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Biofilm production and resistance profile of *Enterobacter* sp. strains isolated from pressure ulcers in Petrolina, Pernambuco, Brazil

Produção de biofilme e perfil de resistência de cepas de Enterobacter *sp. isoladas de úlcera por pressão em Petrolina, Pernambuco, Brasil*

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ABSTRACT

Introduction: The *Enterobacter* genus is formed by lactose-fermenting bacteria. These microorganisms cause a wide range of hospital infections such as pneumonia, urinary tract infections and wounds; and they are associated with the colonization of medical devices. **Objective:** To define the resistance profile and the biofilm production of *Enterobacter* sp. strains isolated from pressure ulcers. **Materials and methods:** A quantitative field research (documentary) type laboratory study performed at a hospital in the municipality of Petrolina (PE). Samples were collected from 30 wounds of internal medicine inpatients with stage II pressure ulcers, using the Z-technique with sterile swabs, from February to May 2014. **Results:** The most prevalent bacteria were *Enterobacter* sp. and *Escherichia coli*. All proved multiresistant to antibiotics. Most strains of *Enterobacter* sp. were classified as moderate biofilm producer. **Conclusion:** The strains of *Enterobacter* sp. showed high resistance to the antimicrobials used in clinical routine.

Key words: *Enterobacter*; biofilms; pressure ulcer; microbial drug resistance.

INTRODUCTION

The *Enterobacteriaceae* family is a heterogeneous group of Gram-negative bacilli, whose natural habitat is the intestinal tract of human beings and animals. They are organisms considered facultative aerobes or aerobes that ferment a wide variety of carbohydrates, presenting a complex antigenic structure. They also produce several toxins and other virulence factors[1-3].

The *Enterobacter* genus is formed by lactose-fermenting bacteria; they have capsule, produce mucoid colonies and are motile. These microorganisms are associated with several nosocomial infections, such as pneumonia and urinary tract infections; with the colonization of wounds in general; and with the contamination of hospital devices (catheters, probes, and pacemakers)[4].

Most *Enterobacter* strains have chromosomic genes for beta-lactamase, AmpC, what makes them intrinsically resistant to antimicrobial agents, such as ampicillin and first- and second-generation cephalosporins. Some mutant strains may hyperproduce beta-lactamase, generating resistance to third-generation cephalosporins[1]. According to Santos (2011)[5] and Pirret (2014)[6], *Enterobacter* sp. is also responsible for the colonization of pressure ulcers (PUs).

PU is a necrosis area resulting from tissue ischemia due to compression of bony prominences against a rigid surface over a given time period[7, 8]. The most common sites of occurrence are the sacral, trochanteric and ischial areas[4, 9]. They are classified into four stages according to tissue involvement: stage I – intact skin with localized non-blanchable erythema; stage II – partial thickness loss of dermis with aspect of superficial ulcer and red pink wound bed, with no slough. They may also present as an intact or open/ruptured serum-filled blister; stage III – full thickness tissue loss. Subcutaneous adiposity may be visible, but there is no exposure of bone, tendon or muscle. Slough may be present, but does not obscure the depth of tissue loss, may

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include undermining and tunneling; stage IV – full thickness tissue loss with bone, muscle or tendon exposure. There may be the presence of slough or eschar in some parts of the wound bed, frequently includes undermining and tunneling[10].

Considered an important problem of public health, this kind of skin wound directly affects patients' recovery, at home and in hospital units, as well as it increases health service costs[11]. Because skin ulcers and ulcers of other tissues affect the skin and adjacent tissues, they may cause chronic secondary infection. These changes favor the multiplication of microorganisms at the lesion due to the exudative, serous, crust or hemorrhagic material present in its surface[9].

When present, these infections can be manifested by means of phlogistic signs, such as heat, erythema, purulent secretion and foul odor. Another factor that may indicate the presence of infection is the slow healing of the wound. The main involved microorganisms are *Enterobacter* sp., *Staphylococcus* sp. and *Enterococcus faecalis*[12].

The development of antimicrobial resistance is nowadays a growing problem worldwide and is intimately linked to infections by bacteria of the *Enterobacteriaceae* family. Resistance occurs in two ways: intrinsic, when it is part of the bacterial chromosome; and extrinsic, when the resistance genes are incorporated to it by other elements, such as the excessive, indiscriminate and disseminated use of antibiotic agents[13].

The resistance mechanism may be genetically inherited by new strains produced by intrinsically resistant bacteria, or they may present mobile elements inside, as integrons, transposons, and plasmids, which encode different factors blocking the action of several drugs[1]. Another factor that causes bacterial resistance among *Enterobacter* sp. strains is biofilm production[14]. Biofilm, defined as complex microbial ecosystems embedded in an organic polymer matrix attached to a surface, contains particles of proteins, lipids, phospholipids, carbohydrates, minerals and vitamins that form a kind of crust, under which microorganisms continue to grow, forming a pure culture or an association of several species of microorganisms[15].

Therefore, the occurrence of microbial resistance by biofilm production in PU is a complication for the adequate treatment and the control of this bacterial product. Further studies about this theme should be conducted due to its scientific relevance. Thus, this research was aimed at defining the resistance profile and the biofilm production of *Enterobacter* sp. strains isolated from PUs at a university hospital in the city of Petrolina (PE), Brazil.

MATERIALS AND METHODS

Study site, period and population

The research was conducted from February to May 2014, with samples from 30 PUs in 13 internal medicine inpatients of Hospital Universitário de Petrolina (HUP), situated in the *sertão* of Pernambuco. This research was approved by the Ethics Research Committee, nº 19641513.1.0000.5207.

Sample collection and microbial identification

The biological samples from PUs were weekly collected by two researchers previously trained, which obeyed the following steps: PUs were flushed with 0.9% saline solution and the edges were dried with sterile gauze sponges. Exudate was collected with a sterile swab, using the Z-technique[16], and this material was immediately transferred to test tubes containing sterile brain heart infusion (BHI) broth, being streaked onto their respective isolation media: blood agar, manitol salt agar (*S. aureus*), MacConkey agar, Teague agar (for Gram-negative bacilli). For identification of Gram-negative bacteria, oxidative fermentation (OF) and oxidase tests were carried out. They underwent the tests of lactose fermentation, indole production, motility, citrate use, urea hydrolysis, sulphydric gas production, phenylalanine deaminase, lysine and ornithine decarboxylase and methyl red reaction. For non-fermenting Gram-negative bacilli the following were used: nitrate reduction, gluconate use, pigment production, lysine decarboxylase activity, indole production, acetamide and esculin hydrolysis. Microbiological identification and the antibiotic susceptibility testing (AST) were performed at Laboratório de Análises Clinicas Especializadas de Petrolina (Lacesp).

Antibiotic susceptibility testing

An assay was developed that measures susceptibility/resistance of a bacterium to one or more antimicrobial agents. The used medium was Mueller-Hinton, with antibiotic-impregnated diffusion disks. This analysis permitted to determine the spectrum of sensitivity/resistance to drugs, as well as the minimal inhibitory concentration (MIC) (Clinical and Laboratory Standards Institute [CLSI], former NCCLS).

Testing for biofilm production

In order to assess and quantify biofilm formation, we used the method described by Christensen *et al*. (1995)[17], with some

changes, at Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CPqAM/Fiocruz) in Recife, Pernambuco. The isolated microorganisms were seeded in BHI agar medium, incubated in an incubator at 37ºC for 24 h and later inoculated in cuvettes with 1 ml BHI broth, being well homogenized, to avoid clot formation. The cuvettes were calibrated according to the optical density (OD) between 00.8 and 1, using the spectrophotometer WPA UV 1101 Biotech Photometer at a wavelength of 600 nm. Next, 96-well U-bottom polystyrene plates were filled with 200 µl of diluted cultures. For sample testing and classification according to biofilm production, the following controls were used as reference: negative control (non-biofilm producer) – sterile BHI broth; positive control (strong biofilm producer) – *Staphylococcus aureus* (ATCC 25923) strain. Plates were incubated at 37ºC during 24 h.

After incubation, the supernatant was delicately removed from the wells with a probe, and the precipitate was kept. Then, the wells were washed with 200 µl tryptone soya broth (TSB), and the plates were poured into Lysoform. Bacteria adhering to the well walls were fixed with 2% sodium acetate, and again poured into Lysoform after 3 minutes and stained with 0.1% crystal violet. Excess stain was removed with deionized water; and the plates were kept upside down until drying. The OD of adherent film was obtained by means of enzyme-linked immunosorbent assay (ELISA) reader, at the wavelength of 600 nm. The experiment was performed in triplicate. For interpretation of biofilm production, the average of the three wells was calculated, and the criterion proposed by Stepanovic (2000)[4] was adopted: non-adherent ($OD < 0.12$), moderate producer ($0.12 < OD < 0.24$) and strong producer ($OD > 0.24$).

Results of ASTs and tests of biofilm quantification are presented as tables and graphs, and compared to related studies. Quantitative data were tabulated and analyzed in the light of descriptive statistics, by means of Microsoft Office Excel® 2010.

RESULTS

Among the 30 samples collected from PU exudate, bacteria from the *Enterobacteriaceae* family were the most prevalent, as observed in **Figure 1**.

Figure 2 shows the resistance and antimicrobial susceptibility profile of the 15 isolated strains of *Enterobacter* sp. Among them, four isolates proved resistant to all tested antibiotics. Aminoglycosides were the only group of antibiotics to which the isolates proved more susceptible.

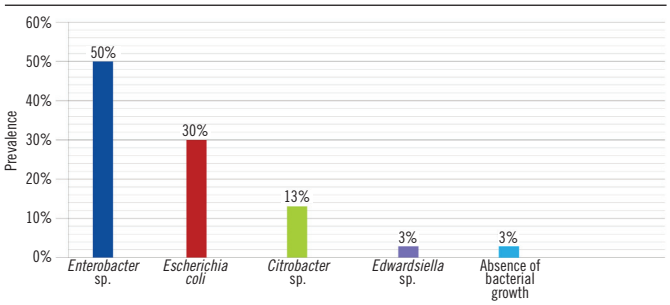

FIGURE 1 – *Prevalence of microorganisms isolated from PU exudate among internal medicine inpatients, Petrolina (PE)*

PU: pressure ulcer.

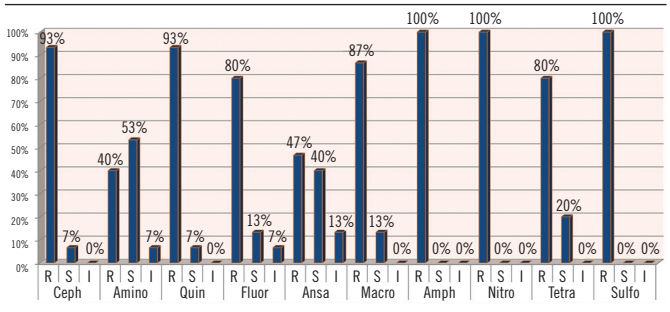

FIGURE 2 – *Resistance and antimicrobial susceptibility profiles of* Enterobacter *sp. isolated from PU exudate among internal medicine inpatients, Petrolina (PE)*

Enterobacter *sp. is intrinsically resistant to penicillin; hence it was not necessary to test this antimicrobial.*

PU: pressure ulcer; R: resistant; S: sensitive; I: intermediate; Ceph: cephalosporins; Amino: aminoglycosides; Quin: quinolones; Fluor: fluoroquinolones; Ansa: ansamycins; Macro: macrolides; Amph: amphenicol; Nitro: nitrofurantoin; Tetra: tetracycline; Sulfo: sulfonamides.

In relation to biofilm production, the 15 analyzed strains of *Enterobacter* sp. were classified as non-adherent, moderate producer, and strong producer; and their characterization can be observed in the **Table**.

TABLE – ***Enterobacter*** **sp. strains isolated from PU exudate among internal medicine inpatients, Petrolina (PE) – classification according to biofilm production**

Classification according to bacterial biofilm production	*n*	%
Non-adherent (non-biofilm producer) ($OD < 0.12$)	7	47
Moderate biofilm producer ($0.12 < OD < 0.24$)	7	47
Adherent (strong biofilm producer) ($OD > 0.24$)	1	6
Total	15	100

Source: CPqAM/Fiocruz.

PU: pressure ulcer; OD: optical density.

DISCUSSION

In the current study there was predominance of bacteria isolates of the *Enterobacteriaceae* family, especially *Escherichia coli* sp. and *Enterobacter* sp. Some studies show predominance of bacteria of the same family in samples of nosocomial infections, with predominance of *Escherichia coli* sp., *Enterobacter* sp. and *Pseudomonas* sp.[9, 13, 18]. In the present study, among the PU collected samples, predominance was observed of bacteria of the Gram-negative group, and all were multiresistant to the used antimicrobials. Braga (2011)[19] and Pirret (2011)[5] stated that in chronic lesions such as PU, the presence of epidemiologically important microorganisms such as Gram-negative bacilli, is common. They can evolve to an infection or work as a reservoir of multiresistant microorganisms. Besides extrinsic and intrinsic factors that induce bacterial resistance, biofilm production is responsible for a large percentage of nosocomial infections[20]. *Enterobacter* sp. is frequently related to infections with the presence of biofilm[14].

The strains isolated in this study proved multiresistant to antimicrobials used in clinical routine, principally cefotaxime, chloramphenicol, cefalotin, cefalexin, and clarithromycin. At a research[19] conducted with isolates from culture of pulmonary secretion present in the orotracheal tube (OTT), tracheostomy and central vascular catheter (CVC) tip cultures, a profile of resistance to cephalosporins similar to that of isolates of *Enterobacter* sp. was observed, confirming the results described in this work.

Roca (2009)[21] conducted a study with *Enterobacter* sp. in isolates from urinary infection, in which he observed 68.7% resistance to third-generation cephalosporins (ceftazidime and ceftriaxone). In the present study, there was greater prevalence of resistance to the same antimicrobials (ceftazidime 93%, and ceftriaxone 60%). Some mutant strains may hyperproduce beta-lactamase, conferring resistance to third-generation cephalosporins[1].

In the current research most strains (47%) of *Enterobacter* sp. revealed to be biofilm producers (adherent). In studies conducted on isolates from orthopedic implants[22], *Enterobacter* sp. strains proved to be biofilm producers, and then, resistant to gentamicin and octenidine hydrochloride. International publications display similar results, in which isolated subcultures of *Enterobacter* sp. produce biofilm of significant intensity[23], besides demonstrating that the *Enterobacter* sp. capacity to form biofilm is correlated with the messenger ribonucleic acid (mRNA) expression level of CSGA and CSGD genes. There is still the presence of curli fimbriae that can play an important role in biofilm formation in *Enterobacter* sp. strains[11, 24].

Although some researches demonstrate a bacterial behavior non-producer of biofilm (non-adherent), the infection sites (infectious environment) – tracheal aspirate[10] and urinary tract[21, 25] – are important variables in the expression of virulence factors, such as biofilm production. Thus, one may infer that such regions are manipulated with greater frequency than PUs.

Bacterial typing is a considerable analysis, because it is based on the idea that bacterial clonal lineages share properties that may be identified and used to distinguish them from the non-similar ones. Strain typing was not performed in this first moment, but it will be done in a future step, once the main objective of this work was to verify the relationship between biofilm production and the resistance profile of the analyzed strains.

CONCLUSION

Enterobacter sp. strains showed high resistance profile to the antimicrobials used in clinical routine of the cited hospital. Moreover, most of them were classified as moderate biofilm producers in the assessed PUs.

The current research is relevant, once identification, analysis and description of bacterial resistance profile, as well as biofilm production, are fundamental for the development of practices, especially in the hospital setting, which minimize the aggravation and the complications caused by this problem.

RESUMO

Introdução: *O gênero* Enterobacter *é formado por bactérias fermentadoras de lactose. Esse microrganismo causa largo espectro de infecções hospitalares, como pneumonia, infecções do trato urinário e feridas, além de associar-se à colonização de dispositivos hospitalares.* ***Objetivo:*** *Definir o perfil de resistência e a produção de biofilme de cepas de* Enterobacter *sp. isoladas de úlcera por pressão.* ***Materiais e métodos:*** *Estudo quantitativo, tipo pesquisa de campo (documental) laboratorial, realizado em um hospital do município de Petrolina (PE). Foram coletadas amostras de 30 feridas de pacientes internados na clínica médica, portadores de úlceras por pressão em estágio II, utilizando a técnica em "Z" com* swab *estéril no período de fevereiro a maio de 2014.* ***Resultados:*** *As bactérias mais prevalentes foram* Enterobacter *sp. e* Escherichia coli*. Todas se mostraram multirresistentes aos antimicrobianos. As cepas de* Enterobacter *sp. em sua maioria foram classificadas como produtoras moderadas de biofilme.* ***Conclusão:*** *As cepas de* Enterobacter *sp. mostraram alto perfil de resistência aos antimicrobianos usados na rotina clínica.*

Unitermos: Enterobacter*; biofilmes; úlcera por pressão; resistência microbiana a medicamentos.*

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