ORIGINAL ARTICLE

Antibiotic resistance, biofilm-forming ability and *fim H* gene in clinical isolates of *Enterobacter spp.*

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Abstract

Background: Enterobacter spp is one of the ESKAPE pathogens, which is an organism causing highly resistant infections mainly due to its ability to produce biofilm. *Aim and Objectives:* To find the antibiotic resistance, biofilm-forming ability and presence of *fimH* gene in clinical isolates of *Enterobacter spp. Material and Methods:* VITEK 2 system was used to identify the organism and determine the antibiogram of the isolates. Biofilm-forming ability was estimated by the modified O'Toole and Kolter method, and *fimH* gene was detected by conventional polymerase chain reaction. *Results:* From various samples, 22 (91.6%) strains of *Enterobacter aerogenes* and two (8.3%) isolates of *Enterobacter cloacae* were isolated, of which 60-100% showed resistance for various beta lactam drugs; 70-75% were sensitive to various aminoglycosides; 54-80% were sensitive to fluoroquinolones; 50-80% were sensitive to carbapenems; and 62.5% isolates were sensitive to tigecycline. Most of the isolates were moderate to strong biofilm producers (OD values > 0.8), especially *Enterobacter* spp. isolated from deep tissue and pus samples. The *fimH* gene was detected in 9 out of 24 (37.5%) isolates, which were strong biofilm producers with an average OD of 1.163 ± 0.293, $p < 0.05$. *Conclusion:* Majority of the isolates were multidrug-resistant and biofilm producers. Isolates possessing *fimH* gene were strong biofilm producers. The findings of the study therefore necessitate a treatment approach that relies on targeted antibiotic therapy based on susceptibility testing and an awareness that biofilm-related complications are highly likely in infections caused by this organism.

Keywords: Antibacterial Agents, Biofilms, Enterobacter, Virulence Factor

Introduction

Genus *Enterobacter* is a non-sporing gram-negative bacillus of the Enterobacteriaceae family. They are widely distributed in soil, water, and sewage [1]. This organism is known for causing nosocomial infections grouped as ESKAPE pathogens, which are predominantly known for their multidrug resistance [2-5]. Whole genome sequencing of multidrug-resistant Enterobacter strains show that they have acquired beta-lactamase and carbapenemase genes from *Klebsiella pneumoniae* belonging to the same family [6-8]. *Enterobacter spp*. are known to cause various infections in humans [9, 10]. Pathogenic mechanisms and factors contributing to infections by *Enterobacter spp.* are not yet understood. Biofilm formation, secretion of various cytotoxins, enterotoxins, hemolysins and pore-forming toxins are the factors that make them pathogenic [11, 12]. Most of the species of Enterobacter are biofilm producers, and this is the reason for their property of antibiotic resistance [13]. Biofilm is defined as a complex microbial ecosystem wherein the persister cells in the matrix are responsible for enhanced resistance to antibiotics and disinfectants [14].

Adhesins of *Enterobacter spp*. contribute to virulence by promoting colonisation and invasion. The uroepithelial bladder lining has the α-d-mannosylated proteins, such as uroplakins and Type 1 fimbriae bind to it by means of the adhesin, namely FimH, which is the main contributing factor for adherence and biofilm formation [15-19]. Hence, this study aimed to determine the antimicrobial resistance pattern, biofilm-forming ability and *fimH* gene in the clinical isolates of *Enterobacter spp.*

Material and Methods

This was a cross-sectional time-bound, prospective study. Twenty-four clinical isolates of *Enterobacter spp.* were included in this study after obtaining institutional ethics committee clearance (IEC MC MLR10-18/350).

Sampling criteria: Clinically significant Enterobacter species isolated from wounds, sputum, pus, and deep tissue were included in the study. Duplicate isolates from the same patient and isolates other than *Enterobacter spp*. were excluded. Sampling was done by a non-probability method (convenience sampling method). Identification and antibiotic sensitivity testing of the isolates were performed via the VITEK-2 system (Biomerieux, USA). Interpretation of antibiotic susceptibility was performed according to the Clinical and Laboratory Standards Institute guidelines [20].

Biofilm detection assay: Biofilm was detected using the modified method of O'Toole [21]. Isolates of *Enterobacter spp*. were inoculated into 1 ml of Brain Heart Infusion broth (BHI) and kept for 18 h at 37˚C. After adjusting the turbidity to 0.5 McFarland standard, 200 µl of the broth culture was dispensed into 96 wells of a microtiter plate in duplicate and kept at 37˚C overnight. Following incubation and aspiration of the contents of the wells, washing was done using phosphate buffer saline (pH 7.4). Then, 100 µl of Bouin fixative was added, and the plates were incubated at 25˚C for 10 min. The contents were discarded and crystal violet (1%) staining was done for 1min and washed with water. This was followed by the addition of glacial acetic acid (33%) and optical density (OD_{570}) was read spectrophotometrically using an Enzyme-Linked Immunosorbent Assay (ELISA) plate reader. Control used was *E.coli* ATCC 25922.

Detection of *fimH* gene by Polymerase Chain Reaction (PCR)

Extraction of DNA

DNA extraction was done by boiling method. Briefly, 3 colonies were suspended in 1ml BHI broth. After overnight incubation at 37º C, and centrifugation (5 min at 12,000 rpm), the supernatant was discarded, the pellet washed with saline, after which it was mixed with 500 µl of distilled water and boiled in a dry bath (10 min) to lyse the cells. This was briefly centrifuged and the supernatant (2 µl) was used as crude DNA.

Amplification

PCR amplification was done by using primers-

F: 5′-TACTGCTGATGGGCTGGTC-3′

R: 5′-GCCGGAGAGGTAATACCCC-3′

PCR mixture consisted of 10 µmol/l of each primer, 200 µM each of deoxynucleotide triphosphates, 1U of Taq DNA polymerase and 1X buffer with 25 mM $MgCl_2$ in a final volume of 25 µl reaction mixture made up with nuclease-free water. DNA was amplified in a thermal cycler (Corbett Research, Qiagen, Germany) with cycling conditions, initial denaturation - 94˚C for

2 min, followed by 40 cycles of denaturation-94˚C for 40 sec, annealing- 50˚C for 1min, extension- 72˚C for 1min, final extension -72˚C for 5 min [5,6]. The amplified product was detected by using 1% agarose gel electrophoresis in 1X Tris Acetate EDTA buffer. Staining of the gel was done using ethidium bromide (0.5 mg/ml) and the gel documentation was done. A biofilm-producing and a non-biofilm-producing isolate were used as the positive and negative controls, respectively, for validation of PCR [17, 22].

Statistical analysis

P-value was calculated using the Statistical Package for the Social Sciences version 20.0 (IBM, USA). Statistical analysis was performed by using the Student's unpaired t-test and Chi-Square test for comparison of biofilm formation and *fimH* gene with antibiotic resistance pattern.

Results

Out of 24 isolates of *Enterobacter spp*, 14 (58%) were isolated from males, while 10 (42%) were isolated from females. Most of the isolates were from pus 9 (37.5%) followed by urine, and deep tissue 5 (20.8%) each, sputum 3 (12.5%), bile and blood one each (4.1%) as shown in Figure 1. Majority of the isolates were from cases of wound infection.

Antimicrobial susceptibility pattern

Figure 2 shows the antibiogram pattern of the clinical isolates of *Enterobacter spp*. with maximum resistance (60-100%) to beta-lactam drugs; 70-75% of the isolates were sensitive to aminoglycosides; 54-80% were sensitive to fluoroquinolones; 50-80% were sensitive to carbapenems; and 15 (62.5%) isolates were sensitive to tigecycline.

Biofilm formation

Most of the isolates were biofilm producers, with OD_{570} values greater than 0.8, except for one strain each of *Enterobacter spp*., isolated from blood and bile. *Enterobacter spp.* isolated from deep tissue and pus samples exhibited strong biofilm production (OD_{570} values 0.9 to 2) (Figure 3). Majority of the clinical isolates exhibited resistance to beta-lactam drugs and were also biofilm producers.

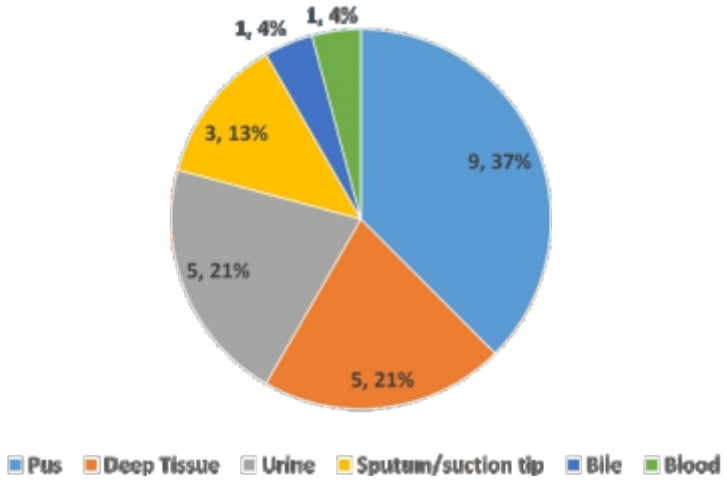

Figure 1: Clinical specimens wise distribution of isolates of *Enterobacter* species

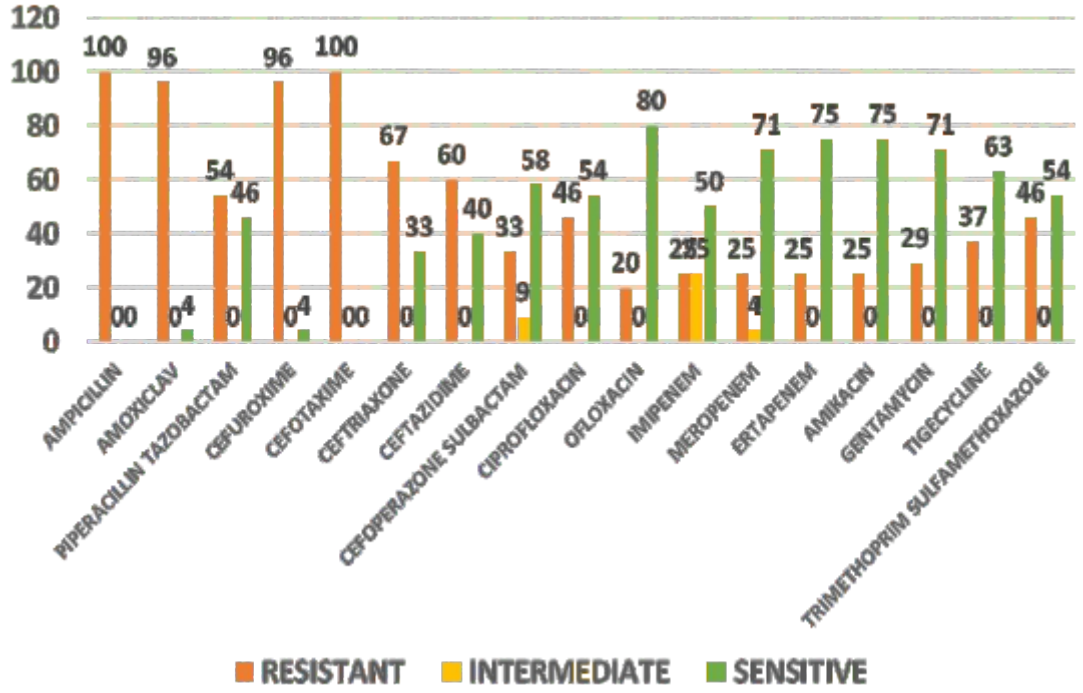

Figure 2: Antibiotic susceptibility pattern of clinical isolates of *Enterobacter spp*.

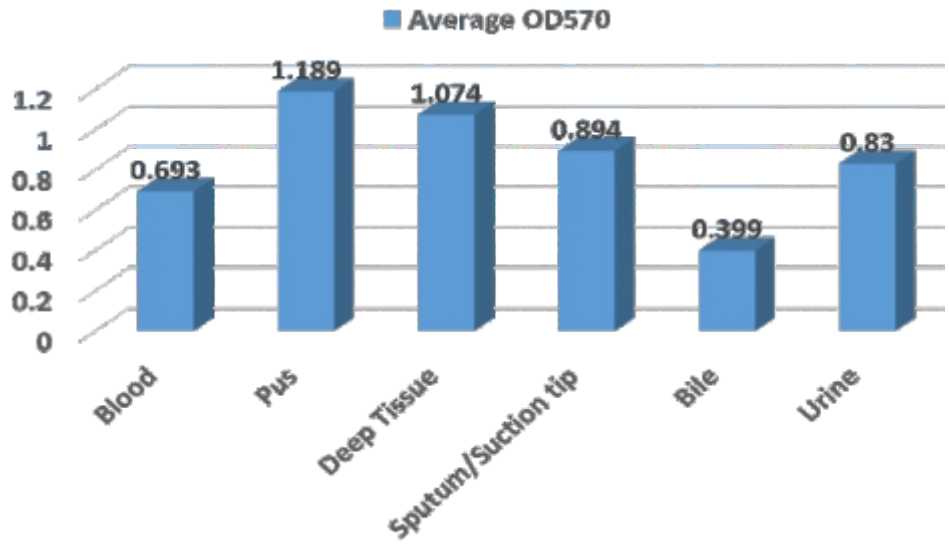

Figure 3: Production of biofilm by clinical isolates of Enterobacter species

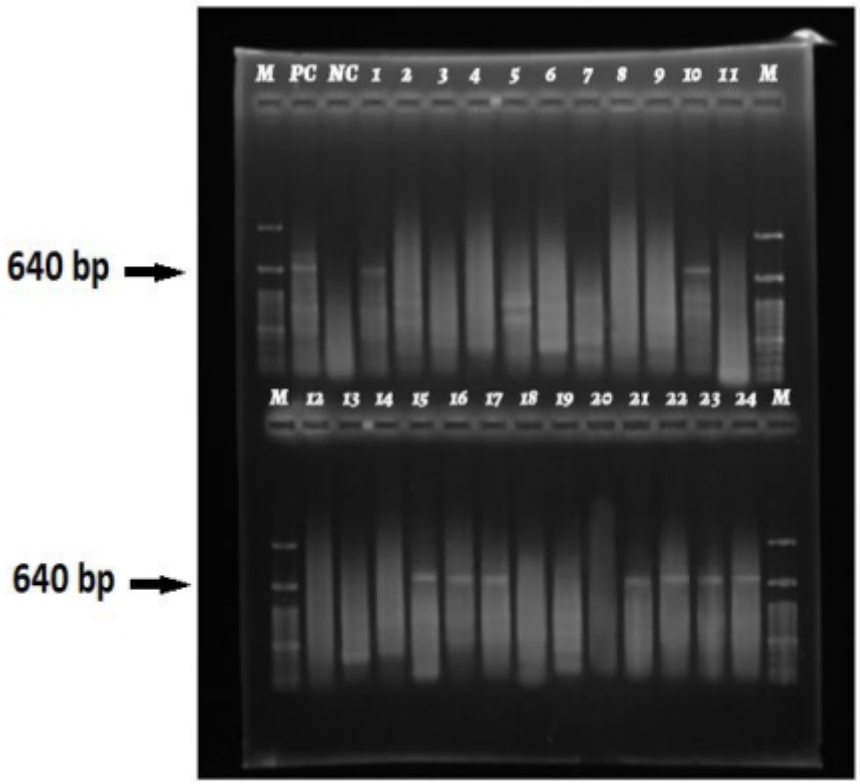

Figure 4: *Enterobacter* spp. *fimH* gene detection by conventional PCR

Presence of *film H* gene

Of the 24 isolates, 9 (37.5%) possessed the *fimH* gene. Those isolates that possessed *fimH* gene were strong biofilm producers with an average OD_{570} of 1.163 ± 0.293, while those that lacked *fimH* gene produced moderate to low levels of biofilm (OD_{570} of 0.9 ± 0.305). There was a strong correlation between *fimH* gene and biofilm formation $p < 0.05$. Figure 4 shows the results of *fimH* gene detection by conventional PCR.

Discussion

Enterobacter spp. is an important nosocomial pathogen causing a number of infections as well as bacteremia, particularly in patients with compromised host defenses [1, 2]. These drug-resistant bacteria causing nosocomial infections, are members of ESKAPE group of pathogens [2]. They easily acquire genes coding for drug resistance and virulence by acquiring mobile elements from other Enterobacteriaceae members. Emerging antibiotic resistance among *Enterobacter spp*. is a global concern due to the risk of treatment failure. Therefore, from a public health perspective, it is imperative to understand the mechanisms involved as well as methods to combat this problem of resistance in *Enterobacter spp*. [3,23].

In a particular Italian study, 445 strains of *Enterobacter spp.* were isolated in a span of 3 years from 2014 to 2017. *E. cloacae* (66%) and *E. aerogenes* (30%) were the predominant isolates.

Majority of them showed resistance to III and IV-generation cephalosporins and piperacillin-tazobactam. Isolates of the year 2017 were in addition, resistant to aminoglycosides and fluoro-quinolones [1]. In this study *Enterobacter* spp. isolates showed maximum resistance (60-100%) to beta-lactam drugs and 9 (37.5%) of the isolates showed resistance to tigecycline. Biofilms have a major role in antibiotic resistance, as they can lead to persistent infections. In a Brazilian study, *Enterobacter spp*. and *E.coli* were the major isolates from 30 cases of stage II pressure ulcers and most of them were biofilm producers [5]. In a particular epidemiological study, 17.3% of nosocomial infection cases isolated *Enterobacter spp*. and 44% were *Enterobacter aerogenes* with 50-56% of the isolates being biofilm producers and 58% of the isolates having the *fimH* gene. Also, they found that 28% and 22% of the isolates were extended spectrum beta-lactamase and metallo-beta-lactamase producers respectively [24]. In a study out of 105 urine samples, 24 yielded *Enterobacter spp*. of which 15 were *E. aerogenes* and 9 were *E. cloacae*. Out of the 24 isolates, 71% were biofilm producers and 75% possessed the *fimH* gene. They found a good association between biofilm production and presence of the *fimH* gene with 16 out of 17 biofilm producers possessing the *fimH* gene [25].

As per one of the studies conducted in India on 26 uropathogenic clinical isolates, 19 isolates possessed *fimH* gene. Further genetic studies were conducted on one of the isolates AK-118. They used the CRISPR tool to suppress the expression of the *fimH* gene and found that it reduces biofilm production by this isolate [26]. In a recent study conducted in the democratic republic of Congo, on clinical isolates of *Staphylococcus aureus* and Enterobacteriaceae from urinary tract and surgical site infection, generally, there was no correlation between biofilm production and drug resistance. But, out of the 8 *Enterobacter spp*. isolated, all were drug-resistant and biofilm formers [25]. Yet another study conducted on 130 clinical isolates of *Enterobacter cloacae* complex in China, the antibiotic susceptibility pattern, biofilm production and virulence genes were detected [27]. The strains were divided into 12 subgroups by partial gene sequencing based on the hsp60 gene, cluster analysis. Most of them belonged to clusters I, II, III, VI, VIII, and IX. Cluster VIII and IX were highly drug-resistant and possessed genes coding for virulence. Maximum biofilm-producing ability was seen in cluster II but it did not possess genes coding virulence. However, the study included detection of *fimA* gene and not *fimH* gene [28].

In a recent study conducted in Iran, out of 786 urine samples, 50 *E. aerogenes* strains were isolated. The isolates showed maximum resistance for co-trimoxazole, ampicillin, amikacin, tetracycline, kanamycin, erythromycin and nalidixic acid and maximum sensitivity for nitrofurantoin, imipenem, ceftriaxone. Drug resistance-conferring genes qnrC, qnrB, qnrA, tetA, tet B, acc, IIa, acc, IIa, ant, Ia and Su were detected in 100%, 80.95%,% 58.14, 87.5%, 81.58%, 86.67%, 82.14, 81.48% and 90% of the strong biofilm producing isolates, respectively. Virulence genes *csgA*, *ybtS*, *markD*, *rmpA*, *csgD* and *fimH* were detected in 84%, 83.33%, 80%, 80%, 80% and 66% of the strong biofilm-producing isolates, respectively. In brief, the correlation between the production of biofilm and

the presence of genes coding for virulence and drug resistance was significant [29, 30]. Similar results were found wherein most of the *Enterobacter spp*. isolates were multi-drug resistant and biofilm producers. The isolates that possess *fimH* gene were very strong biofilm producers.

Limitations of the study: The present study looked for the *fimH* gene coding for the type 1 fimbrial adhesin. Biofilm formation involves complex, multifactorial processes and many other genes and pathways also need to be studied. The study does provide a view regarding one gene, but suggests future scope for looking into the other genes and mechanisms involved in the process.

Conclusion

The majority of the *Enterobacter spp*. isolates were multidrug-resistant and were also producers of biofilms. Isolates possessing the *fimH* gene were strong biofilm producers. The study, therefore, concludes that the triad consisting of biofilm production, gene, and resistance plays a key role in making the organism an important nosocomial pathogen.

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