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Extended-spectrum beta-lactamases (ESBLs) detection in some uropathogenic bacteria and their correlation with biofilm formation

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Abstract

Background and objective: The tolerance of biofilms to antibiotics results in the dissemination of resistance. Many of the recurrent urinary tract infections are assumed to be caused by biofilm producing uropathogenic isolates. This study aimed to investigate the correlation between the ability of biofilm formation and extended spectrum beta lactamase producing uropathogens.

Methods: A total of 438 urine specimens were collected from Rizgary Teaching hospital in Erbil, Iraq, from September to December 2017. Extended spectrum beta lactamase was determined using the Vitek-2 automated system and confirmed by using the combination disk diffusion test. Biofilm formation was checked using 96-well flat bottom microtiter plates.

Results: Out of the 438 urine specimens, only 37.89% (n =166) developed an infection, the most common isolate was *Escherichia coli.* The distribution of the bacterial species according to the patients' gender found to be significant (*P* = 0.014). The proportion of betalactamase producing isolates was 29% (n = 31). The strength of biofilm formation among *Klebsiella* species was significantly higher than in *Escherichia coli* (*P* <0.001), while a non-significant difference (*P* = 0.163) was observed between beta lactamase production and ability of biofilm formation. The sensitivity and specificity of VITEK-2 in the detection of extended-spectrum beta-lactamase were 79.48% and 80.95%, respectively.

Conclusion: The study revealed that *Klebsiella* species were stronger biofilm producers. Beta lactamase producing isolates do not have a greater ability of biofilm formation.

Keywords: Biofilm; ESBL; *E. coli*; *Klebsiella pneumonia.*

Introduction

It is predicted that 150 million urinary tract infections (UTI) develop annually on a global level. As a consequence, approximately 6 billion dollars are spent on health care.[1] Different species of bacteria are able to cause infections, and in all populations, the majority of infections are caused by the gram negative uropathogenic Enterobacteriacae *Escherichia coli (E. coli)*. Other causative bacterial species with varying frequency include gram negative *Klebsiella* species (spp), *Pseudomonas aeroginosa*, *Proteus* spp, *Staphylococcus saprophyticus*, and *Streptococcus agalataciae*.[2,3] UTIs are the second most common cause for prescription of empirical antibiotics, which results as a leading cause for antibiotic usage and resistance. The World Health Organization has classified resistant bacteria according to critically of the need for new antibiotics into three groups; critical, high, and medium priority. Enterobacteriaceae extended spectrum β-lactamase enzymes (ESBLs) producing bacteria are classified within the critical priority.[4,5] Beta-lactams have been massively used since 1940s. They destroy a broad spectrum of bacteria and have very little toxicity to humans; therefore resistance to this group of antibiotics is a remarkable threat. It is reported that the most common site for ESBL production

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is UTIs.[6] Biofilm can be defined as an aggregation of bacterial cells that attach to each other or to a number of surfaces like medical devices. It is demonstrated that biofilm is difficult to eliminate and usually resistant to antibiotic levels that are 10-1000 greater than levels required to eradicate free living bacteria.[7-9] Studies indicate that about more than 60% of bacterial infections in developed countries are supposed to be biofilm producers. The tolerance of the structured communities to antimicrobial agents results in the dissemination of resistance.[7] Antibiotics fail to penetrate the polymeric matrix which surrounds the bacterial cells as a consequence the embedded bacterial cells have time enough to acquire resistance through horizontal gene transfer. Also, the high cell density within the biofilm contributes to gene transfer.[7,10] Many of the recurrent UTIs are assumed to be caused by biofilm producing uropathogenic isolates.[11] Several studies revealed a significant association between biofilm formation and ESBLs in *E. coli*, *P. aeruginosa* and *Acinetobacter baumanii* from different specimens.[12-14] This study aimed to investigate the correlation between biofilm formation and resistance in ESBLs, specifically in uropathogens, and to evaluate the proportion of ESBL producing isolates among *E. coli* and *Klebsiella* spp.

Methods

Study design and specimen Collection

In this cross-sectional study, a total of 438 urine specimens were collected from patients who attended the Urology Department, Rizgary Teaching Hospital in Erbil city with suspicion of having UTI, from September to December 2017. Aseptic morning mid-stream urine was collected from these patients in a sterile container. Patients under antibiotic treatment were excluded from the study.

Diagnosis of bacterial isolates

Urinalysis was done for detection of pyuria, with emphasis on the counting leukocyte ≥5 per HPF in the sediment after centrifugation at 3000 rpm for 5 minutes. All urine specimens showed positive pyuria were cultivated on MacConkey and blood agar. Following incubation at 37Cº for 24 - 48 hrs, the plates checked for bacterial growth. The patient indicated UTI when the plate counts showed a growth of 10^5 cfu/ml by adjusting the bacterial suspension to 0.5 McFarland tube.[15] The Identification and antibiotic sensitivity test were done using VITEK-2 automated system.

Biofilm assay

Biofilm formation was determined by the 96 well tissue culture plate (TCP) assay (Caplugs, Evergreen, USA), as described in other studies.[16] Briefly, 200 µL Luria Bertani (LB) broth was added to wells of a sterile 96-well flat bottom poly-styrene plate with a cover. All wells inoculated with 10 µL of bacterial culture adjusted to 0.5 McFarland tube. Following incubation at 37°C for 24 hrs, the cultures were re-moved, and the wells were washed twice with 200 µL of phosphate buffered saline (PBS, pH = 7.4) and dried at room temperature. Biofilms were stained with 0.1% crystal violet solution for 15 minutes, and the plates were washed in distilled water and dried at room temperature. The optical density (OD) of the bio-films was measured at 630 nm using an ELISA reader. For comparative analysis, the OD630 values were used to classify the degree of biofilm production for the bacterial isolates. Briefly, the cut-off OD (ODc) was classified as follows: OD < ODc = non biofilm producer; ODc < OD < 2 × Odc = weak biofilm producer; 2 × Odc < OD < 4 × Odc = moderate biofilm producer; and OD > 4 × Odc = strong biofilm producer.

Detection of extended spectrum beta lactamases

VITEK-2 test system

Each isolate was tested using the VITEK- 2 system with the ESBL test panel. The system determined in the first step the ESBL isolates according to the sensitivity

profile. The ones which demonstrated resistance to third generation cephalosporin and aminoglycosides were identified as ESBLs.[17] Quality control strains (*E. coli* ATCC 25922) was included in each run. The sensitivity and specificity for the VITEK- 2 test system were calculated against the results of the phenotypic methods described below.

Phenotypic confirmatory combination disk diffusion test (PCDDT)

The ESBL production was initially tested with the CLSI confirmatory test using both Cefotaxime CTX (30 mg) and Ceftazidime CAZ (30 mg) disks alone and in combination with Clavulanic acid CA (10 mg). The CTX or CAZ disk and CTX+CA or CAZ+CA disk (Becton Dickinson Company/ USA) were placed at a distance of 3 cm from each other on the inoculated Muller Hinton agar plates. Following incubation for 16-18 hours, the test was considered positive when an increase in the growth-inhibitory zone around either the CTX or the CAZ disk with CA was 5 mm or greater of the diameter around the disk containing CTX or CAZ.[17]

Statistical analysis

Data analysis was performed using the statistical package for the social sciences (version 23). Chi-square and Fischer's exact tests were used to evaluate any association between two variables. A *P* value equal to or less than 0.05 was considered statistically significant.

Ethical approval

Approval was obtained from the ethical committee at the College of Pharmacy, Hawler Medical University. Also, all the attended patients were asked for verbal consent.

Results

Out of the 438 mid-stream urine specimens, only 166 (37.89%) showed significant growth for UTI. A non significant association was detected between UTI and gender *P* = 0.391; 117 (39.26%) females developed infection compared to 49 (35%) males, as shown in Table 1.

Table 1: Prevalence of UTI and gender.

UTI	Female No. %	Male No. %	Total No. %	*P* value
Positive	117 (39.26)	49 (35)	166 (37.89)	0.391
Negative	181 (60.73)	91 (65)	272 (62.1)	
Total	**298 (100)**	**140 (100)**	**438 (100)**	

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Of the total 166 positive bacterial cultures, *E. coli* showed to be the most common isolate (95, 57.2%), followed by *Streptococcus agalactiae* (14, 8.4%), Staphylococci coagulase negative spp (12, 7.2%), and *Klebsiella pneumoniae* (11, 6.6%). The distribution of the bacterial species according to the patients' gender found to be significant (*P* = 0.014). However, only *K. pneumoniae* was significantly more prevalent in females than males (*P* = 0.034), as shown in Table 2.

Table 2: Association between gender and the bacterial species of the UTIs.

Bacterial species	Gender		Total	*P value*
	Female	Male		
	No. %	No. %	No. %	
E. coli	63 (53.8)	32 (65.3)	95 (57.2)	0.173
K. pneumonia	11 (9.4)	0 (0)	11 (6.6)	0.034
S. agalactiae	13 (11.1)	1 (2)	14 (8.4)	0.067
Staph. coagulase negative	9 (7.7)	3 (6.1)	12 (7.2)	1
Proteus mirabilis	3 (2.6)	0 (0)	3 (1.8)	0.555
Sphingomonas paucimobilis	3 (2.6)	2 (4.1)	5 (3)	0.632
Candida species	3 (2.6)	0 (0)	3 (1.8)	0.555
Salmonella species	2 (1.7)	0 (0)	2 (1.2)	1
Acinetobacter baumanii	1 (0.9)	1 (2)	2 (1.2)	0.504
Klebsiella oxytoca	1 (0.9)	0 (0)	1 (0.6)	1
Acinetobacter lwoffli	1 (0.9)	2 (4.1)	3 (1.8)	0.208
Staphylococcus intermedius	1 (0.9)	0 (0)	1 (0.6)	1
Enterococcus faecalis	1 (0.9)	0 (0)	1 (0.6)	1
Shigella species	1 (0.09)	0 (0)	1 (0.6)	1
Serratia fonticola	1 (0.9)	0 (0)	1 (0.6)	1
Pseudomonas aeroginosa	1 (0.9)	3 (6.1)	4 (2.4)	0.07
Pseudomonas fluoresence	1 (0.9)	1 (2)	2 (1.2)	0.504
Staphylococcus aureus	1 (0.9)	0 (0)	1 (0.6)	1
Enterobacter cloace	0 (0)	1 (2)	1 (0.6)	0.295
Citrobacter freundii	0 (0)	2 (4.1)	2 (1.2)	0.085
Yersinia pseudotuberculosis	0 (0)	1 (2)	1 (0.6)	0.295
Total	**117 (100)**	**49 (100)**	**166 (100)**	

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The study focused on the most common isolates of Gram negative bacteria as ESBLs producers. Consequently, *E. coli* and *K. pneumoniae* showed to be the majority of these isolates. The VITEK-2 method yielded results for 39 (36.8%) of total65 isolates as ESBL-producers. On the bases of the (PCDDT) method identified correctly 31 (29%) ESBL-produces, which means the VITEK-2 test yielded eight false positive results. Therefore, the sensitivity and specificity of VITEK-2 are 79.48% and 80.95%, respectively. Among 34 isolates of non-ESBL produces, TCP detected 18 (52.9%) non-biofilm producers, while most of the 31 isolates of ESBL-producers showed weak biofilm formation 17 (54.8%). The results found a non-significant difference between biofilm and ESBL production *P* = 0.163, as shown in Table 3. In total, 58 isolates of *E.coli*, 29 (50%)showed weak biofilm, while 4 (57.1%) of the total seven isolates of *K. pneumoniae* were moderate. Therefore, the correlation between biofilm formation and the bacterial species was highly statistically significant (*P* <0.001), as shown in Table 4.

Table 3: Correlation between biofilm formation and ESBL.

Biofilm formation	ESBL		Total	*P* value
	Positive	**Negative**		
	No.%	**No. %**	**No. %**	
Non-biofilm	9(29)	18(52.9)	27 (41.5)	0.163
Weak	17(54.9)	12(35.3)	29 (44.6)	
Moderate	4(12.9)	2(5.9)	6 (9.2)	
Strong	1(3.2)	2(5.9)	3 (4.6)	
Total	**31(100)**	**34 (100)**	**65 (100)**	

E. coli ATCC (25922) moderate biofilm formation

Table 4: Correlation between biofilm formation and bacterial species.

Biofilm formation	Bacterial isolates		Total	*P* value
	E.coli	***K. pneumoniae***		
	No. %	**No. %**	**No. %**	
Non-biofilm	25 (43.1)	2 (28.6)	27 (41.5)	<0.001
Weak	29 (50)	0 (0)	29 (44.6)	
Moderate	2 (3.4)	4 (57.1)	6 (9.2)	
Strong	2 (3.4)	1 (14.3)	3 (4.6)	
Total	**58 (100)**	**7 (100)**	**65 (100)**	

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Discussion

Phenotypic characteristics of pathogens and their correlation with biofilm and ESBL producing isolates in patients with UTI needs to have attention about since biofilm related infections are often associated with antibiotic resistance and difficult to screen them in routine diagnostic laboratories.[7] The precise detection of ESBLs in regular clinical laboratories remains a challenge for microbiologists. We compared two phenotypic methods, VITEK-2, and PCDDT as widely used methods specifically developed to detect ESBL production. The ability of the VITEK-2 system as a routine method to detect ESBL production was rather low, as the sensitivity and specificity were 79.48% and 80.95%, respectively. A previous study reported VITEK-2 sensitivity peaked to 92% ESBL detection in *E. coli* and *K. pneumoniae.*[18] Our results agreed with previous work reported, the ability of the combined disk method to detect ESBL was very satisfactory, and sensitivity can reach 100% when testing both cefotaxime and ceftazidime against isolates of Enterobacteriaceae.[19] On the bases of our results, the ESBL-producing isolates showed to be about 29%. Studies revealed a higher rate of ESBL in southern and eastern European countries than northern ones. On the other hand, researchers from Denmark reported a prevalence below 1%, while investigations from Turkey, Hungary, and Romaine, described the total proportion of ESBL production was over 10%.[20] The high rate of ESBL-isolates in the present study might have resulted from the overuse of broad spectrum antibiotics in the region and a consequence of prescribing antibiotics by physicians without an antibiotic sensitivity test. Out of the total ESBL isolates (n=31), about 23 were biofilm producers, 54.87% (n=17) showed to be weak, and 16.1% (n=5) were moderate or strong biofilm producers. Different studies indicated a significant association between biofilm formation and ESBL producers in isolates of *E. coli,*[14,21–23] *P. aeruginosa*[12] and *A. baumanii.*[13] The same results were found in the present study, even though the difference was not statistically significant (*P* = 0.163). A possible explanation for the findings is that *E. coli* can be classified into four commensals phylogenetic groups, two groups were considered as non-pathogenic while the other two groups were virulent.[24] However, the small sample size tested for biofilm formation (n=65) might be another justification. On the other hand, the present study showed that the association between biofilm formation and the bacterial species was highly significant (*P* <0.001), and the strength of biofilm was significantly higher among *K. pneumoniae*. In total, most of the *E. coli* isolates were weak biofilm producers 50% (n=29), while most of the *K. pneumoniae* isolates 71.4% (n=5) were moderate or strong biofilm producers. Similar results have been observed in a study from China that stated that biofilm forming *K. pneumoniae* strains had a greater ability of ESBL producing; from the 44.7% biofilm producer strains, 45.3% were ESBL producers.[25] A possible explanation for the results might be that *K. pneumoniae* strains harbor both type 1 and type 3 fimbriae however *E. coli* strains lack type 3 fimbriae, studies showed that type 3 fimbriae contribute in strong biofilm formation.[26] The incidence rate of UTI during the study was 37.84%, the most common UTI causative agent in the present study was *E. coli* with 57.2% which is in agreement with previous investigations.[27-30] In addition to that, a significant difference was observed between the bacterial species and gender (*P* = 0.014), and similar findings were observed in other studies.[31,32] The prevalence of UTI between males and females was notably different. One of the reasons is that the female urethra is shorter compared to the male urethra, which allows bacteria colonize to reach the bladder. The second cause is the urethral opening is proximate to both openings of the genital and intestinal tract, which

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shelters large bacterial communities.[33] The limitation of the study included that ESBLs and biofilm producing uropathogenic isolates were not confirmed by molecular methods. One of the strength is that per our knowledge it was the first study to investigate the correlation between biofilm production and ESBLs in Erbil city.

Conclusion

The incidence rate of UTI over three months was 37.89%. The most common isolate was *E. coli*. The distribution of the bacterial species according to the patients' gender found to be statistically significant. The strength of biofilm formation was significantly higher among *K. pneumoniae* strains. The study showed that ESBL- producing isolates did not have a significantly greater ability of biofilm formation.

Competing interests

The authors declare no competing interests.

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