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RESEARCH ARTICLE

Study of Various Virulence Genes, Biofilm Formation and Extended-Spectrum β-lactamase Resistance in *Klebsiella pneumoniae* Isolated from Urinary Tract Infections

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Abstract:

Objective:

The aims of the current study were to evaluate the capacity of *K. pneumoniae* isolated from hospital-acquired urinary tract infection to form biofilm, the relation of this capacity to various virulence genes and the prevalence of Extended Spectrum β-lactamases (ESBL) among these isolates by phenotypic and genotypic methods.

Material and Methods:

The study included 100 non-duplicate strains of *K. pneumoniae* isolated from 100 different urine samples from patients with hospital-acquired urinary tract infection. The isolated strains were studied for biofilm formation, ESBL production by phenotypic methods. Molecular studies were applied for the detection of ESβLs genes bla_{TEM}, bla_{SHV}, bla_{CTX-M} and for detection of virulence genes *fim*H, *uge, rmp*A, *mag A, wzy, kfa* and aerobactin genes.

Result:

The majority of the isolates had the capacity to form a biofilm (81%), with ESBL prevalence rate 41%. The most prevalent gene among ESBL producing *K. pneumoniae* was bla_{CTX-M} (73.2%) followed by bla_{SHV} (53.6%) and bla_{TEM} (51.2%). Among the virulence genes studied in *K. pneumoniae* isolates, the most prevalent gene was *fimH* (76%), *uge* (70%). There was significant association between ESBL production, and resistance to amikacin, cefepime, ceftazidime, gentamicin, imipenem and meropenem and biofilm production in *K. pneumoniae* isolates. There was significant association between bla_{CTX-M}, bla_{SHV}, *fim*H, *mag*, *kfa*, *wzy, rmp*A and aerobactin and biofilm production in *K. pneumoniae*.

Conclusion:

The present study highlights the prevalence of virulence genes among biofilm-forming strains of *K. pneumoniae* isolated from hospital-acquired urinary tract infection. Moreover, there was association between biofilm formation and ESBL production. Further studies are required to elucidate the clinical impact of the association of these different mechanisms.

Keywords: ESBL, Biofilm, *K. pneumoniae*, PCR, Virulence genes, Phenotypic methods.

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1. INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*), as a gram-negative bacillus, is known to be associated with various infections such as Urinary Tract Infection (UTI) especially hospital-acquired Catheter-Associated Urinary Tract Infection (CAUTI), sepsis and pneumonia [1, 2].

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There are many virulence factors in *K. pneumoniae* associated with its pathogenicity. Among these factors is the capacity of *K. pneumoniae* to form a biofilm that protects it from the host immune response as well as from the antibiotics [3, 4]. The formation of biofilm leads to the prolonged persistence of *K. pneumoniae* on the epithelium and on the medical devices with difficulty in its eradication. There are genes related to biofilm formation in *K. pneumoniae* such as capsular genes like *rmp*A gene associated with K1/K2 virulent

type, fimbriae genes such as *fim*H and *mrk*D that regulate fimbrial types I and 3, respectively and genes encoding the enzymes used for the biosynthesis of lipopolysaccharide such as *uge* gene that encodes for the uridine diphosphate galacturonate 4-epimerase [5, 6] and genes that regulate iron uptake by *K. peumoniae* including aerobactin and *kfu* genes [7]. Moreover, quorum sensing system especially type 2 and pga ABCD operon that regulates the synthesis and translocation of poly-b-1,6-N-acetyl-D-glucosamine adhesin are involved in the biofilm formation through enhancement of cell to cell communication and intercellular adhesion process [8].

Beside biofilm formation, clinical isolates of *K. pneumoniae* are known to possess resistance to third generation of cephalosporines through the production of Extended Spectrum β-Lactamase (ESBL).

Extended-spectrum beta-lactamase is associated with the mutation of genes that regulate β-lactamases mainly bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes. More than 300 different ESBLs variants have been described [9].

There are few reports about the association of biofilm and ESBL within isolates of *K. pneumoniae* from hospital-acquired urinary tract infection.

Therefore, the present study aimed to study the capacity of *K. pneumoniae* isolated from hospital-acquired urinary tract infection to form biofilm and to correlate this capacity to various virulence genes and finally detect the prevalence of ESBL among these isolates by phenotypic and genotypic methods.

2. MATERIALS AND METHODS

The study included 100 non-duplicate strains of *K. pneumoniae* isolated from 100 patients with hospital-acquired urinary tract infections from Mansoura University Hospital, Egypt as defined by CDC in a period from December 2016 till January 2018. The study was approved by Mansoura Faculty of Medicine ethical committee and approval was obtained from each participant.

2.1. Microbiological Culture

Urine samples from catheterized patients were collected as part of the routine microbiological investigation, after disinfection of the catheter collection part using 70% ethyl alcohol solution discarding the first few drops of urine and collecting 5-10 ml urine using a sterile syringe. Urine samples were immediately sent to the laboratory to be processed and examined within 2 hours. Quantitative urine culture was performed by the use of sterile calibrated loop with a culture of I micron of the urine onto MacConkey agar plate and incubation was performed at 37°C for 24-48 hours. The colonies were identified by gram staining and biochemical identification was performed by VITEK 2 system according to the instructions of the manufacturers. The isolated *K. pneumonia*e were subjected to antibiotics susceptibility testing by disc diffusion method, determination of ESBL done by combined disc diffusion method and study of biofilm formation by Microtiter plate method. Molecular studies were carried out for the detection of virulence genes and *bla* genes.

2.2. Antibiotic Susceptibility Testing

Antibiotic susceptibility was performed by discs diffusion method according to Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI 2018) [10]. The used discs were: amikacin, cefotaxime, ceftazidime, cefepime, ciprofloxacin, levofloxacin, gentamicin, piperacillin-tazobactam, trimethoprim/sulfamethoxazole, imipenem, and meropenem (Oxoid-UK). The interpretation of the results was done according to CLSI 2018 [10].

2.3. ESBL Production

Determination of ESBL by Combined disk diffusion confirmatory test.

Klebsiella pneumoniae strains resistant to ceftazidime (30 µg) and/ or cefotaxime (30 µg) were subjected to the screening of ESBL enzymes production by combined disk diffusion confirmatory test. The principle of the test depends upon the use of ceftazidime and cefotaxime discs alone and conjugated with clavulanic acid. The increase of the inhibition zone diameter around discs conjugated with clavulanic acid ≥5mm was interpreted as positive ESBL production.

The suspension of overnight growth of *K. pneumoniae* in Muller-Hinton broth was prepared with adjusted turbidity to 0.5 McFarland and was subculture on Muller-Hinton agar medium and antibiotic discs containing ceftazidime, ceftazidime (30 µg)+clavulanic acid (10 µg), cefotaxime, and cefotaxime (30 µg + clavulanic (10 µg) pairs were placed with 20 mm space between them. Plates were incubated at 37ºC for 24 hours. The increase of growth inhibition zone ≥ 5 mm around ceftazidime/clavulanic acid and cefotaxime/clavulanic compared to ceftazidime and cefotaxime respectively was regarded as an ESBL producing isolate [11]. The strain of *Klebsiella pneumoniae* (ATCC 700603) was used as a positive quality control strain for ESBL production.

2.4. Biofilm Assay

Biofilm formation by *K. pneumonia*e isolates was assessed by the semi-quantitative method [12].

Briefly, 0.5 McFarland suspensions of *K. pneumoniae* isolates were prepared using brain heart infusion broth (Oxoid-UK) and 100 microliters of the suspensions were inoculated onto wells of 96-well polystyrene plate and incubated at 37°C for 18 hours. Negative control well was inoculated with sterile brain heart infusion broth. Then, wells were stained with 200 microliters of crystal violet with concentration 0.5% for 20 minutes and the excess stain was washed by 200 microns sterile distilled water for three times. Then the stain was eluted with 200 microliters of 95% of ethanol and the optical density was measured at 570 nm by enzyme-linked immunoassay reader. The positive Optical Density (OD) was defined as three standard deviations above the mean OD of the negative control. The *K. pneumonia*e NTUH-K2044 strain was used as positive control.

2.5. Molecular Studies of *K. pneumoniae*

2.5.1. Extraction of DNA

DNA of the isolated *K. pneumoniae* was prepared by boiling method. Briefly, colonies were suspended in 100 microliters of sterile distilled water and boiled at 100ºC in the water bath for 15 minutes then rapidly cooled at -20°C for one hour, then centrifugation and the supernatant were preserved for the use in the amplification processes [13].

2.5.2. PCR for Virulence Genes of K. pneumoniae

PCR was performed for the detection of virulence genes for *K. pneumoniae*. The genes detected and the sequences of the used primers were summarized in Table **1**. The conditions of PCR were performed according to Shah *et al*., 2017 [13] for each gene. Negative controls were included as sterile distilled water and *K. pneumoniae* ATCC13883 was used as positive control. The products of the amplifications were analyzed by the use of gel electrophoresis 1.5% stained with ethidium bromide and the bands were visualized by UV.

2.5.3. Multiplex PCR for ESBL Genes

The extracted DNA was used for multiplex PCR for the detection of ESBL genes bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$. The sequences of the used primers were shown in Table **1**. The amplification steps were primary denaturation at 94°C for 4 minutes, then 35 cycles of amplification (94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute) with final extension at 72°C for 7 minutes. *K. pneumoniae* ATCC 7881 was used as a positive control. The products of the amplifications were analyzed by the use of gel electrophoresis 1.5% stained with ethidium bromide and the bands were visualized by UV [13].

2.5.4. Statistical Analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (SPSS) version 24. The quantitative data were presented as mean, standard deviations and ranges and qualitative data was presented by number and percentages. The comparison between qualitative data was done by the use of chi-square. P-value was considered significant if it was <0.05.

3. RESULTS

The study included non-duplicate 100 isolates of *K. pneumoniae*. The majority of the isolates had the capacity to form biofilm (81 isolates, 81%) with ESBL prevalence rate 41% (41 isolates). Forty-one isolates had the combined capacity to form biofilm and were ESBL, 40 isolates had the capacity to form biofilm and were non ESBL producers and 19 isolates were non ESBL and lack the capacity to form biofilm (Fig. **1**).

Table 1. Genes, sequences of the used primers and the bp of the used products.

Gene	Sequence	bp	Positive Control
*Fim*H-1	F: 5′-ATG AAC GCC TGG TCC TTT GC-′3 R: 5′-GCT GAA CGC CTA TCC CCT GC-′3	688	*E. coli* 700336
*mrk*D	F: 5′-CCA CCA ACT ATT CCC TCG AA -′3 R: 5′-ATG GAA CCC ACA TCG ACA TT-′3	240	*K. pneumoniae* ATCC BAA-2473
*mag*A (K1)	F: 5′-GGT GCT CTT TAC ATC ATT GC-′3 R: 5′-GCA ATG GCC ATT TGC GTT AG-′3	1282	*Klebsiella pneumoniae* subsp. *pneumoniae* Strain K6 ATCC 700603
wzy (K2)	F: 5′-GAC CCG ATA TTC ATA CTT GAC AGA G-′3 R: 5′-CCT GAA GTA AAA TCG TAA ATA GAT GGC-′3	641	*Klebsiella pneumoniae* subsp. *pneumoniae* Strain K6 [ATCC® 700603™
*rmp*A	F: 5′-ACT GGG CTA CCT CTG CTT CA -′3 R: 5′-CTT GCA TGA GCC ATC TTT CA-′3	535	*Klebsiella pneumoniae* 1100770
uge	F: 5′-TCT TCA CGC CTT CCT TCA CT-′3 R: 5′-GAT CAT CCG GTC TCC CTG TA-′3	534	*K. pneumoniae* ATCC BAA2473
kfu	F: 5′- GAA GTG ACG CTG TTT CTG GC -′3 R: 5′-TTT CGT GTG GCC AGT GAC TC-′3	797	*Klebsiella pneumoniae* subsp. *pneumoniae* (Schroeter) Trevisan ATCC13882
Aerobactin	F: 5′-GCA TAG GCG GAT ACG AAC AT-′3 R: 5′- CAC AGG GCA ATT GCT TAC C T-′3	556	*Klebsiella pneumoniae* subsp. *pneumoniae* Strain K6 [ATCC 700603
bla_{TEM}	F: 5′-ATA AAA TTC TTG AAG ACG AAA -′3 R: 5′- GAC AGT TAC CAA TGC TTA ATC -′3	1080	*K. pneumoniae* ATCC 7881
bla_{SHV}	F: 5′- GGG TTA TTC TTA TTT GTC GC -′3 R: 5′-TTA GCG TTG CCA GTG CTC-′3	930	*K. pneumoniae* ATCC 7881
$bla_{CTX\text{-}M}$	F: 5′-GCT ATG TGC AGT ACC AGT AA-′3 R: 5′-ACC AGA ATG AGC GGC GC-′3	585	*K. pneumoniae* ATCC 7881

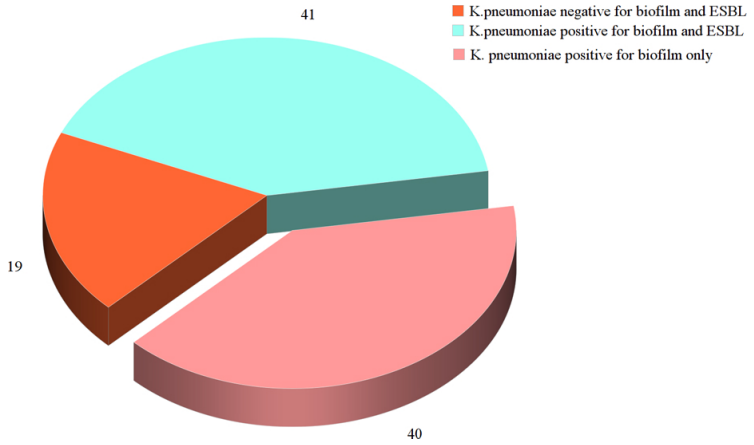

Fig (1). The frequency of ESBLs production and biofilm formation among isolated *K. pneumoniae*.

Fig (**2**). shows the optical density of biofilm formed by each isolate. The genes detected among ESBL producing *K. pneumoniae* was bla_{CTX-M} in 30 isolates followed by bla_{SHV} 22 isolates and bla_{TEM} in 21 isolates. Multiple genes were present in 25 of the isolates. Among the virulent genes' studies in *K. pneumoniae* isolates, the most prevalent gene was *fimH* (76%), *uge* (70%), *rmpA* (62%), *mag* (59%), *wzy* (59%) and aerobactin gene (51%). Lower prevalence was detected for and *kfa* gene (33%).

There was significant association between ESBL production, and resistance to amikacin, cefepime, ceftazidime, gentamicin, imipenem and meropenem and biofilm-producing *K. pneumoniae* data not shown. There was a significant association between bla_{CTX-M}, bla_{SHV}, *fimH,magA*, *kfa*, *wzy, rmpA* and aerobact and biofilm-producing *K .pneumoniae* (Table **2**).

Table 2. Comparison between biofilm producing and non-biofilm producing *K. pneumoniae* species regarding genes associated with ESBLs production and other virulence genes.

ESBLs and Other Virulence Genes	Biofilm forming *K. pneumoniae*(n=81) No. (%)	Non-Biofilm forming *K. penumoniae* (n=29) No. (%)	*P*
bla_{TEM}	12 (14.8%)	9 (31%)	*P*=0.007
bla_{SHV}	11 (13.6%)	11 (37. 9%)	*P*=0.005
bla_{CTX-M}	30 (37%)	0 (0%)	*P*=0.0002
fimH	72 (88.9%)	4 (13.8%)	*P*=0.0001
magA	49 (60.5%)	10 (34.4%)	*P*=0.0001
wzy	49(60.5%)	10(34.4%)	*P*=0.0001
rmpA	58 (71.6%)	4 (13.8%)	*P*=0.0001
uge	59 (72.8%)	11 (37.9%)	*P*=0.2
kfa	33 (40.7%)	0 (0%)	*P*=0.0001
aerobact	48 (59.3%)	3 (10.3%)	*P*=0.0001

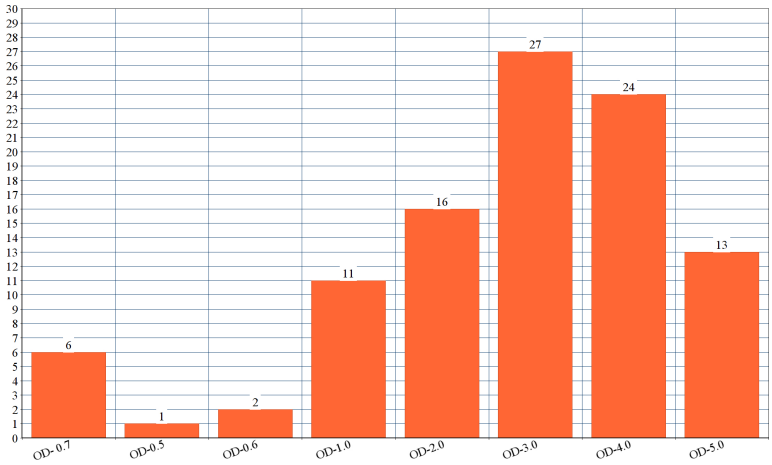

Fig. (2). Distribution of OD of biofilm formation among isolated *K. pneumoniae*.

4. DISCUSSION

Klebsiella pneumoniae has emerged as an important pathogen causing around 20% of infections in the hospitals worldwide [14].

There are several factors that increase the virulence of *K. pneumoniae*, of which the capacity to form biofilm is prevalent. The majority of the isolates in the present study had the capacity to form biofilm (81%). This finding was similar to the previous report concerning isolates from urinary tract infections [15]. The formation of biofilm protects *K. pneumoniae* species against the host immune responses, the action of antibiotics and enhance its persistence [16, 17].

One of the other factors associated with infection by *K. pneumoniae* is the emergence of resistance to antibiotics by different mechanisms. The common mechanism is ESBL production. In the present study, the prevalence of ESBL production was 41%. The prevalence of ESBL production among *K. pneumoniae* species varied from 30% up to 65% in different studies [18, 19]. The variations of the prevalence of ESBL depend upon the empirical use of antibiotics in local regions with positive pressure on *K. pneumoniae* leading to the selection of resistant strains.

The most prevalent genes among ESBL producing *K. pneumonia*e were $bla_{\text{CTX-M}}$ followed by bla_{SHV} and bla_{TEM}. Several reports had a similar finding with $bla_{\text{CTX-M}}$ was the most prevalent gene followed by bla_{TEM} and bla_{SHV} [20, 21]. The high prevalence of $bla_{\text{CTX-M}}$ among *K. pneumoniae* may represent selective pressure due to the wide prescriptions of cephalosporins, particularly cefotaxime and ceftriaxone in many geographical regions including Egypt [22]. However, the prevalence rates vary not only between different countries but even between different hospitals in the same country [23].

The ESBL enzymes are encoded by plasmid genes, therefore, it is expected for one isolate to carry different genes as the mixed genes were present in 65.8% of the isolates in our study. Similar findings were reported by [20]. Further studies are required to understand the mechanisms mediated by multiple *bla* genes on the same isolate.

The present study revealed that the most prevalent virulence genes among *K. pneumoniae* species were *fimH* (76%), *uge* (70%), *rmpA* (62%), *mag* (59%) and*wzy* (59%) and the lowest prevalence was detected for aerobactin gene (50%) and *kfa* gene (33%). Similar prevalence rates were reported for *fimH*, *uge* and *rmpA* genes [23, 24]. The prevalence of aerobactin gene varies in different studies from 3% up to 92.8% [25, 26]. The difference in the prevalence of virulence genes appears to be associated with the difference of the clinical sources of the isolated *K. pneumoniae* species.

Several studies demonstrated an association between the aerobactin gene and the regulatory genes of mucous in *K. pneumoniae* [26, 27]. There are cumulative data that the virulence of *K. pneumoniae* is enhanced by the presence of aerobactin due to the presence of other multiple virulence genes on the same plasmid carrying it [27].

There was a significant association between resistance to amikacin, cefepime, ceftazidime, gentamicin, imipenem and meropenem and biofilm production by *K. pneumoniae*. The finding was explained by several mechanisms such as the adherent bacterial cells are resistant to the actions of different antibiotics due to the limitation of the penetration of the antibiotics, beside the slow growth of the bacterial cells inside the biofilm [16]. Moreover, the exchange of plasmids that carry antibiotics resistance genes between contact microbial cells may play role [28, 29]. ESBL production by isolated *K. pneumoniae* species was significantly associated with biofilm formation. This finding was similar to previous reports [29, 30]. Again, this finding can be explained by the formation of protective environment to *K. pneumoniae* away from the antibiotics and the transmission of resistant genes. This was confirmed in the present study by significant association of bla_{SHV} and $bla_{\text{CTX-M}}$ genes with biofilm formation.

Virulence genes of *K. pneumoniae*, *fimH, magA, kfa, rmpA* and aerobactin had a significant association with biofilm formation. Several studies correlated the association of genes of fimbriae, capsule and lipopolysaccharides with the biofilm formation by *K. pneumoniae* through initial adhesion by fimbriae and lipopolysaccharide followed by coverage of substratum and construction of mature biofilm architecture by the capsule [31, 32].

CONCLUSION

The present study highlights the prevalence of virulence genes among biofilm-forming strains of *K. pneumoniae* isolated from hospital-acquired urinary tract infections. Moreover, there was an association between biofilm formation and ESBL production. Further studies are required to elucidate the clinical impact of the association of these different mechanisms.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by Mansoura Faculty of Medicine ethical committee, Egypt.

HUMAN AND ANIMAL RIGHTS

No Animals were used in this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was obtained from all the participants prior to publication.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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