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ORIGINAL ARTICLE

Klebsiella pneumoniae in Patients with Acute Exacerbation of Chronic Obstructive Pulmonary Disease in Menoufia University Hospitals

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Key words:
Chronic obstructive pulmonary disease (COPD), Acute Exacerbation of COPD (AECOPD), Plasmid mediated quinolone resistance (PMQR)

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ABSTRACT

Background: *Klebsiella pneumoniae is an important etiological agent of Acute Exacerbation of Chronic Obstructive Pulmonary Disease (AECOPD) which is a progressive, debiliting lung disease with multiple co-morbidities. Infections with multi-drug resistant (MDR) K. pneumoniae strains are usually associated with high morbidity and mortality, long hospital stay and high healthcare costs.* ***Objectives:*** *to determine the incidence of aerobic bacteria isolated from patients with COPD, to detect plasmid-mediated quinolones resistance genes (PMQR); qnrA1-6, qnrB1-3, 5, 6, 8, qnr B4 and qnrS1-2 among K. pneumoniae isolates and to investigate the relation between antimicrobial susceptibility patterns and biofilm production.* ***Methodology:*** *The study enrolled 25 patients with stable COPD (group 1), 50 patients with AECOPD (group 2) and 25 patients with other chest diseases as control group (group 3). K. pneumoniae isolates were identified by standard microbiological methods and their antibiograms were tested by the modified Kirby Bauer disk diffusion method. Biofilm production was detected by congo red agar and modified congo red agar methods. Quinolone resistance and distribution of PMQR encoding genes were detected by multiplex PCR.* ***Results:*** *Klebsiella spp. represented 35.4% of all isolates from the studied groups. By vitek-2system, K. pneumoniae was the predominant spp. (94.3%). K. pneumoniae isolates were highly resistant to Amoxicillin (84.9%), Amoxicillin/clavulanic acid (75.8%) followed by ciprofloxacin (63.6%) and levofloxacin (57.6%). Biofilm production was found among78.8% and 84.8% by CRA and MCRA methods respectively. About 57.6% of K. pneumoniae isolates had PMQR genes by Multiplex PCR.* ***Conclusion:*** *The presence of quinolone resistance in the majority of K. pneumoniae isolates emphasizes the need for establishing tactful policies associated with infection-control measures to prevent dissemination of the multidrug resistant strains*

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common chronic and slowly progressive inflammatory disease of the airway**.** Acute exacerbations of COPD are associated with substantial morbidity and mortality**.** *K.pneumoniae* is an important Gram-negative organism that commonly play pathogenic role in AECOPD[1] This opportunistic pathogen can generate a thick layer of biofilm enabling the bacteria to attach to living or abiotic surfaces, to trap of antibiotics and impair of drugs and plasmid exchange which contributes to drug resistance [2]**.**

Quinolones are among the most commonly administered antimicrobials commonly used for treatment of serious infections caused by *K. pneumoniae*. Plasmid-mediated quinolone resistance (PMQR) can arise from the expression of proteins encoded by the qnrA, -B, and -S genes that are able to protect the DNA gyrase [3]. The current study aimed to determine the incidence of multi-drug resistant *K. pneumoniae* isolated from patients with COPD, to detect biofilm-producing *K. pneumoniae* and to investigate the presence of PMQR genes using multiplex PCR.

METHODOLOGY

Collection of samples:

This study was conducted over the period from November 2016 to October 2017 and its protocol was approved by the local ethics committee of the Menoufia University. This study included three groups; group (1), 25 patients with stable COPD were presented to Chest Out patient's Clinic at Menoufia University Hospital [MUH} (with no history of exacerbations in last three months), group (2), 50 patients with AECOPD and group (3), 25 patients with different chest diseases as a control group admitted to Chest departments at MUH. All the selected patients were subjected to full history taking and thorough clinical examination. The Global

Initiative for Chronic Obstructive Lung Disease (GOLD) classification was used for evaluating disease severity [4].

One hundred sputum samples were evaluated based on Bartlett's grading system

Bartlett's criteria used[5]:

No. of Neutrophils /10 X LPF	Grade
<10	**0**
10-25	**+1**
>25	**+2**
Presence of mucus	**+1**
No. of Epithelial Cells /10 X LPF	
10-25	**-1**
>25	**-2**

A final score of 0 or less indicated contamination (non-acceptable sample).

A score of 1 and above indicated an acceptable sample.

Identification of *K. pneumoniae* isolates (Acceptable sample):

Sputum samples were processed through Gram staining and inoculation onto nutrient, blood, chocolate, MacConkey's and mannitol agars (Oxoid, UK) and incubated aerobically at 37 °C for 24 hours.The grown *K. pneumoniae* isolates were identified by standard methods [6] and Vitek -2 compact system**:** (bioMerieux, France) [7]. Confirmed *K. pneumoniae* isolates were stored in nutrient broth with 16% glycerol and frozen at -80°C.

Antimicrobial susceptibility testing:

Disk diffusion method*:*

The disk diffusion method was performed for isolated *K. pneumoniae* against antimicrobial agents (Oxoid) that recommended by CLSI,[8]; amoxicillin (AML, 20µg), amoxicillin /clavulanate (AMC, 30µg) (20µg amoxicillin +10 µg clavulanic acid), piperacillin/tazobactam (TZP, 100/10µg), cefoxitin (FOX,30µg), cefepime (FEP ,30µg), cefotaxime (CTX ,30µg), ceftriaxone (CRO ,30µg), ceftazidime (CAZ ,30µg), imipenem (IPM,10µg), meropenem (MEM,10µg), ertapenem (ETP,10µg), aztreonam (ATM,30µg), gentamicin (CN,l0µg), amikacin (AK,30µg), tobramycin (TOB,10µg), Doxycycline (DO,30µg), ciprofloxacin (CIP,5µg), Levofloxacin (LEV) (5 µg), trimethoprim-sulfamethoxazole (TMP/ SMX, l,25µg - 23.75µg)**.**

Minimal inhibitory concentration (MIC) of ciprofloxacin and levofloxacin by agar dilution method:

K .pneumoniae isolates were tested for MIC of ciprofloxacin and levofloxacin by agar dilution method as recommended by CLSI [8].

Detection of biofilm production by *K.pneumoniae* isolates:

- Biofilm formation assay was determined by Congo red agar and modified Congo red agar methods

Congo red agar (CRA) method [9]:

Phenotypic characterization of biofilm production was performed by culturing of *K.pneumoniae* isolates on CRA plates aerobically for 24 h at 37°C. Isolates presenting two tons of black, bright black (BB) or dry opaque black (OB), were classified as biofilm producers (positive), whereas red, pink or Bordeaux colonies were classified as negative. Red and Bordeaux colonies arose in the center of black colonies should be subcultured onto CRA to obtain pure isolates of the producer and non-producer variants**.**

Modified Congo red agar (MCRA) method[9]:

The modifications include changing the concentration of Congo red dye and sucrose, omission of glucose and replacement of BHIA by an alternative agar, Blood Base Agar-2 (BAB-2). **{9}**

Detection of PMQR genes by multiplex PCR:

This table illustrates sequences of specific primers for PMQR genes (qnrA1-6, qnrB1-3, 5, 6, 8, qnr B4 and qnrS1-2) [10]:

Primer name	Sequence	Annealing Temp (_C)	Product size(bp)
qnrA1-6	**F:**ACG CCAGGATTTGAGTGAC **R:** CCAGGCACAGATCTTGAC	**53**	**571**
qnrB1-3, 5, 6, 8	**F:**GGCACTGAATTT ATCGGC **R:**TCCGAATTGGTCAGATCG	**49**	**594**
qnrB4	**F:**AGTTGTGATCTCTCCATGGC **R:** CGGATATCTAAATCGCCCAG	**53**	**594**
qnrS1-2	**F**:CCTACAATCATACATATCGGC **R:** TTCGAGAATCAGTTCTTGC	**53**	**388**

Plasmid DNA was extracted using Thermo Scientific gene JET™ genomic DNA Purification kit. The PCR program involved an initial denaturation at 94°C for 5min, followed by repeated 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing for 1min, and primer extension at 72°C for 1.5min. The optimum annealing temperature was determined for each primer pair in the optimization step.

Finally, an extended 72°C step for 10min was recorded to ensure that all of the products are full-length**.** For both multiplex PCR reactions, each reaction mixture (25 µl) consisted of 25µl Taq green PCR Master Mix, 1µl forward primer, 1µl reverse primer (Qiagen, Germany), 1µl template DNA in addition to 22µl nuclease-free distilled water. The PCR programs were performed in a thermal cycler (Biometra, Germany). The amplified DNA products were detected on 1.5% agarose gels by ethidium bromide staining (Sigma, USA). A DNA ladder (100-1000bp) (Fermentas, Germany) was used to estimate allele sizes in base pairs (bp) for the gel.

Statistical analysis:

Computer SPSS program version 17 was used. The results were expressed as ranges and mean± SD. Chisquare test was done and p value <0.05 was considered as significant.

RESULTS

The mean age of group (1) (stable COPD) and group (2) (AECOPD) were 55.8±7.9 and 61.1±6.7 respectively with a high statistically significant difference (P<0.001). Males were more affected than females in AECOPD (82.0%) and stable COPD (68%) compared to other different chest diseases (group III) with a high statistically significant difference (P<0.001).

Smoking and associated Co- morbidities were a highly statistically significant risk factor for stable COPD and AECOPD. Also, higher rates of exacerbation (52%) were found in admitted patients to hospital with a high statistically significant difference (P<0.001). There was a high statistically significant difference between AECOPD and different chest diseases regarding a history of antibiotic administration (p<0.0001) (table 1).

Table 1: Socio-demographic and clinical characteristics of the studied groups:

Socio-demographic and clinical characteristics	Group I (No. =25)	Group II (No. =50)	Group III (No. =25)	Test of significance	P value	Post hoc test
Age (years)						P1=0.003*
Mean±SD	55.8±7.9	61.1±6.7	44.0±4.2	**ANOVA =**	**<0.001****	P2<0.001**
Range	42 – 68	52 – 75	38 – 50	57.09		P3<0.001**
Gender	**No. (%)**	**No. (%)**	**No. (%)**	**χ^2 test=**		P1=0.17
Male	17 68.0	41 82.0	9 36.0	15.96	**<0.001***	P2=0.02*
Female	8 32.0	9 18.0	16 64.0			P3<0.001**
Smoking-index	**(No.= 16)**	**(No.= 36)**	**(No.= 4)**	**Kruskal Wallis test**=27.46	**<0.001****	P1<0.001**
Mean±SD	265.6±99.5	462.5±168.8	250.0±70.7			P2=0.77
						P3=0.12
Associated comorbidities	**No. (%)**	**No. (%)**	**No. (%)**			P1=0.08
Yes	14 56.0	38 76.0	11 44.0	**χ^2 test=**	0.02*	P2=0.39
No	11 44.0	12 24.0	14 56.0	8.02		P3=0.006*
Previous hospital admission	**No. (%)**	**No. (%)**	**No. (%)**	**χ^2 test=**		P1=0.001** P2=0.04*
Yes	3 12.0	26 52.0	9 36.0	11.37	**0.003***	P3=0.19
No	22 88.0	24 48.0	16 64.0			
Antibiotic administration						P1=0.02*
Yes	9 36.0	32 64.0	3 12.0	**χ^2 test=**	**<0.001****	P2=0.05*
No	16 64.0	18 36.0	22 88.0	19.16		P3<0.001**

*significant difference **highly significant difference

P1--- Group I versus II P2--- Group I versus III P3--- Group II versus III

Smoking Index: cumulative smoking exposure quantitatively.

A total of 97/100 specimens (97%) showed positive cultures and only **2** specimens were mixed (2 isolates for each) as shown in table 2. The most predominant isolates in AECOPD were *Klebsiella* spp. representing 27.3% from all isolates (*K. pneumoniae* 94.3% and *K. oxytoca* 5.7% by Vitek2 system), followed by *S. aureus* (12.1%) *and P aeureginosa (*9.1%).

Table 2: Distribution of isolated organisms among the studied groups:

Sputum culture (n=100)								
Growth (positive culture)			97		97%			
No growth (Negative culture)			3		3%			
Isolated bacteria (n=99)	**Group I**		**Group II**		**Group III**		**Total**	
	No.	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**
S. aureus	13	(13.1)	12	(12.1)	4	(4.0)	29	29.3%
S. pneumoniae	6	(6.1)	1	(1.0)	8	(8.1)	15	15.1%
***Klebsiella* spp.**	2	(2.0)	27	(27.3)	6	(6.1)	35	35.4%
• ***K. pneumoniae*(33)**	2	(2.0)	25	(75.7)	6	(6.1)		
• ***K. oxytoca*(2)**	0	(0.0)	2	(100)	0	(0.0)		
P. aeruginosa	0	(0.0)	9	(9.1)	4	(4.0)	13	13.1%
E. coli	4	(4.0)	1	(1.0)	0	(0.0)	5	5.1%
***Acinetobacter* spp.**	0	(0.0)	2	(2.0)	0	(0.0)	2	2.0%
Total	**25**	**(25.3)**	**52**	**(52.5)**	**22**	**(22.2)**	**99**	**100**

A highly significant relation was found between severity of COPD (according to GOLD classifications) and *K. pneumoniae* isolation. In group I, *K. pneumoniae* was isolated only from patients with very severe stage (2/27, 7.4%).while, in group II, *K. pneumoniae* was isolated in all stages, but the highest rate in severe stage of AECOPD (70.4%) as shown in table 3.

Table 3: Relation between *K. pneumoniae* isolates and GOLD Classification of group I and II

GOLD classifications of group I and II		***K. pneumoniae* among group I and II (No. =27)**		**χ^2 test**	**P value**
		No.	**%**		
Group I (No. =25)	**Mild (No. =4)**	0	0	27.00	**<0.001****
	Moderate (No. =4)	0	0		
	Severe (No. =8)	0	0		
	Very severe (No. =9)	2	7.4		
	Total	**2**	**7.4**		
Group II (No. =50)	**Mild (No. =4)**	1	3.7		
	Moderate (No. =12)	5	18.5		
	Severe (No. =34)	19	70.4		
	Total	**25**	**92.6**		

K. pneumoniae was highly resistant to amoxicillin (28/33, 84.9%), amoxicillin/clavulanic acid (25/33 75.8%), followed by ciprofloxacin (21/33, 63.6%) and levofloxacin (19/33. 57.6%). On the other hand, *K. pneumoniae* was sensitive to meropenem (22/33, 66.7%) followed by imipenem (20/33, 60.6%) and ertapenem (18/33, 54.5%) as shown in Table 4.

Table 4: Antimicrobial susceptibility pattern of 33 *K. pneumoniae* isolates by disc diffusion method

Antibiotic groups	Antibiotic	Disk content	*K. pneumonia* (No. =33)		
			S No. (%)	I No. (%)	R No. (%)
Penicillins	Amoxicillin **(AML)**	**20 µg**	3 (9.0)	2 (6.1)	28 (84.9)
	Amoxicillin/clavulanic acid **(AMC)**	**20/10 µg**	3 (9.0)	5 (15.2)	25 (75.8)
	Piperacillin/tazobactam **(TZP)**	**100/10µg**	8 (24.2)	4 (12.1)	21 (63.6)
Cephalosporins	Cefoxitin **(FOX)**	**30 µg**	17 (51.5)	2 (6.1)	14 (42.4)
	Ceftazidime **CAZ)**	**30 µg**	20 (60.6)	0 (0.0)	13 (39.4)
	Cefotaxime **(CTX)**	**30 µg**	18 (54.5)	4 (12.1)	11 (33.3)
	Ceftriaxone **(CRO)**	**30 µg**	17 (51.5)	3 (9.1)	13 (39.4)
	Cefepime **(FEP)**	**30µg**	14 (42.4)	5 (15.2)	14 (42.4)
Monobactam	Aztreonam **(ATM)**	**30 µg**	21 (63.6)	0 (0.0)	12 (36.4)
Carbapenems	Ertapenem **(ETP)**	**10 µg**	18 (54.5)	4 (12.1)	11 (33.3)
	Imipenem **(IPM)**	**10 µg**	20 (60.6)	1 (3.0)	12 (36.4)
	Meropenem**(MEM)**	**10 µg**	22 (66.7)	0 (0.0)	11 (33.3)
Aminoglycosids	Amikacin **(AK)**	**30 µg**	14 (42.4)	3 (9.1)	16 (48.5)
	Gentamicin **(CN)**	**10 µg**	16 (48.5)	0 (0.0)	17 (51.5)
	Tobramycin **(TOB)**	**10 µg**	18 (54.5)	2 (6.1)	13 (39.4)
Quinolones	Ciprofloxacin **(CIP)**	**5 µg**	12 (36.4)	0 (0.0)	21 (63.6)
	Levofloxacin**(LEV)**	**5 µg**	14 (42.4)	0 (0.0)	19 (57.6)
Tetracyclines	Doxycycline **(DO)**	**30 µg**	19 (57.6)	4 (12.1)	10 (30.3)
Anti-metabolites	Trimethoprim/Sulfamethoxazole (Co-trimexazole) **(TMP)**	**1.25/23.75 µg**	19 (57.6)	0 (0.0)	14 (42.4)

S: susceptible R: resistant I: intermediate

Biofilm production was detected among 78.8% and 84.8% of *K. pneumoniae* isolates by CRA and MCRA methods respectively and about 96% of *K. pneumoniae* isolates were in group (2) (AECOPD) as shown in Fig 1, Fig. 2 and Table 5

Table 5: Number and percent of biofilm formation among *K. pneumoniae* isolates by Congo Red Agar (CRA) and Modified Congo Red Agar (MCRA)

K. pneumoniae isolates No=33	Biofilm formation								χ^2 test	P value
	CRA				MCRA					
	Positive		Negative		Positive		Negative			
	No	%	No	%	No	%	No	%	0.41	0.52
	26	78.8	7	21.2	28	84.8	5	15.2		

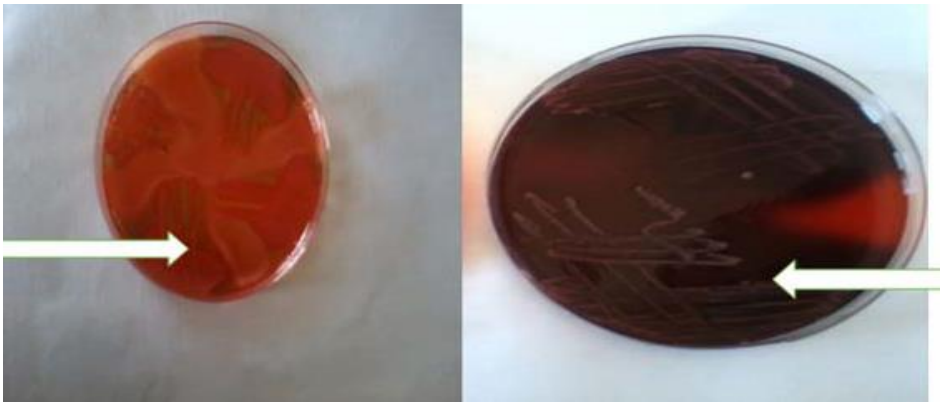

Fig. 1: Modified Congo Red agar plate (MCRA) showing biofilm formation. A) Red colored colonies-negative for biofilm; B) Dry black crystalline colonies-positive for biofilm

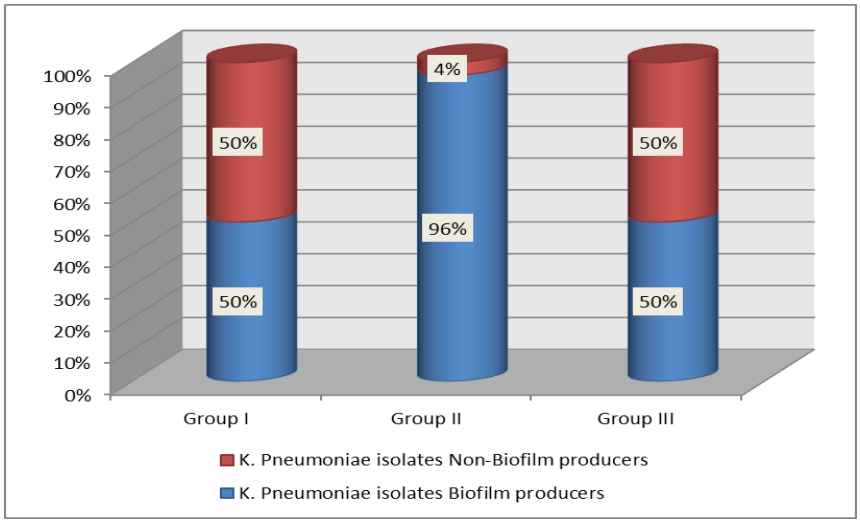

Fig. 2: Biofilm Formation in *K. pneumoniae* isolates by MCRA

A significant relationship was detected between biofilm production and multidrug resistance, where 89.3%, 75.0% and 67.9% biofilm forming *K. pneumoniae* strains were markedly resistant to amoxicillin/clavulanic acid, ciprofloxacin and levofloxacin respectively as shown in table 6.

Table 6: Antibiotic resistance pattern of biofilm-producing and non-biofilm producing *K. pneumoniae* isolates as determined by Modified Congo red agar (MCRA)

Resistance to antimicrobial agents by disk diffusion method	MCRA				Z test	P value
	Biofilm producers (No. =28)		Non-biofilm producers (No. =5)			
	No.	%	No.	%		
Amoxicillin/clavulanic acid (25)	25	89.3	0	0.0	3.73	<0.001**
Piperacillin/tazobactam (21)	20	71.4	1	20.0	1.70	0.09
Cefoxitin (14)	13	46.4	1	20.0	0.61	0.54
Ceftazidime (13)	13	46.4	0	0.0	1.46	0.14
Cefotaxime (11)	10	35.7	1	20.0	0.17	0.86
Ceftrioxone (13)	13	46.4	0	0.0	1.46	0.14
Cefepimen (14)	12	42.9	2	40.0	0.37	0.71
Aztronam (12)	12	42.9	0	0.0	1.33	0.18
Ertapenam (11)	11	39.3	0	0.0	1.20	0.23
Imipenam (12)	12	42.9	0	0.0	1.33	0.18
Meropenam (11)	11	39.3	0	20.0	1.20	0.23
Amikacin (16)	16	57.1	0	0.0	1.87	0.06
Gentamycin (17)	16	57.1	1	20.0	1.04	0.29
Tobramycin (13)	12	42.9	1	20.0	0.47	0.64
Ciprofloxacin (21)	21	75.0	0	0.0	2.71	0.007*
Levofloxacin (19)	19	67.9	0	0.0	2.34	0.02*
Doxycycline (10)	9	32.1	1	20.0	0.01	0.99

*Significant difference **highly significant difference

Only 15/33 *K. pneumoniae* isolates were susceptible to both ciprofloxacin and levofloxacin by MIC but only one isolate had qnrB1-3, 5, 6, 8 gene by multiplex PCR. In 18/33 resistant *K. pneumoniae* isolates to both ciprofloxacin and levofloxacin by MIC, qnrB1-3, 5, 6, 8 (as a single gene) was presented only in 6 *K. pneumoniae* isolates by multiplex PCR. While combined genes qnrA1-6 & qnrB1-3, 5, 6, 8 and **qnrA1**-6 & qnrB1-3, 5, 6, 8 & qnrS1-2 were detected in 6 *K. pneumoniae* isolates by multiplex PCR (Table 7)

Table 7: Relation between PMQR genes detected by multiplex PCR and ciprofloxacin and levofloxacin susceptibility pattern by MIC of *K. pneumoniae* isolates

Genotype	*K. pneumoniae* isolates (No.= =33)			
	Ciprofloxacin and levofloxacin susceptible isolates (No.=15)		Ciprofloxacin and levofloxacin resistant isolates (No.=18)	
	No.	%	No.	%
Single gene				
• qnrB1-3, 5, 6, 8 **(No.=7)**	1	6.7	6	33.3
Combined genes				
• qnrA1-6 and qnrB1-3, 5, 6, 8 **(No.=6)**	0	0.0	6	33.3
• qnrA1-6 and qnrB1-3, 5, 6, 8 and qnrS1-2 **(No.=6)**	0	0.0	6	33.3

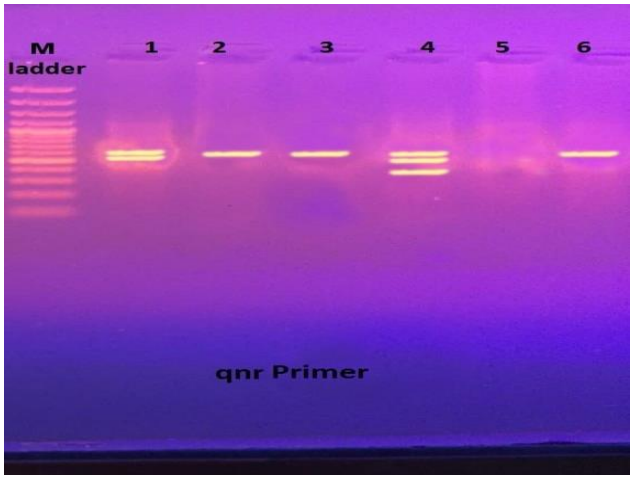

Fig. 3: Agarose gel electrophoresis for the multiplex PCR amplified products of *K. pneumoniae PMQR* genes

- Lane M: DNA molecular size marker (1000 bp)
- Lane 1 represents positive *qnrA*1-6 (571 bp) and positive *qnrB2,3,6,8* (594 bp) genes lanes 2,3, 6 represents positive *qnrB* (594 bp) genes
- Lane 4: represents positive *qnrA*1-6 (571 bp) and positive *qnrB* 2,3,6,8(594 bp) genes and positive qnrS1-2 (388bp) genes
- Lane 5: represent negative sample

DISCUSSION

Acute Exacerbation of COPD is the most frequently triggered by bacterial infection [{1}]. In this study the most frequent isolated organisms in AECOPD were *K. pneumoniae* (94.3%), followed by *S. aureus* (12.1%) *and P. aeureginosa* (9.1%). This result was correlated with Chakraborty [11] in Tripura, Lin [12] in Taiwan and Kuwal [1]. On the other hand, ***Shashibhushan*** [13] in India and ElKorashy and El sheriff [14] and ElFeky [15] in Egypt reported that the most prevalent isolated organism was *S. pneumoniae*.

A highly significant relationship was detected between severity of COPD and *K. pneumoniae* isolation in this research. As, *K. pneumoniae* was isolated only from patients with very severe stage of stable COPD (7.4%). While, in AECOPD *K. pneumoniae* was isolated in all stages with the highest rate in its severe stage (70.4%). ***Aydemir*** [{16}] in turkey found that, *K. pneumoniae* isolates were isolated from mild stage, moderate and severe stages of COPD by percentages of 0.0%, 2.0% and 6.0% respectively. This finding was in contrary to Lin [12] who found a higher incidence of *K. pneumoniae* in patients with mild COPD (39.1%) as compared with moderate (16.6%) and severe (13.8%) COPD.

The dramatic increase of antibiotic resistance in *K. pneumoniae* has become a significant and growing threat to public and environmental health [{17}]. This fact has emerged clearly in this research and many other researches. In this study, *K. pneumoniae* was highly resistant to amoxicillin (84.9%), amoxicillin/clavulanic acid (75.8%) followed by ciprofloxacin (63.6%) and levofloxacin (57.6%). Seyedpour and Eftekhar [18] found that *K.pneumoniae* resistance rates to ceftazidime, levofloxacin and ciprofloxacin were 13.5%, 11.5% and 7.7% respectively. In India *K. pneumoniae* strains were mainly resistant to ampicillin, ceftazidime, amikacin, tetracycline and ciprofloxacin [2]. This was in contrary to Lal[19] in India who found that *K. pneumoniae* was sensitive to levofloxacin (92%) and ciprofloxacin (85%). Shreshra[20] in Kathmandu University Hospital found that; co-trimoxazole, ciprofloxacin and amikacin were highly effective against *K. pneumoniae* isolates. By 100%, 100% and 88% respectively.

In this study, 78.8% and 84.4% of *K. pneumoniae* displayed a positive biofilm phenotypically by CRA and MCRA methods respectively., This was nearer to the studies done by Seifi[21] in Tehran and *by* Pramodhini[22] in India. They demonstrated that, *93.6*% and 60% of *K. pneumoniae* strains were *in vitro* positive for biofilm formation examined by MCRA.

In spite of, biofilm acts as a diffusion barrier to many antimicrobials, ciprofloxacin is able to penetrate evenly into biofilms, but unfortunately, it is unable to eradicate them completely. So, ciprofloxacin treatment of biofilms could induce resistance at even higher frequencies [25]. In our study, about (89.3%), (89.3%), (67.9%) and 20/33 (71.4%) of resistant *K. pneumoniae* strains to amoxicillin/clavulanic acid, Ciprofloxacin, Levofloxacin, and piperacillin/tazobactam respectively were biofilm producers. Similar findings were reported by Subramanian [{23}], they found that *K. pneumoniae* strains forming biofilm were highly resistant to levofloxacin (88.2%), ciprofloxacin (88.2%) and cefotaxime (82.4%) as compared to non-biofilm producing strains, Fàbrega [24] in Spain found that resistance of *K. pneumoniae* strains to ampicillin (83.3%), cefotaxime (73.3%), levofloxacin (80%) and ciprofloxacin (93.3%) was comparatively higher among biofilm producers than non-biofilm producers. Also, Yang and Zhang [25] reported that biofilm-producing *K. pneumoniae* isolates were highly resistant to antimicrobials.

PMQR genes have been found worldwide in multiple species of enterobacteria being particularly frequent in *K. pneumoniae* [6]. In the present study 57.6% (19/33) of *K. pneumoniae* isolates were positive for PMQR genes by multiplex PCR. Among them, 36.8% had qnrB1-3, 5, 6, 8 gene, 36.8% had qnrA1-6 and qnrB1-3, 5, 6, 8 and 36.8% had qnrA1-6 and qnrB1-3, 5, 6, 8 and qnrS1-2, while no isolates had qnr B4. Our results agreed with Seyedpour and Eftekhar [18] who found that 59.5% of the *K. pneumoniae* isolates carried the PMQR determinants and among them, 50.5% harbored *qnrB*, 4.2% harbored qnrS and none had qnrA. Majlesi [26] in Saudi Arabia found that, qnrB gene and qnrS gene were present in 47.8% and 4.34% of *K. pneumoniae* respectively, while no isolate had qnrA. Wang [27] found that 42% of *K. pneumoniae* strains had *qnr A* gene. On the other hand, lower percentages were reported by Shams [28], ***qnrA*** gene was detected only in 3.2% ciprofloxacin resistant *K. pneumoniae* isolates**.** Higher rates were reported by Hammad et al.[29] in Egypt who detected PMQR determinants in 100% of *K. pneumoniae* isolates. The positive rates of qnrB and qnrS were (83.7 %), (81.4 %) and respectively. No qnrA was detected in any isolate. It is probably associated with the geographical distribution of *PMQR* genes[6].

CONCLUSION

K. pneumoniae play a major role in acute exacerbation of COPD. Very high percentage of them (84.4%) were biofilm producers. Moreover, finding a relationship between biofilm forming *K. pneumoniae* strains and antibiotic resistance, suggests that gene transfer mechanisms within the biofilm environments are likely to occur. Existence of quinolone resistance is a serious public health problem and requires continuous surveillance, monitoring and strict applications of infection control policies.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

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REFERENCES

1. Kuwal A, Joshi V and Dutt NA. Prospective Study of Bacteriological Etiology in Hospitalized Acute Exacerbation of COPD Patients: Relationship with Lung Function and Respiratory Failure, Turk Thorac J 2018; 19: 19-27.
2. Navon-Venezi S, Kondratyeva S and Carattoli A: K. pneumoniae: a major worldwide source and shuttle for antibiotic resistance, FEMS Microbiology Reviews 2017; 41(3): 252–275.
3. Minarini LA, Poirel L and Cattoir V: Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. J Antimicrob Chemother 2008; 62(3):474e8.
4. Global Initiative for Chronic Obstructive Lung Disease (GOLD): A collaborative project of the National Health, Lung, and Blood Institute, NIH and the World Health Organization. 2014, Available at: www.goldcopd.com
5. Mokkapati A and Yalamanchili M: Correlation of Sputum Gram's Stain And Culture In Lower Respiratory Tract Infections. IOSR Journal of Dental and Medical Sciences 2013 (IOSR-JDMS) e-ISSN: 2279-0853, p-ISSN: 2279-0861. 8(1). 06-09.
6. Vàzquez C, Lago N, Legido JL, Arias I and Mato MM : Microcalorimetric study of the growth of E. faecalis, K. pneumoniae and their mixtures in an enriched culture medium. J Therm Anal Calorim 2013;113: 1415–1420

7. Espinar MJ, Ribeiro M and Rodrigues AG (2011): Extended-spectrum β-lactamases of E. coli and K. pneumoniae screened by the VITEK 2 system. Journal of Medical Microbiology 2011, 60: 756-760
8. Clinical and Laboratory Standards Institute (CLSI): Performance standards for antimicrobial susceptibility testing; informational Supplement. CLSI supplement M100S.2018 Wayne, PA. 36: 66-130.
9. Panda PS, Chaudhary U and Dube SK. Comparison of four different methods for detection of biofilm formation by uropathogens. Indian J Pathol Microbiol 2016; 59:177-9
10. Bouchakour M, Zerouali K, Amarouch H, El Mdaghri N and Courvalin P: Plasmid-mediated quinolone resistance in expanded spectrum beta lactamase producing Enterobacteriaceae in Morocco. J Infect Dev Ctries.2010 4:779-803.PubMed PMID: 21252459
11. Chakraborty A, Choudhury A, Debnath J and Saha N: Bacteriological Profile and Antibiotic sensitivity Pattern in Acute Exacerbation of advanced cases of Chronic Obstructive Pulmonary Disease (COPD). J Evid Based Med Healthc 2016; 3(1), 20-23.
12. Lin S-H, Kuo P-H, Yang P-C and Kuo S-H (2007): Sputum bacteriology in hospitalized patients with acute exacerbation of chronic obstructive pulmonary disease in Taiwan with an emphasis on K. pneumoniae and P aeruginosa:. Respirology 2007, 12. 81–87.
13. Shashibhushan B. L, Nagaraja C, Arun B. J, Nagaraj N: Bacteriological profile and antibiotic sensitivity pattern in sputum culture of chronic obstructive pulmonary disease patients. Int J Adv Med 2016, 3 (3):671-674.
14. ElKorashy RIM and El-Sherif RH: Gram negative organisms as a cause of acute exacerbation of COPD. Egyptian Journal of Chest Diseases and Tuberculosis 2014; 63, 345–349.
15. ElFeky DS, Elmandory HM, Galal M and Abdel Hakim M (2016): Sputum Bacteriology in Patients with Acute Exacerbation of Chronic Obstructive Pulmonary Disease. Int. J. Curr. Microbiol. App. Sci 2016, 5(1): 289-305.
16. Aydemir Y, Aydemir O and Kalem F: Relationship between the GOLD combined COPD assessment staging system and bacterial isolation, International Journal of COPD 2014; 9 1045–1051.
17. Giannella M, Lewis R, Trecarichi EM, Petrosilla N and Tambarello M: Predictors of mortality in multidrug-resistant K. pneumoniae bloodstream infections 2013; 11(10). 1053- 1063.
18. Seyedpour SM and EftekharF: Quinolone Susceptibility and Detection of qnr and aac(6')-Ib-cr Genes in Community Isolates of Klebsiella pneumoniae. Jundishapur J Microbiol.2014; 7(7): e11136
19. Lal SB, Kumar KR and Sudeena D: Profile of bacterial culture and sensitivity from the sputum of chronic obstructive pulmonary patients with acute exacerbations. Asian Pacific Journal of Health Sciences2017; 4(1):173-182.
20. Shrestha R, Shrestha B, Shakya Shrestha S, Prajapati B, Karmacharya BM: Study of Pre-disposing Factors of Acute Exacerbation of Chronic Obstructive Pulmonary Disease and Antibiotic Prescribing Pattern with Reference to Antibiotic Sensitivity Test. Kathmandu Univ Med J.2015;51(3):255-60.
21. Seifi K, Kazemian H, Rezagholizadeh F, Saee Y, Shirvani F and Houri H: Evaluation of Biofilm Formation Among K. pneumoniae Isolates and Molecular Characterization by ERIC-PCR, Jundishapur J Microbiol 2016; 9(1): e30682
22. Pramodhini S, Niveditha, Umadevi S, Kumar S and Stephen S: Antibiotic resistance pattern of biofilm forming uropathogens isolated from catheterized patients in Pondicherry, india, AMJ.2012; 5(7), 344-348.
23. Subramanian P, Umadevi S, Kumar S and Stephen S: Determination of correlation between biofilm and extended spectrum β lactamases producers of Enterobacteriaceae. SchoRes J 2012.; 2:2-6.
24. Fa`brega A, Soto SM, Balleste´-Delpierre C, Ferna´ndez-Orth D, and Vila J: Impact of quinolone-resistance acquisition on biofilm production, J Antimicrob Chemother 2014; 69: 1815–1824.
25. Yang D and Zhang Z: Biofilm-forming Klebsiella pneumoniae strains have greater likelihood of producing extended-spectrum beta-lactamases. J. Hosp. Infect. 2008, 68, 369–371.
26. Majlesi A, Kakhki RK, Nejad M, Mashouf RY, Roointan A, Abazari M, Alikhani MY: Detection of plasmid-mediated quinolone resistance in clinical isolates of Enterobacteriaceae strains in Hamadan, West of Iran, Saudi Journal of Biological Sciences 2018; 25. 426–430
27. Wang A, Yang Y, Lu Q, Wang YI, Chen Y, Deng LI, Ding H, Deng Q, Zhang H, Wang C, Wang L and Shen X: Presence of qnr gene in Escherichia coli and K. pneumoniae resistant to ciprofloxacin isolated from pediatric patients in China. BMC Infectious Diseases 2008; 8:68.

28. Shams F, Hasani A, Pormohammad A, Rezaee MA, Nahaie MR, Hasani A and Arbatan AE: qnrA implicated quinolone resistance in Escherichia coli and Klebsiella pneumoniae clinical isolates from a University Teaching Hospital, Life Science Journal 2014;11(12s)

29. Hammad H, Hadiya S, EL-Feky M and Aly S : Co-occurrence of Plasmid-mediated Quinolone Resistance and Carbapenemases in Klebsiella pneumoniae Isolates in Assiut, Egypt, Egyptian Journal of Medical Microbiology 2017, 26 (4)