression).

Materials and methods

Study design

Part of this work was done in the Faculty of Natural and Life Sciences, Mustapha Stambouli University, Algeria. Another part of this study was carried out in the Training and research unit (UFR) of Pharmacy, University of Picardy Jules Verne, France from March 2016 to June 2018.

The patients included in the study suffered from infectious complications in the lower limbs and were hospitalized for surgical management of lower-extremity wounds which were considered for the study. The study was conducted in accordance with Good Clinical Practices. All procedures performed involving human participants were in accordance with the ethical standards (JAMA, N20; 2013) of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards [12].

All patients signed a written informed consent prior to the inclusion in the study procedure. Once their informed consent was obtained, demographic details as well as the type and duration of diabetes were documented from each patient's medical records.

Clinical diagnosis of infection was defined by the presence of at least 2 of the following indicators: local swelling or

indurations, erythema around the wound ($\varnothing > 0.5$ cm), local tenderness or pain, local warmth, and purulent discharge [13]. Common skin infections and wounds without clinical signs of infection should never be taken.

Specimen collection

The sampling procedures have taken into account the level of infection which can be superficial or deep. Before any sampling for bacteriological or histological purposes, a preparation of the wound was required (debridement followed by wound cleansing with sterile physiological serum to avoid contamination by commensal flora that colonizes the skin) [14], but no antimicrobial or antiseptic agent was introduced into the wound before specimen collection. For superficial wounds, swabbing using a sterile dry swab (CITOSWAB®) was performed on the wound surface for 5 s, using a pressure sufficient to extract the fluid from the inner part of the wound [15]. For deep wounds, samples were collected by needle aspiration (introduced 0.5 cm–2 cm deep) to collect all the germs responsible for the infection. If no liquid could be obtained by this procedure, 1 to 2 mL of sterile physiological serum were injected then retrieved using a second needle [14].

Bacterial identification of isolated strains

First, all samples were quickly transported and cultured on an enrichment broth which was the sterile nutritive broth (Pasteur Institute of Algeria) at 37 °C for 24–48 h. Next, they were subcultured on either non selective media, such as Columbia blood agar and Trypticase soya agar (TS), or selective ones. These latter comprised Chapman agar for *Staphylococci*, M17 agar for *Lactococci*, Eosin methylene blue (EMB), Hektoen and Cetrimide agars for Gram negative bacteria (the supplier of all these culture media is the Pasteur Institute of Algiers, Algeria). After 18 to 24 h incubation at 37 °C, all isolates underwent phenotypic identification using API systems (BioMérieux, France) according to the manufacturer's instructions based on the color change of each test. *Staphylococcus* isolates were additionally tested for their coagulase enzyme production using rabbit plasma (Pasteur Institute of Algiers, Algeria). Coagulase is an enzyme produced by *S.aureus* that converts fibrinogen (soluble) in plasma to fibrin (insoluble).

The strains were then identified by MALDI-TOF (MS) mass spectrometry according to manufacturer's instructions (Bruker Daltonics), using a matrix-assisted laser ionization source (MALDI = matrix-assisted laser desorption/ionization) and a flight time analyzer (TOF = Time-of-Flight). For each sample, a mixture containing a colony of the strain with 1 μ L of 80% formic acid and 1 μ L of the CHCA (α -cyano-4-hydroxycinnamic acid) matrix was prepared. The recorded data is presented in the form of a spectrum (Fig. 1) where each peak corresponds to a type of molecule. Thus, a search in the database was carried out for the

Fig. 1 Example spectrum Maldi-ToF of *Klebsiella pneumoniae* species

identification of the corresponding germ using Maldi Biotyper V 4.0 software.

Antibacterial susceptibility test

All identified organisms were subjected to antibiotic sensitivity testing by the standard disc diffusion method, using commercially purchased antibiotic discs by the suppliers (Himedia, Liofilchem or Cypress diagnostics). [Amoxicillin (AMX 25 µg), Amoxicillin+Clavulanic acid (AMC 20/10 µg), Ampicillin (AMP 10 µg), Penicillin G (P 10 µg), Cefotaxim (CTX 30 µg), Cefoxitin (FOX 30 µg), Chloramphenicol (C 30 µg), Gentamicin (GEN 10 µg), Colistin (CS 10 µg), Erythromycin (E 15 µg), Nalidixic Acid (NA 30 µg), Tetracyclin (TE 30 µg), Ciprofloxacin (CIP 5 µg), Levofloxacin (LEV 5 µg) and Vancomycin VA 30 µg)]. Results were interpreted according to the 2016 European Committee for Antibiotic Susceptibility Testing Guidelines [16]. In this study, we have classified bacterial resistance to antibiotics by the character multidrug resistance (MDR) according to standards from the European Center for Disease Prevention and Control [17].

Resistance mechanisms detection

a) Extended-spectrum β -lactamase Detection (ESBL)

The double synergy method was performed under standard conditions using the disc of amoxicillin-clavulanate disc (AMC 20/10 µg) positioned at a distance of 30 mm to a disc of 3rd generation cephalosporin (C3G) disc (cefotaxime (CTX) or aztreonam (ATM)). The presence of an ESBL was

inferred when the inhibition zone around the C3G disk was enhanced on the side of the AMC disk, resulting in a characteristically shaped zone referred to as a “champagne-cork,” “keyhole,” “ellipsis,” or “phantom” image [18].

b) Methicillin-resistant *Staphylococcus aureus* Detection (MRSA)

The cefoxitin disk diffusion method was performed, using a 30 µg disk. After 18 h incubation at 37 °C, strains with an inhibition zone diameter ≤ 21 mm and those with a diameter were ≥ 22 mm, were considered to be methicillin-resistant (MRSA) and methicillin-sensitive *S.aureus* (MSSA), respectively [19].

c) The research of penicillinase

The iodometric color reaction, based on the destruction of the starch-lugol complex by the penicillic acid resulting from the penicillin attack by the *Staphylococcal* penicillinase that can be visualized by the discoloration of a dark blue iodine-starch complex, was used [20].

d) Phenotypic Detection of Efflux Pump-mediated Resistance in non-fermenter Gram-negative Bacilli

For the first attempt, *P.aeruginosa* and *A.baumannii* strains isolated in this study (DFI) were further submitted to a phenotypic test to evaluate the part of efflux pumps (EPs) in the resistance to fluoroquinolones, which are good substrates for a majority of EPs of the Resistance-Nodulation-Division family.

First, Minimum Inhibitory Concentrations (MICs) for ciprofloxacin and levofloxacin were determined using E-Test strips (BioMérieux, Dalvagen, Solna, Sweden), according to the manufacturer's instructions.

Resistant isolates were further subjected to ciprofloxacin MIC determination by the microbroth dilution method on 96-well microplates in the presence and absence of efflux pump inhibitors (EPIs), namely 1-(1-naphthylmethyl)-piperazine (NMP) and Carbonyl-Cyanide-3-Chlorophenyl hydrazone (CCCP) for *A. baumannii* isolates [21] or Phenylalanine-Arginine- β -Naphthylamide (PA β N)(Sigma-Aldrich, Saint-Quentin-Fallavier, France) for *P. aeruginosa* [22]. The cut-off value usually accepted as a signature of an efflux-driven resistance is a 4-fold reduction in the original MIC for a given antibiotic when an efflux pumps inhibitor is added [22].

Cascading dilutions of the antibiotic (CIP) were performed starting with a concentration of 128 μ g/ml to 0.0625 μ g/ml (this concentration was increased to 512 μ g/ml for some highly resistant strains). For the EPIs, we started with a concentration of 100 μ g/ml to 0.05 μ g/ml.

This method is not the only one for efflux pumps detection, because we have a project in the future concerning a molecular method by RT-qPCR to select the genes coding for this resistance system.

Statistical analysis

The quantitative results were expressed as mean $\pm$ standard deviation; while the qualitative variables were expressed as frequency (%). The statistical analysis was performed using SPSS (version 24). A value of $p < 0.05$ was taken as statistically significant.

Results

Population characteristics

Table 1 summarized the demographic characteristics of selected patients included in this study grouped $n = 117$ patients with sex ratio = 1.72, with dominance of male sex.

Table 1 Demographic characteristics of patients included in the study

	Overall	Male	Female	<i>P</i> value
Number (%)	117 (100)	74 (63.2)	43 (36.8)	/
Age (yrs)	63.23 $\pm$ 11.15 ^a	62.56 $\pm$ 10.43 ^a	64.37 $\pm$ 12.34 ^a	0.68
Weight (kg)	77.21 $\pm$ 6.51 ^a	77.68 $\pm$ 6.39 ^a	76.39 $\pm$ 6.70 ^a	0.85
Body Mass Index (kg/m ²)	26.35 $\pm$ 2.92 ^a	25.95 $\pm$ 2.70 ^a	27.04 $\pm$ 3.19 ^a	0.01
Type 2 diabetes	93%	63.63%	36.37%	/
Length of diabetic affection (yrs)	13.56 $\pm$ 3.61 ^a	13.64 $\pm$ 3.80 ^a	13.41 $\pm$ 3.31 ^a	0.45

^a: Results are given as mean $\pm$ standard deviation

Bacteriological results

A bacteriological examination was performed on 117 pus samples collected from DFIs: 62 superficial samples (53%), 35 deep ones (30%) and 20 pus aspirations (17%). We have chosen samples to be taken depending on the type of wound.

a. Bacterial Characteristics

Overall, 100 out of 117 (85.47%) samples were positive for bacterial culture. DFIs were mainly polymicrobial (47%, 55/117) while mono-microbial infections were seen in 38% cases (Table 2). We have no significant difference between the mono-or polymicrobial combination between woman and man ($p = 0.9687$). But, there is a significant relationship ($P = 0.000$) between the type of wound (superficial or deep) and mono-microbial or polymicrobial ecology.

A total of 231 isolates was obtained from the 100 positive culture samples, averaging 2 species per patient. Gram negative pathogens represented 62.77% (145) of the isolates. Among those, 39.83% (92) were Enterobacteriaceae with *E.coli* being the most commonly isolated 19.91% (46), followed by *Klebsiella oxytoca* 6.06% (14). Non-fermenting Gram-negative bacilli represented 22.95% (53) isolates with 15.59% (36) *P. aeruginosa* strains and 7.36% (17) *A.baumannii* ones. For Gram positive bacteria, the most commonly isolated species was *Staphylococcus aureus* 25.97% (60), followed by coagulase negative *Staphylococci* 5.20% (12), *Streptococcus sp* 3.89% (9), *Enterococcus faecalis* 2.16% (5).

Antimicrobial susceptibility test

The results of tested antibiotics resistance are shown in Table 3.

According to ECDC data, multidrug-resistance (MDRs) were defined as resistance to one or more antibiotics of three or more categories [23]. In our study, we have found that multidrug-resistant organisms (MDR) were more frequently found in *A.baumannii*,

Table 2 Distribution of samples according to the association character of identified bacteria ($n = 234$)

	Number (117)	Percentage (%)
Mono-microbial	45	38.49
Bi-microbial	10	8.54
Tri-microbial	24	20.51
≥4 germs	21	17.94
Negative culture	17	14.52

followed by *Staphylococci*, *E.faecalis*, Enterobacteriaceae, *P.aeruginosa*, and *Streptococcus* respectively (Fig. 2). There was a significant correlation ($P = 0.004$) between deep wound and growth of MDR.

And, we have observed that there may be a relationship between the respective mechanisms of resistance and the prescriptions of antibiotics in patients. For example, the resistance of non-fermenting Gram negative bacteria to fluoroquinolones as well as Enterobacteriaceae and *Staphylococci* to β -lactams.

a. Detection of ESBL

In this study, 43.4% of Enterobacteriaceae were ESBL producers. The frequency of ESBL production varied from one species to another (Fig. 3).

b. Detection of Penicillinase and MRSA

In *S.aureus* isolates, 57 out of 60 (95%) produced a penicillinase, while this production was never detected for coagulase negative *Staphylococci*. Methicillin resistance was also present in 18.3% of *S.aureus* isolates, where we observed that

there is a significant correlation ($P = 0.000$) between deep wounds and the presence of MRSA.

c. Detection of Efflux Pump

E-test results revealed that all *A.baumannii* (17 strains, 100%) had a MICs for ciprofloxacin and levofloxacin greater than 32 $\mu\text{g/ml}$. Among the 36 *P.aeruginosa* isolates, 17 (47.2%) were resistant to ciprofloxacin and levofloxacin.

In *P. aeruginosa* isolates, antimicrobial susceptibility to ciprofloxacin showed that 41.7% of the isolates had an MIC reduction of at least 4-fold in the presence of Phenylalanine-Arginine- β -Naphthylamide, hence accounting for a phenotypic resistance at least partly mediated by efflux. In *A.baumannii*, a significant reduction in MICs was witnessed in 70% and 23% of the strains for CCCP and NMP, respectively (Fig. 4).

Discussion

The emergence of bacterial resistance with different mechanisms presents a public health problem, as is the case in this study carried out on diabetic foot infections.

Present study revealed that DFIs were mainly polymicrobial (47%). This result was in accordance with those obtained by Saseedharan et al [24] and Thokur et al [25] which found 22.7% and 58% respectively. In their studies they focused on the Epidemiology of DFI and the characterization of antibiotic resistance in patients suffering from DFI.

But Anvarinejad M et al [26] reported in 2015 that monomicrobial strains were most common with 79% in their study put the stress on isolation and antibiotic susceptibility of the microorganisms isolated from DFI. The source of the infection, the depth of the wound, the use of antibiotics for treatment, tissue perfusion, and different types of infection can influence the diversity of pathogens in the DFIs [27–29].

Table 3 Number of antibiotic resistance of isolated bacteria from selected diabetic foot infection samples

	P	AMX	AMP	CTX	FOX	GEN	C	CIP	LEV	COL	TE	E	VA	NA	Total
<i>Enterobacteria</i>	nt	86	nt	58	nt	49	41	35	30	nt	nt	nt	nt	32	92
<i>S.aureus</i>	56	nt	nt	nt	11	24	32	nt	nt	nt	43	25	nt	nt	60
CN <i>Staphylococci</i>	3	nt	nt	nt	0	0	0	nt	nt	nt	2	0	nt	nt	12
<i>Enterococcus sp.</i>	nt	nt	5	nt	nt	2	3	nt	nt	nt	nt	3	2	nt	5
<i>Streptococcus sp.</i>	6	nt	nt	nt	nt	4	2	nt	nt	nt	4	4	5	nt	9
<i>A.baumannii</i>	nt	nt	nt	16	£	13	nt	17	17	17	10	nt	nt	nt	17
<i>P.aeruginosa</i>	nt	nt	nt	nt	nt	23	nt	17	17	36	nt	nt	nt	nt	36

nt: not tested; CN *Staphylococci*: coagulase negative *Staphylococci*; P: Penicillin G; AMX: Amoxicillin; AMP: Ampicillin; CTX: Cefotaxim; FOX: Céfoxitin; GEN: Gentamicin; C: Chloramphenicol; CIP: Ciprofloxacin; LEV: Levofloxacin; CS: Colistin; TE: Tetracyclin; E: Erythromycin; VA: Vancomycin; NA: Nalidixic Acid.

Fig. 2 Frequency of MDR profile of the strains isolated from DFIs

We have also found a difference in bacterial ecology between wound types. It appears that the superficial wounds were generally mono-microbial presented by *Staphylococcus* species, and deep ones were characterized by the presence of more than two germs including Gram-negative bacilli and Gram-positive cocci. In severe DFI, there was more than one bacteria including Enterobacteriaceae, *S.aureus*, and *P.aeruginosa* [30].

Grams negative were more prevalent (62.77%) in our study, and the predominant isolated organisms were Enterobacteriaceae. Various previous works confirmed our results with the following values 51.2% and 59% respectively [1]. The frequency of Gram positive organisms' isolation (37.23%) was also similar to that found by **Hefni et al** [31] who found 30.52% of Gram positive isolates. The International Working Group on the Diabetic Foot (IWGDF) which indicated that *Staphylococcus aureus* was the most common and virulent pathogen in DFIs. Even when it was not the only isolated bacterial species, it was usually part of a mixed infection [32].

The rates of resistance against antibiotics of isolated bacteria from diabetic foot lesions varied according to studies. This was due to the different habits in matters of antibiotics

prescription despite the existence of recommendations that have tried to standardize prescriptions [33]. The proportion of *S.aureus* strains producing a Penicillinase was also consistent with data from the previous literature on the bacterial profile and antibiotic resistance in patients with DFI [31, 33, 34]. Our susceptibility rates to Gentamicin (40%) and erythromycin (41.66%) were also in alignment with those of the study of **Zubair et al** [35] with the values of Gentamicin and erythromycin (37.8%, 45.9%), respectively. The high resistance to Penicillin can be explained by the use of β -lactams in the treatment of DFIs in our selected population according to patient records.

Strains of Coagulase Negative *Staphylococci* (CONS) were more susceptible to the tested antibiotics including Methicillin. However, **Zubair et al** [35] in a clinical microbiological study and antimicrobial resistance and the profile of DFI found that the Coagulase negative *Staphylococcus* were all resistant to Erythromycin and 75% resistant to Amoxicillin, **Banashankari et al** [36] indicated that only 15% were Methicillin-resistant. This difference could be explained by the duration of hospitalization, as well as the antibiotic prescription which differs from one region to another, as we mentioned recently.

Fig. 3 Frequency of ESBL production in *Enterobacteriaceae*

Fig. 4 Determination of efflux pump frequency in *A.baumannii* and *P.aeruginosa* Isolates

Resistance patterns of Enterobacteriaceae showed a significant resistance to β -lactams which was similar to the findings reported earlier by **Zubair et al** [35]. It is the most used molecule in the population of our study. There was a direct relationship between the total amount of antibiotics used in a particular hospital over a period of time and the number of resistant strains that emerge [37].

For *P.aeruginosa*, the susceptibility of isolated strains to Fluoroquinolones (47.22%), Colistin (100%) and Gentamicin (63.88%) was partially in accordance with the results of **Perim et al** [38] regarding resistance to Gentamicin (25%) and consistent with that of **Saseedharan et al** [24] regarding the rate of resistance to Ciprofloxacin (49%). But **Hefni et al** [31] found that all *P.aeruginosa* isolates were sensitive to Gentamicin and 90% to Polymyxin and Levofloxacin. Our findings correlated with the study of **Nora et al** [39], which showed that *P.aeruginosa* exhibited a (MDR) pattern who is defined by bacteria resistant to more than one antimicrobial agent.

Treatment of *A.baumannii* has become a medical challenge due to the increasing prevalence of MDR species and the lack of viable treatment alternatives [40]. In our study, the majority of isolated *A.baumannii* strains were resistant to all tested antibiotics (Cefotaxim 94.11%, Gentamicin 76.4%, Tetracyclin 58.82%) and a totally resistance to Ciprofloxacin, Levofloxacin, and colistin. Our results along with previous ones showed a high level of resistance of non-fermenting Gram-negative bacilli to Fluoroquinolones and others. A similar trend that was reported by **Thokur et al** [25] indicated that *Acinetobacter* have similar resistance rates (Cefotaxim 94%, Ciprofloxacin 84%, Gentamicin 75%). This may be due to the use of these antibiotics in the treatment of Multi-drug resistant strains in DFIs which caused the emergence of resistance [41]. The majority of clinical *A. baumannii* strains with high resistance to different classes of antibiotics have become a global problem [42].

Enterococci isolated in this study were fully resistant to Ampicillin (100%), but variable levels to the other antibiotics Chloramphenicol 60%, Erythromycin 60%, Gentamycin 40%. These results were partially consistent with those of other similar studies of **Thokur et al** [25]. Although, *Enterococcus* was totally referred to as commensals, in people with diabetes they can act as opportunistic pathogens. Our results revealed a frequency of 40% resistance to Vancomycin which was in accordance with the literature which indicates that the emergence of antibiotic resistant *Enterococci* and the high prevalence of virulence traits in a clinical study is considered an alarming situation [43].

The results of our study and what was found by **Chaudhry et al** [44] showed a similarity in the frequency of *Streptococcus* resistance to Vancomycin and Chloramphenicol. Several studies have described the emergence of microorganisms' resistance against antibiotics as being large due to their inappropriate use [45].

We found that all *A.baumannii* were MDR (100%), while 82.6% *Staphylococci* were MDR and 47.2% *P.aeruginosa*. These were in agreement with **Sekhar et al** [46] in a study of antimicrobial susceptibility pattern in diabetic foot ulcer who indicated that *Acinetobacter* isolates showed full resistance to all common antibiotics tested and **Shankar et al** [47] in a similar study, who found a frequency of 44% of MDR *P. aeruginosa*. Our study revealed that MDR is widespread in our population, especially in patients with deep wounds. In addition, prolonged use of antibiotics, as well as manipulation with non-sterile instruments leads to the multiplication of MDR germs [33]. MDR rates of isolates in 2004 were 18%, while the rate reported in 2014 was 63.4% [26].

Previously, the antibiotic resistance problem of great concern has been Gram-negative organisms which produce ESBLs [48]. In this study, the frequency of ESBL producing strains was 43.4% of Enterobacteriaceae, mainly *K. pneumoniae*, *E.coli*, *E.cloacae*, and *K.oxytoca*. Other studies

on DFI [24–26] were in accordance with our results and showed that the frequency of positive ESBL strains was Mostly in *E.coli*, *K.pneumoniae* and *E.cloacae* species. In our study, the prevalence of strains producing ESBLs was higher. Over the last decade, other MDR organisms, particularly Gram negatives with ESBLs, have been more often isolated in DFIs [37–39]. This requires treatment of DFIs covering ESBL producing Gram negative bacteria [37]. Our ESBL producing strains are generally isolated from patients with complicated wounds that need long hospitalization, which is consistent with the literature that indicates that in patients with ESBL infection, higher mortality, long hospitalization, higher costs and lower cure rate are observed [49].

Regarding MRSA, our results were converged to those obtained by other studies [34–54] with frequencies of 23%, 13%, 20.47%, 23.07% and 23.15% respectively. However other studies found higher frequencies of MRSA in DFIs as Al Benwan et al [50] and Chaudhry et al [44] respectively 60% and 88%. According to the literature MRSA rates are constantly increasing in DFI [51, 52]. This may be due to massive prescription of β -lactams [53]. In our study, all MRSA were also resistant to all tested antibiotics. The literature indicated that MRSA is also not sensitive to aminoglycosides, macrolides, tetracycline, chloramphenicol and lincosamides [54]. In our case, MRSA was isolated from patients hospitalized for a long period of time with deep wounds. MRSA infection can be caused by several factors such as inappropriate or long-term use of antibiotics, length of hospital stay, and nasal MRSA carriage [55].

Our study represented the first attempt to demonstrate efflux pump in fluoroquinolones resistance in non-fermenting Gram negative bacilli isolated from hospitalized patients with DFI. Many studies examining the MDR phenotype have shown that the efflux system is an important mechanism for bacterial resistance against various antibiotics [56, 57]. Active efflux might be responsible for the selection of high-level MDR strains causing therapeutic impasses [58], prolonged hospitalization and increased risk of both morbidity and mortality [59]. Reports on the prevalence of efflux-mediated resistance in *P.aeruginosa* clinical strains not (DFI) varied from 39.6% up to 92.6% [22–42, 44–65]. The results obtained in this study are therefore on the lower side of this range. As PA β N was largely active against Mex type systems [66], it could be interesting to further investigate the fluoroquinolone resistant strains using RT-qPCR to confirm the overexpression of these EPs.

As for *A.baumannii*, a significant difference in the mechanisms of action of the two compounds CCCP and NMP was observed in this study. EPIs were not similarly active on all efflux systems and/or do not have the same activity with all antibiotics [67]. For example, MICs of fluoroquinolones, chloramphenicol and gentamicin have been shown to decrease after the addition of CCCP [68, 69]. Only 23% of our

A. baumannii strains showed a reduction of at least 4-fold of their ciprofloxacin MICs in the presence of NMP. However, other studies found efflux-mediated resistance to ciprofloxacin in 48 to 93.3% of their *A.baumannii* isolates in presence of NMP [70, 71].

Our study and others confirmed a better restoration of the activity of ciprofloxacin in the presence of CCCP EPI [72], up to 86.1% [73]. There were several types of efflux systems in *A.baumannii*, but the expression of the AdeABC EP, member of the Resistance-Nodulation-Division (RND) family has been well characterized in the species resistant to fluoroquinolones, aminoglycosides, and in some β -lactams [21, 74]. The different rates of resistance of the isolated bacteria is probably the result of the source of the infections, as well as the geographical distribution of infections in the world [75]. Knowledge and determination of the microorganisms (such as bacteria) causative of DFI and their antimicrobial resistance profiles is essential for a proper treatment and thus for the eradication of infections [76].

Conclusion

In this study, we observed the diversity of isolated bacteria from DFI including Gram-negative bacilli and Gram-positive cocci. As is the case with DFIs, antibiotics therapy has been used to treat a large number of bacterial infections and improve life expectancy, but the large prescription and inappropriate use of these molecules have led to the emergence and dissemination of antibiotic-resistant bacteria, which translated into a therapeutic impasse. Part of this resistance can be supported by the production of ESBL in *Enterobacteriaceae*, and methicillin-resistance in *Staphylococcus aureus*, but the phenomenon encountered for the first attempt in non-fermenting Gram-negative bacilli in diabetic foot infection was the resistance by the efflux pump system. These results should be taken into account by the clinician in the prescription of probabilistic antibiotic therapy in this context.

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Authors' contributions BB: technical handling of the samples and article writing; CM: design and supervision of efflux pump inhibition experiments and article writing; NC.; BM.; ATT: design and supervision of the clinical study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.”

Informed consent Informed consent was obtained from all individual patients included in the study article.

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