

RESEARCH ARTICLE

Prevalence, Antimicrobial Resistance and Biofilm Formation of *Klebsiella Pneumoniae* Isolated from Human and Cows

Ahmed M Ammar¹, Marwa I Abd El-Hamid¹ and Noha A Gomaa^{*2}

¹Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Sharkia, Egypt

²Directorate of Veterinary Medicine, Zagazig, Sharkia, Egypt

E-mail: dr.noha_3li@yahoo.com

Running title: Biofilm of multidrug resistant *Klebsiella pneumoniae*

Abstract

Klebsiella pneumoniae (*K. pneumoniae*) is an important multidrug-resistant (MDR) and biofilm producing pathogen that causes diverse infections in human and animals. This study aimed to evaluate the prevalence, antimicrobial resistance, and biofilm forming ability of *K. pneumoniae* isolates from diseased humans sputum and urine samples and mastitic cows milk samples in Zagazig city, Sharkia Governorate, Egypt. A total of 100 human and animal samples were collected and examined for the presence of *Klebsiella* species. The recovered isolates were identified by conventional bacteriological procedures and confirmed by the traditional PCR at the genus and species levels using *gyrA* and *16S-23S ITS* genes, respectively. The confirmed *K. pneumoniae* isolates were subjected for testing their antimicrobial susceptibility and biofilm formation ability. The results revealed that 10 out of 50 human samples were positive for *K. pneumoniae* (20%); 28% (7/25) from sputum samples and 12% (3/25) from urine samples. Additionally, the prevalence rate of *K. pneumoniae* in milk samples from mastitic cows was 4% (2/50). The antimicrobial susceptibility results showed that *K. pneumoniae* isolates had high resistance rates to ampicillin and ampicillin/clavulanate (100% each) with multiple antibiotic resistance (MAR) index of 0.083, followed by azithromycin, cefepime, and trimethoprim/sulphamethoxazole (91.7% each). Meanwhile, these isolates revealed high sensitivity levels to chloramphenicol (83.3%) with MAR index of 0.014. Furthermore, 91.7% of *K. pneumoniae* isolates were able to produce the biofilm, where 8.3% was a non-biofilm producer. Out of 11 *K. pneumoniae* isolates, 6 (54.5%), 3 (27.3%) and 2 (18.2%) were moderate, strong and weak biofilm producers, respectively. The present study emphasized the high prevalence of MDR and biofilm producing *K. pneumoniae* isolates in human sputum and urine and cow's milk samples. Therefore, more attentions should be taken against antimicrobials usage and for providing new antibiofilm approaches against *Klebsiella* species biofilms.

Key-words: *Klebsiella pneumoniae*, Prevalence, Antimicrobial resistance, Biofilm.

Introduction

Klebsiella species are Gram-negative, non-motile, facultative anaerobic, encapsulated and rod shaped bacteria those belonged to the family *Enterobacteriaceae* [1]. These bacteria are pervasive in nature, where they present in the environment and also they exist as normal flora in the gastrointestinal tract of animals and humans [2]. Despite this, they are opportunistic pathogens and can cause serious nosocomial infections like pneumonia, septicemia,

meningitis, urinary tract and soft tissue infections [3] and pyogenic liver abscesses in mammals [4] besides bovine mastitis [5] and mare metritis [6].

Klebsiella spp. have many virulence factors those are incriminated in their pathogenesis and infectivity comprising capsule, lipopolysaccharide, fimbrial and non-fimbrial adhesins, siderophores and the biofilm formation capability [7].

The biofilm is an aggregation of microbial cells those are irretrievably

combined with abiotic and/or biotic surfaces and are enclosed in a self-formed exopolysaccharide matrix of extracellular materials including polysaccharides, proteins and DNA [8,9]. Biofilm is considered the most crucial virulence factor of *Klebsiella* spp. [10], where it protects the microbes from opsonization by antibodies, phagocytosis and removal via the ciliary action of epithelial cells [11]. This leads to an elevation in the resistance of *Klebsiella* spp. to the host immune defenses and to the antimicrobial molecules [8].

Within the biofilm, there are high horizontal gene transmission and plasmid transfer rates and this exaggerates the resistance problem and renders the treatment of infections with classical methods very complicated [12], where multidrug-resistant (MDR), extensively drug-resistant (XDR) [13] and pandrug-resistant (PDR) [14] isolates of *K. pneumoniae* have been registered all over the world.

Multidrug-resistant *K. pneumoniae* has the tendency to form the biofilm [15]. The resistance of antibiotics in mature bacterial biofilm is 10–1,000 times than that of planktonic bacteria [16]. Furthermore, the capability of *K. pneumoniae* for the production of biofilm, especially on indwelling medical apparatus and the endurance of these biofilm producing *klebsiella* strains isolated from medical devices to many antibiotics will lead to chronic urinary and respiratory infections, hardness in remedy and high fatality rates [17,18].

Many studies were applied on patients in Egyptian hospitals for isolation and detection of antibiotic susceptibility and biofilm formation ability of *Klebsiella* spp. Meanwhile, there are limited published reports focusing on the resistance and biofilm status of *Klebsiella* spp. in the veterinary sector in Egypt.

So, this study aimed to determine the prevalence, antimicrobial sensitivity and biofilm production ability of *Klebsiella* spp.

in humans and animals at Sharkia Governorate, Egypt.

Materials and methods

Sampling

A total of 100 samples were collected from Zagazig city, Sharkia Governorate, Egypt. The samples were recovered from both human (50) and animal (50) origins. The human samples including sputum (25) and urine (25) were collected from patients attending various hospitals and laboratories, while 50 milk samples were collected from clinical cases of bovine mastitis from private farms during the winter season. The animal study was endorsed by the committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University. Regarding the human samples, it was approved by the research ethical committee of Faculty of Medicine, Zagazig University and the work was conducted in compliance with the Ethics of the world Medical Association (Declaration of Helsinki) for studies involving humans. Written informed permissions were taken from patients taking part in the research study after a complete explanation of the aim of the study.

Isolation and identification of *Klebsiella* species

All the collected samples were streaked onto HiCrome *Klebsiella* Selective Agar base (HIMEDIA, M1573, India) supplemented with *Klebsiella* Selective Supplement (HIMEDIA, FD225, India) for selective isolation and identification of *Klebsiella* species. The plates were incubated aerobically at 37°C for 24h. The suspected colonies of *Klebsiella* species were selected and subcultured onto MacConkey's agar (Oxoid, UK) and Eosin Methylene Blue (EMB) agar (Oxoid, UK) plates to confirm the isolation of this bacterium and the plates were incubated

aerobically at 37°C for 24h. The presumptive isolates were identified by Gram's staining and then they were confirmed by biochemical tests involving indole, methyl red, Vogas - Proskauer, citrate utilization, catalase, oxidase and urease in addition to examining their motility [19].

Molecular confirmation of *Klebsiella* species

All recovered klebsiella isolates were molecularly confirmed at the genus level via the PCR amplification of the genus specific *gyrA* gene. Moreover, PCR assays targeting the 16S-23S internal transcribed spacer (ITS) and the *pehX* gene were carried out to detect *K. pneumoniae* and *K. oxytoca*, respectively.

Extraction of genomic DNA was carried out according to QIAamp DNA mini kit guidelines (Catalogue no. 51304), (Qiagen, Netherlands).

PCR assay was achieved in a 25 µL reaction mixture containing 12.5 µL of Emerald Amp GT PCR master Mix (code No. RR310A kit, Takara, Japan), 1 µL of each primer (20 pmol; Biobasic, Canada), 3 µL of each template DNA and 7.5 µL of PCR-grade water.

Oligonucleotide specific primers for the amplification of genus *Klebsiella* and species specific genes were used. The sequences of the selected primers from the previous published papers are as followings:

gyrA-F: 5'
CGCGTACTATACGCCATGAACGTA 3'
and *gyrA*-R: 3'
ACCGTTGATCACTTCGGTCAGG
5' [20], 16S-23S ITS-F: 5'
ATTTGAAGAGGTTGCAAACGAT
3' and 16S-23S ITS-R: 3'
TTCACCTCTGAAGTTTTCTTGTGT
TC 5' [21] and *pehX*-F: 5'
GATACGGAGTATGCCTTTACGG
TG 3' and *pehX*-R: 3'
AGCCTTTATCAAGCGGATACTG
G 5' [22]. The amplification conditions

for *gyrA* were performed as following: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 30 sec and a final extension step at 72°C for 5 min [20]. Moreover, the cyclic conditions for PCR assay based on 16S-23S ITS were done as following: initial denaturation at 94°C for 10 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 20 sec and extension at 72°C for 20 sec and a final extension phase at 72° for 10 min [21]. Finally, the PCR cyclic conditions for *pehX* gene were applied as following: initial denaturation at 95°C for 2 min, then 30 cycles of denaturation at 94°C for 20 sec, annealing at 59°C for 20 sec and extension at 72°C for 30 sec and a final extension stage at 72°C for 10 min [22]. The PCR amplified products were analyzed by agarose gel electrophoresis using 1.5% agarose gel (Sigma-Aldrich, USA). Ethidium bromide dye (Sigma-Aldrich, USA) was added to the agarose gel for staining of the DNA during examination and imagination under the ultra violet (UV) transilluminator (Spectroline, USA) [23].

Antimicrobial susceptibility testing

Detection of antibiotic susceptibility profiles of *K. pneumoniae* isolates was executed by Kirby Bauer disc diffusion procedure [24] and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) recommendations [25]. For examination of *K. pneumoniae* isolates susceptibility, subculturing of the preserved isolates in brain heart infusion (BHI) broth (Oxoid, UK) with glycerol was performed onto MacConkey's agar and the inoculated plates were incubated at 37°C for 24 h. After that, spreading of the prepared 0.5 MacFarland bacterial suspension onto Mueller Hinton agar (MHA) (Oxoid, UK) plates was carried out by a sterile cotton

swab and then the antimicrobial discs (Oxoid, UK) were placed onto those plates. After incubation of the plates at 37°C for 24 h, the inhibition zone around each antimicrobial disc was measured. The used antimicrobial discs (Oxoid, UK) were ampicillin (AMP, 10 µg), amoxicillin/clavulanate (AMC, 20/10 µg), amikacin (AK, 30 µg), azithromycin (AZM, 15 µg), aztreonam (ATM, 30 µg), cefepime (FEP, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), imipenem (IPM, 10 µg), nalidixic acid (NA, 30 µg), tetracycline (TE, 30 µg) and trimethoprim/ sulphamethoxazole (SXT, 1.25/23.75µg).

Determination of multiple antibiotic resistance (MAR) index [26]

$$\text{MAR index for the antimicrobial} = \frac{\text{Number of antimicrobial resistant isolates}}{\text{Number of antimicrobials} \times \text{Number of isolates}}$$

$$\text{MAR index for the isolate} =$$

$$\frac{\text{Number of antimicrobials to which the isolate was resistant}}{\text{Number of antimicrobials to which the tested isolate was exposed}}$$

Detection of biofilm formation by *K. pneumoniae*

Tissue culture plate method

Quantitative biofilm formation method was done as previously described [27, 28]. Briefly, three wells (for each isolate) of the sterile 96-well flat bottomed polystyrene tissue culture microtiter plate (Cleanway, Egypt) were filled with 200 µL of a 0.5 MacFarland (1.5×10^8 CFU/mL) klebsiella suspension prepared in trypticase soy broth (TSB) (Oxoid, UK).

Negative control wells with sterile TSB were also involved. After incubation of the plate at 37°C for 24 h under aerobic conditions, the wells were emptied from the media and then they were washed three times with sterile phosphate-buffered saline (PBS) (Al-Gomhorya Company, Zagazig, Sharkia, Egypt). The plate was then air

dried for an hour and 200 µL of 0.1% crystal violet solution (Al-Gomhorya Company, Zagazig, Sharkia, Egypt) was added to each well for 15 min for staining the formed biofilm. After that, the excess stain was rinsed off by placing the plate under running tap water. Crystal violet bound to the biofilm was extracted after adding 200 µL of 95% ethanol (Cleanway, Egypt) per well. Finally, the absorbance of the extracted crystal violet in each well was measured at 570 nm on an ELISA reader (BioTek, USA). All biofilm assays were performed in triplicate.

The cut-off optical density (OD_c) for the microtiter - plate test was defined as three standard deviations above the mean OD of the negative control.

Adherence capabilities of *K. pneumoniae* isolates were classified into four groups: non-adherent, weakly, moderately or strongly adherent depending on the OD of klebsiella biofilms as

- $OD \leq OD_c$: Non-adherent
- $OD_c < OD \leq 2 \times OD_c$: Weakly adherent
- $2 \times OD_c < OD \leq 4 \times OD_c$: Moderately adherent
- $4 \times OD_c < OD$: Strongly adherent

Statistical analysis

The results were analyzed by SPSS version 25 (Armonk, NY: IBM Corp). Categorical results were reported as frequencies and percentages. Chi-square test of significance was applied to test the differences among different categories. Fisher's exact test was also applied to test the association between sources and /or types of samples and infection with *K. pneumoniae*. P -value < 0.05 was considered statistically significant.

Results

Characterization of klebsiella isolates

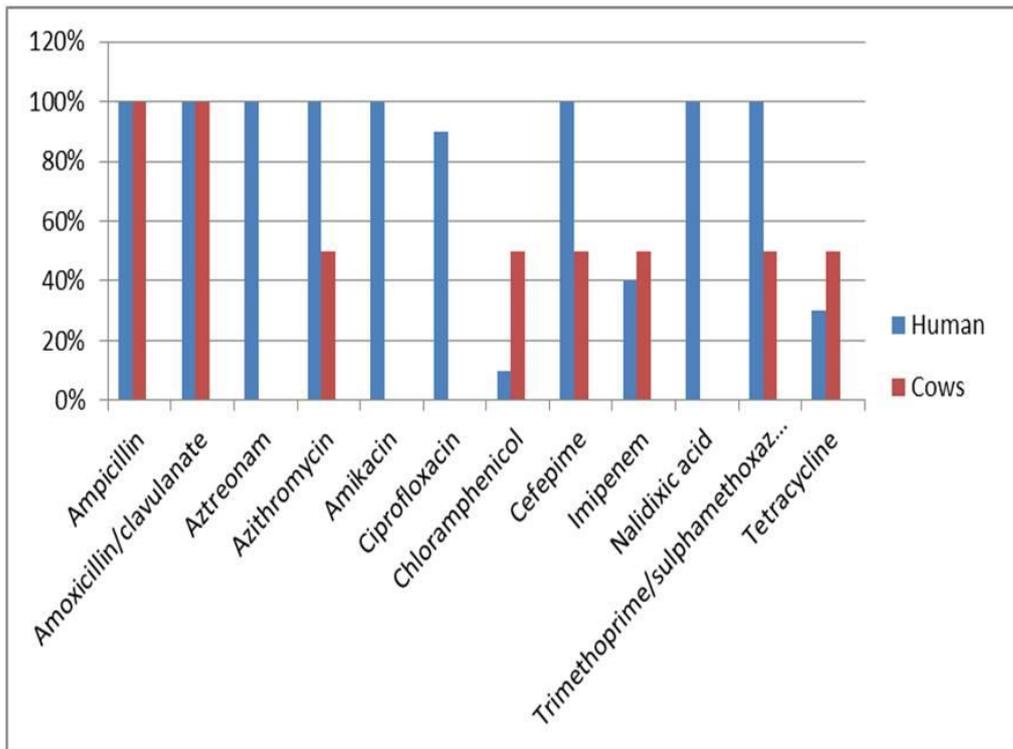
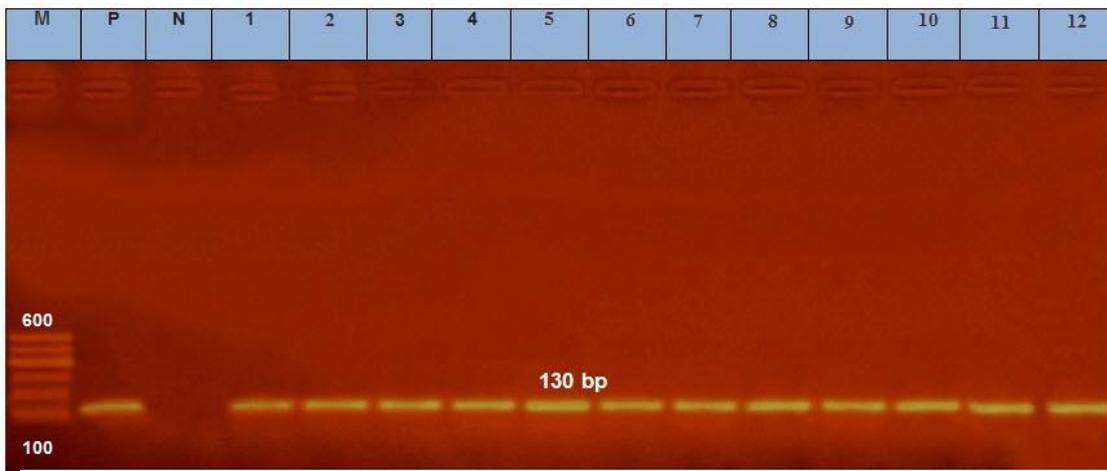
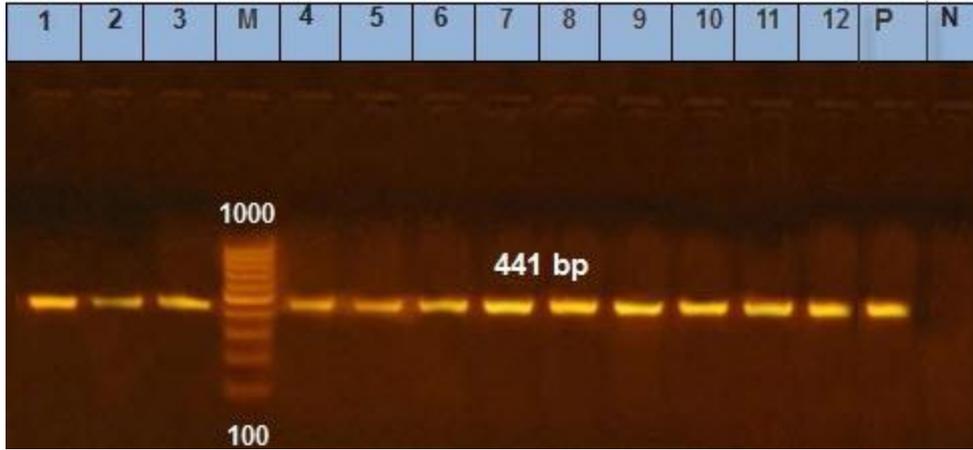
Distinctive klebsiella colonies were purple-magenta coloured colonies

on HiCrome Klebsiella Selective Agar base medium. Moreover, large shiny pink lactose fermenter mucoid dome shaped colonies on MacConkey's agar and non-green metallic dark pink to purple colored mucoid colonies on EMB agar were also observed for *Klebsiella* species. *Klebsiella* isolates gave positive reactions for Vogas-Proskauer, citrate utilization, urease, and catalase tests, but they gave negative reactions for indole, methyl red, oxidase and motility tests. Relevant results from the previous tests gave a preliminary confirmation for *Klebsiella* spp.

Molecular confirmation of Klebsiella species

A total of 12 *klebsiella* isolates was confirmed at the molecular level by PCR amplification of the genus specific gene, *gyrA*. The results revealed that all the examined isolates (100%) harbored *gyrA* gene giving rise to an amplified products at 441 bp as shown in Figure 1. Furthermore, the confirmed *klebsiella* isolates were distinguished at the species level by PCR amplification of species specific genes, 16S-23S ITS for *K. pneumoniae* and *pehX* gene for *K. oxytoca*. The results displayed that all the 12 isolates (100%) possessed 16S-23S ITS gene and yielded the amplified products at 130 bp and therefore they were identified as *K. pneumoniae* as shown in (Figure 2).

Prevalence of K. pneumoniae in human and cow samples



The overall prevalence of *K. pneumoniae* in human samples was 20% (10/50), where 7 out of 25 (28%) sputum samples and 3 from 25 (12%) urine samples were positive for *K. pneumoniae*. Moreover, the isolation rate of *K. pneumoniae* in milk samples from mastitic cows was 4% (2/50). Statistical analysis of our results showed significant variations between *K. pneumoniae* infection and samples origins ($P = 0.01$) as well as samples types ($P = 0.028$).

Antimicrobial susceptibility results

The antimicrobial resistance profiles of the 12 klebsiella isolates from humans and cows are shown in (Table 1). Klebsiella isolates revealed high rates of resistance to ampicillin and amoxicillin/clavulanate (100% each) with MAR index of 0.083, followed by azithromycin, cefepime and trimethoprim/sulphamethoxazole (91.7% each) and amikacin, aztreonam and nalidixic acid (83.3% each). Meanwhile, klebsiella isolates exhibited high sensitivity rates to chloramphenicol (83.3%), followed by imipenem (33.3%). *K. pneumoniae* isolates of human origin were more resistant to most antimicrobial agents than those isolated from the animal origin. *K. pneumoniae* isolates of human origin showed high resistance rates to ampicillin, amoxicillin/clavulanate, aztreonam, azithromycin, amikacin, cefepime, nalidixic acid and trimethoprim/sulphamethoxazole (100% each) and ciprofloxacin (90%), but they displayed high sensitivity rates to chloramphenicol (90%). Meanwhile,

K. pneumoniae isolates of animal origin exhibited high resistance rates to ampicillin and amoxicillin/clavulanate (100% each), followed by azithromycin, chloramphenicol, cefepime, imipenem, trimethoprim/sulphamethoxazole and tetracycline (50% each), but they revealed high sensitivity rates to amikacin and nalidixic acid (100% each) (Figure 3).

Majority of *K. pneumoniae* isolates from different sources (91.7%) showed a high level of MDR pattern, since they displayed resistance to at least five diverse antimicrobial classes (Table 2). The highest MDR rate was recorded for human *K. pneumoniae* isolates (70%, 7/10), where they showed resistance to 5 different groups of antimicrobials. Overall, a total of 7 different antimicrobial resistance patterns of the 12 *K. pneumoniae* isolates were identified. The most prevalent pattern was the resistance to ampicillin, ciprofloxacin, imipenem, amoxicillin/clavulanate, azithromycin, cefepime, aztreonam, amikacin, nalidixic acid and trimethoprim/sulphamethoxazole being identified by 4 (33.3%) *K. pneumoniae* isolates (Table 3). Statistical analysis displayed various significance levels of *K. pneumoniae* resistance to the used antimicrobials, where there was a highly significant difference when using aztreonam, cefepime, ciprofloxacin, azithromycin and trimethoprim/sulphamethoxazole ($P < 0.01$), but there was a significant difference when nalidixic acid, amikacin, and chloramphenicol were used ($P < 0.05$) (Table 1).

Table (1): Antimicrobial susceptibility profiles of 12 klebsiella isolates against 12 antimicrobial agents with their respective MAR indices

Antimicrobial class	Antimicrobial agent (symbol)	No. of klebsiella isolates (%)			MAR index	χ^2
		R	I	S		
Lactams	Ampicillin (AM)	12 (100)	0 (0)	0 (0)	0.083	NA
	Amoxicillin/ clavulanate (AMC)	12 (100)	0 (0)	0 (0)	0.083	NA
	Aztreonam (ATM)	10 (83.3)	1 (8.3)	1 (8.3)	0.069	0.001**
	Cefepime (FEP)	11 (91.7)	1 (8.3)	0 (0)	0.076	0.004**
	Imipenem (IPM)	5 (41.7)	3 (25)	4 (33.3)	0.035	0.779 ^{NS}
Fluorinolones	Ciprofloxacin (CIP)	9 (75)	2 (16.7)	1 (8.3)	0.063	0.009**
	Nalidixic acid (NA)	10 (83.3)	0 (0)	2 (16.7)	0.069	0.021*
Aminoglycosides	Amikacin (AK)	10 (83.3)	0 (0)	2 (16.7)	0.069	0.021*
Macrolides	Azithromycin (AZM)	11 (91.7)	0 (0)	1 (8.3)	0.076	0.004**
Phenicolones	Chloramphenicol (C)	2 (16.7)	0 (0)	10 (83.3)	0.014	0.021*
Tetracyclines	Tetracycline (TE)	4 (33.3)	5 (41.7)	3 (25)	0.028	0.779 ^{NS}
Dihydrofolate pathway inhibitors	Trimethoprim/ sulphamethoxazole	11 (91.7)	0 (0)	1 (8.3)	0.076	0.004**

MAR: multiple antibiotic resistance

 χ^2 : denotes chi-square value; NA: not applicable; NS: non-significant ($P > 0.05$); * denotes a statistical significance ($P < 0.05$); ** denotes highly significant difference ($P < 0.01$)**Table (2): The resistance rates of klebsiella isolates from different sources and their frequency of multiple antibiotic resistance indices**

No. of antimicrobial classes	No. of antimicrobial agents	MAR index of isolates	No. of resistant klebsiella isolates (%)		
			Humans (10)		Cows milk (2)
			Sputum (7)	Urine (3)	
1	2	0.17	0	0	1 (50)
5	8	0.67	7 (100)	0	1 (50)
	9	0.75			
6	10	0.83	0	2 (66.7)	0
	10	0.83			
7	11	0.92	0	1 (33.3)	0

MAR: multiple antibiotic resistance

Table (3): Antimicrobial resistance patterns of 12 klebsiella isolates against 12 antimicrobial agents

No.	Antimicrobial resistance pattern	No. of klebsiella isolates (%)	
		Humans (10)	Cows (2)
1	AM, CIP, IPM, AMC, AZM, FEP, SXT, ATM, AK, NA	4 (40)	0
2	AM, TE, CIP, AMC, AZM, FEP, SXT, ATM, AK, NA	2 (20)	0
3	AM, TE, CIP, AMC, C, AZM, FEP, SXT, ATM, AK, NA	1 (10)	0
4	AM, CIP, AMC, AZM, FEP, SXT, ATM, AK, NA	2 (20)	0
5	AM, AMC	0	1 (50)
6	AM, AMC, AZM, FEP, SXT, ATM, NA, AK	1 (10)	0
7	AM, TE, IPM, AMC, C, AZM, FEP, SXT	0	1 (50)

AM: ampicillin, AMC: amoxicillin/ clavulanate, AK: amikacin, ATM: aztreonam, AZM: azithromycin, C: chloramphenicol, CIP: ciprofloxacin, NA: nalidixic acid, FEP: cefepime, IPM: imipenem, SXT: trimethoprim/sulphamethoxazole, TE: tetracycline

MAR index

The frequency of MAR indices in *K. pneumoniae* isolates of human and animal origins is clarified in (Table 2). The results illustrated that *K. pneumoniae* isolates were resistant to 2 - 11 of 12 tested antimicrobials with MAR indices ranging from 0.17 to 0.92. Generally, plurality of the isolates (91.7%) were MDR with MAR indices greater than 0.2. Analysis of MAR index results for *K. pneumoniae* isolates of human and animal origins demonstrated that the level of MDR exhibited by the isolates of human origin is alarming such that 100% of these isolates were resistant to three or more different antimicrobial classes. Furthermore, the MAR index of 0.83 was the most prevalent among *K. pneumoniae* isolates of human origin (60%).

Biofilm formation

Biofilm formation of *K. pneumoniae* isolates by tissue culture plate method is shown in Table 4. Among 12 tested *K. pneumoniae* isolates, 11 (91.7%) were positive for the production of biofilm, where one isolate (8.3%) was non-biofilm producer. Among the 11 biofilm producing *K. pneumoniae* isolates, 6 (54.5%) were found to be moderate biofilm producers, 3 (27.3%) were strong biofilm producers and 2 (18.2%) were weak biofilm forming isolates. Five (50%) out of 10 human *K. pneumoniae* isolates were moderate biofilm formers, 3 (30%) were strong biofilm producers and one isolate was weak biofilm producer. Meanwhile, one animal *K. pneumoniae* isolate (50%) was moderate biofilm former and the other isolate (50%) was weak producer of biofilm.

Table (4): Biofilm formation of klebsiella isolates by tissue culture plate method

Biofilm degree	No. of klebsiella isolates (%)	
	Humans (10)	Cows (2)
Tissue culture plate	Strong	3 (30)
	Moderate	5 (50)
	Weak	1 (10)
	Non-adherent	1 (10)

Discussion

Klebsiella pneumoniae are serious MDR and biofilm forming bacteria that lead to difficult treated infections in human and animals and many mortalities [29]. So, in our study, we discussed the prevalence, antimicrobial resistance, biofilm forming ability and molecular confirmation of *K. pneumoniae* isolates in Zagazig city, Sharkia Governorate, Egypt. Our survey found that the prevalence rate of *K. pneumoniae* isolates in human was 20% (10/50). This prevalence rate was higher than those recorded in the study of Al-Zahraa University Hospital, Cairo, Egypt (6.95%, 143/2058) [30]. Meanwhile, it was lower than the *K. pneumoniae* prevalence rates recorded in the survey carried out in Vidarbha region, India (40.43%, 437/1081) [31]. The highest record of *K. pneumoniae* in this study was found in sputum samples with a percentage of 28% (7/25), which was close to that recorded in a recent study conducted in Egypt (20%) [30]. Meanwhile, this was dissimilar with that recorded in a previous study conducted in India, where 45% of sputum samples were contaminated with *K. pneumoniae* isolates [31]. In the present study, 4% (2/50) of milk samples collected from mastitic cows were contaminated with *K. pneumoniae*. This prevalence rate was lower than those recorded in previous studies in Kafr EL-Sheikh Governorate, Egypt (20.93%, 36/172) [32]. The differences in these prevalence rates may be

associated with several factors such as alterations in locality, samples sources, samples numbers and unhygienic conditions during milking.

High spread of antimicrobial resistance is a worrying emergent affair in human and veterinary fields. In the current study, the results of antimicrobial sensitivity testing displayed that *K. pneumoniae* resistance was observed most commonly to ampicillin and amoxicillin/clavulanate (100% each). This was in agreement with a previous study conducted in Iran [33], where higher resistance rate to ampicillin and amoxicillin/clavulanate (95-100%) were observed. On the other hand, lower resistance rates to amoxicillin/clavulanate were recorded previously in Morocco (51%) [34]. In our study, a higher resistance rate was also observed for *K. pneumoniae* isolates against azithromycin, cefepime and trimethoprim/sulphamethoxazole (91.7% each). This result was conflicting with those of previous research in Iran [35], where lower resistance rates of *K. pneumoniae* isolates were noticed to cefepime and azithromycin (43.14% and 41.18%, respectively). Earlier studies also reported relatively higher resistance rates of *K. pneumoniae* isolates to trimethoprim/sulphamethoxazole; 88.2% in Egypt [36]. In our study, higher resistance rates of *K. pneumoniae* isolates were also recorded for amikacin, aztreonam and nalidixic acid (83.33% each).

Meanwhile, previous studies reported

lower resistance rates to amikacin; 37.5% in Egypt [37] and 0% in Iran [33]. Similar resistance rates of *K. pneumoniae* isolates to aztreonam were observed previously in Mexico (81.5%) [38]. Regarding the resistance of *K. pneumoniae* isolates to nalidixic acid, various levels were reported in different countries; 71.7% in India [39] and 2% in Iran [33].

On the other hand, our *K. pneumoniae* isolates showed a higher sensitivity rate to chloramphenicol (83.33%), but this result was higher than those reported previously in Nigeria (15.4%) [40]. The following sensitivity rate in the current result was for imipenem (33.3%), but this result was lower than those recorded in Iran (98%) [33].

In the present study, 11 out of 12 *K. pneumoniae* isolates (91.7%) were MDR. This high percentage of MDR *K. pneumoniae* isolates was similar to that observed in another study carried out in Egypt (95.77%) [41], but it was higher than those reported previously in Nepal (66.7%) [42].

The variations in prevalence of MDR *K. pneumoniae* isolates in diverse studies may be attributed to the dissimilarity in clinical conditions of patients and animals, the samples involved in the survey, the used antibiotics and the application of the infection control measures.

Additionally, antibiogram consequences displayed that the MDR patterns in *K. pneumoniae* isolates of human origin were higher than those reported for the isolates of animal origin, which was harmonic with another survey in China [43]. This could be due to the massive use of antimicrobials in Egypt, which made the antimicrobials ineffectual in *K. pneumoniae* infections chemotherapy.

It is known that *K. pneumoniae* isolates have the ability to produce biofilm, which is considered the most important virulence factor in the pathogenicity of

this bacterium. In our study, we found that 91.7% of *K. pneumoniae* isolates produced biofilm. The present result was similar to that of a previous study conducted in Iran [10], where 93.6% of *K. pneumoniae* strains formed biofilm, but it was higher than those reported in previous research carried out in Nepal (73.3%) [44]. This variation may be due to differences in the geographic region, specimens` kinds, samples` sizes, samples` numbers and procedures of biofilm detection.

In conclusion, the present study has revealed the elevated emergence of MDR and biofilm forming *K. pneumoniae* isolated from human and mastitic cow milk sources, which gives rise to many serious infections. These results indicate the misusing of antimicrobials in human and veterinary fields. So, future studies are needed to innovate new effective antimicrobials and antibiofilm techniques against MDR and biofilm forming *K. pneumoniae* isolates.

Conflict of interest

None of the authors have any conflict of interest to declare.

References

- [1] Umeh, O. and Berkowitz, L.B. (2009): Klebsiella infections. eMedicine. Com, accessed on 27 January, 2020.
- [2] Podschun, R.; Pietsch, S.; Höller, C. and Ullmann, U. (2001): Incidence of *Klebsiella* species in surface waters and their expression of virulence factors. J Appl Environ Microbiol, 67(7): 3325-3327.
- [3] Podschun, R. and Ullmann, U. (1998): *Klebsiella* spp. as nosocomial pathogens: Epidemiology, taxonomy, typing methods and pathogenicity factors. Clin Microbiol Rev, 11(4): 589-603.
- [4] Wang, J.H.; Liu, Y.C.; Lee, S.S.J.; Yen, M.Y.; Chen, Y.S.; Wang, J.H.; Wann, S.R. and Lin, H.H. (1998): Primary liver abscess due to *Klebsiella pneumoniae* in Taiwan. Clin Infect Dis, 26(6): 1434-1438.

- [5] Munoz, M.A.; Ahlstrom, C.; Rauch, B.J. and Zadoks, R.N. (2006): Fecal shedding of *Klebsiella pneumoniae* by dairy cows. *J Dairy Sci*, 89(9): 3425–3430.
- [6] Kikuchi, N.; Hiramune, T.; Taniyama, H. and Yanagawa, R. (1987): Difference of virulence in causing metritis in horses between heavily encapsulated, less heavily encapsulated and non-capsulated strains of *Klebsiella pneumoniae* capsular type 1. *Jpn J Vet Res*, 35(4): 263-273.
- [7] Magesh, H.; Kumar, A.; Alam, A.; Priyam; Sekar, U.; Sumantran, V.N. and Vaidyanathan, R. (2013): Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae*. *Indian J Exp Biol*, 51(9): 764-772.
- [8] Hoiby, N.; Bjarnsholt, T.; Givskov, M.; Molin, S. and Ciofu, O. (2010): Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents*, 35(4): 322-332.
- [9] Piperaki, E.; Syrogiannopoulos, G.A.; Tzouvelekis, L.S. and Daikos, G.L. (2017): *Klebsiella pneumoniae*: Virulence, biofilm and antimicrobial resistance. *Pediatr Infect Dis J*, 36(10): 1002-1005.
- [10] Seifi, K.; Kazemian, H.; Heidari, H.; Rezagholizadeh, F.; Saeed, Y.; Shirvani, F. and Hourii, H. (2016): Evaluation of biofilm formation among *Klebsiella pneumoniae* isolates and molecular characterization by ERIC-PCR. *Jundishapur J Microbiol*, 9(1): e30682.
- [11] Costerton, J.W.; Montanaro, L. and Arciola, C.R. (2005): Biofilm in implant infections: its production and regulation. *Int J Artif Organs*, 28(11): 1062–1068.
- [12] Diaz, M.A.; Hernandez, J.R.; Martinez-Martinez, L.; Rodrifuez-Bano, J.; Pascual, A. and Grupo de Estudio de Infección Hospitalaria (GEIH) (2009): Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Spanish hospitals: 2nd multicenter study (GEIHBLEE project, 2006). *Enferm Infecc Microbiol Clin*, 27(9): 503-510.
- [13] Bi, W.; Liu, H.; Dunstan, R.A.; Li, B.; Torres, V.V.L.; Cao, J.; Chen, L.; Wilksch, J.J.; Strugnell, R.A.; Lithgow, T. and Zhou, T. (2017): Extensively drug-resistant *Klebsiella pneumoniae* causing nosocomial bloodstream infections in China: Molecular investigation of antibiotic resistance determinants, informing therapy, and clinical outcomes. *Front Microbiol*, 8: 1230.
- [14] Guducuoglu, H.; Gursoy, N.C.; Yakupogullari, Y.; Parlak, M.; Karasin, G.; Sunnetcioglu, M. and Otlu, B. (2018): Hospital outbreak of a colistin-resistant, NDM-1- and OXA- 48-producing *Klebsiella pneumoniae*: high mortality from pandrug resistance. *Microb Drug Resist*, 24(7): 966–972.
- [15] Sanchez, J.R.C.J.; Mende, K.; Beckius, M.L.; Akers, K.S.; Romano, D.R.; Wenke, J.C. and Murray, C.K. (2013): Biofilm formation by clinical isolates and the implications in chronic infections. *BMC Infect Dis*, 13: 47.
- [16] Lebeaux, D.; Ghigo, J.M. and Beloin, C. (2014): Biofilm-related infections: Bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol Rev*, 78(3): 510–543.
- [17] Hatt, J.K. and Rather, P.N. (2008): Role of bacterial biofilms in urinary tract infections. *Curr Top Microbiol Immunol*, 322: 163–192.
- [18] Bellifa, S.; Hassaine, H.; Balestrino, D.; Charbonnel, N.; M'hamedi, I.; Terki, I. K.; Lachachi, M.; Didi, W. and Forestier, C. (2013): Evaluation of biofilm formation of *Klebsiella pneumoniae* isolated from medical devices at the University Hospital of Tlemcen, Algeria, *Afr J Microbiol Res*, 7(49): 5558-5564.
- [19] Ewing, W. H. (1986): Edwards and Ewing's identification of

Enterobacteriaceae, 4th ed. Elsevier Science Publishing Co., Inc., New York, N.Y.

[20] Bauer, A.W.; Kirby, W.M.; Sherris, J.C. and Turck, M. (1966): Antibiotic susceptibility testing by a standardized single disk method, *Am J Clin Pathol*, 44: 493-496.

[21] Clinical and Laboratory Standards Institute (CLSI) (2019): Performance Standards for Antimicrobial Susceptibility Testing: Twenty-ninth informational supplement (eds.), CLSI document M100-S29. West Valley Road, Wayne, Pennsylvania, USA.

[22] Krumperman, P. H. (1983): Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl Environ Microbiol*, 46(1): 165–170.

[23] Stepanović, S.; Vukovic, D.; Dakic, I.; Savie, B. and Svabic-Vlahovic, M. (2000): A modified microtiter plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods*, 40(2): 175-179.

[24] Tendolkar, P.M.; Baghdayan, A.S.; Gilmore, M.S. and Shankar, N. (2004): Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. *Infect Immun*, 72(10): 6032–6039.

[25] Brisse, S. and Verhoef, J. (2001): Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* genes sequencing and automated ribotyping. *Inter J Syst Evol Microbiol*, 51(Pt 3): 915–924.

[26] Liu, Y.; Liu, C.; Zheng, W.; Zhang, X.; Yu, J.; Gao, Q.; Hou, Y. and Huang, X. (2008): PCR detection of *Klebsiella pneumoniae* in infant formula based on 16S–23S internal transcribed spacer. *Int J Food Microbiol*, 125(3): 230–235.

[27] Kovtunovych, G.; Lytvynenko, T.; Negrutka, V.; Lar, O., Brisse, S. and Kozyrovska, N. (2003): Identification of *Klebsiella oxytoca* using a specific PCR assay targeting the polygalacturonase *pehX* gene. *Res Microbiol*, 154(8): 587–592.

[28] Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989): Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, NY.

[29] Effah, C.Y.; Sun, T.; Liu, S. and Wu, Y. (2020): *Klebsiella pneumoniae*: an increasing threat to public health. *Ann Clin Microbiol Antimicrob*, 19(1): 1.

[30] Abo Samra, M.A.; Ali, N.K. and El-Madboly, A.A. (2019): Detection of multi-drug resistant *Klebsiella pneumoniae* in Al-Zahraa University Hospital. *Egypt J Hosp Med*, 75(6): 3006-3012.

[31] Thosar, M.G. and Kamble, V.A. (2014): Prevalence of *Klebsiella* species isolated from clinical samples in vidarbha region of Maharashtra state, India. *Int J Pharm Bio Sci*, 5(3): B40 – B45.

[32] Ahmed, H.F.; Asfour, H.A.E.; Sobeih, A.M.K.; Darwish, S.F.; Abu El-Magd, M.; Darwish, S.A. and El Saka, H. I.A. (2016): Phenotypic and molecular identification of *Klebsiella* and *Salmonella* species isolated from subclinical mastitis milk of Egyptian buffalo. *Global Veterinaria*, 16 (5): 500-507.

[33] Dallal, M.M.S.; Khalifeh-Gholi, M.; Yazdi, S.S., Mehrani, F.; Hadayatpour, A. and Yazdi, M.K.S. (2018): Antimicrobial resistance pattern of *Klebsiella* spp isolated from patients in Tehran, Iran. *J Surg Med*, 2(2): 84-86.

[34] El Bouamri, M.C.; Arsalanea, L.; El Kamounia, Y. and Zouhaira, S. (2014): Antimicrobial susceptibility of urinary *Klebsiella pneumoniae* and the emergence of carbapenem-resistant strains: A retrospective study from a

university hospital in Morocco, North Africa. Afr J Urol, 21(1): 36–40.

[35] Babakhani, S.; Shokri, S. and Baharvand M. (2015): Antibiotic resistance pattern of *Klebsiella pneumoniae* isolated from nosocomial infections in Aleshtar hospital, Lorestan province. Report of Health Care, 1(2): 55-59.

[36] EL-Ganiny, A.M.; Areej Mostafa EL-Mahdy, A.M.; Abd EL-Latif, H.K.; Ibrahim, R.H. and Abdelsabour, H.I. (2016): Phenotypic and genotypic detection of β -lactams resistance in *Klebsiella* species from Egyptian hospitals revealed carbapenem resistance by OXA and NDM genes. Afr J Microbiol Res, 10(10): 339-347.

[37] Aly, S.A.; Tawfeek, R.A. and Mohamed, I. S. (2016): Bacterial catheter-associated urinary tract infection in the Intensive Care Unit of Assiut University Hospital. Al Azhar Assiut Med J, 14(2): 52-58.

[38] Alcántar-Curiel, M.D.; Ledezma-Escalante, C.A.; Jarillo-Quijada, M.D.; Gayosso-Vázquez, C.; Morfín-Otero, R.; Rodríguez-Noriega, E.; Cedillo-Ramírez, M.L.; Santos-Preciado, J.I. and Girón, J.A. (2018): Association of antibiotic resistance, cell adherence, and biofilm production with the endemicity of nosocomial *Klebsiella pneumoniae*. Biomed Res Int, 7012958.

[39] Kumar, A.R. (2013): Antimicrobial sensitivity pattern of *Klebsiella pneumoniae* isolated from pus from tertiary care hospital and issues related to the rational selection of antimicrobials. J Chem Pharm Res, 5(11): 326-331.

[40] Romanus, I.I. and Egwu, O.A. (2011): Analysis of antibiotic susceptibility of *Klebsiella pneumoniae* strains isolated from different clinical specimens in Enugu state. Can J P Appl Sci, 5(3): 1609-1614.

[41] Kamel, N.A.; El-tayeb, W.N.; El-Ansary, M.R.; Mansour, M.T. and Aboshanab, K.M. (2018): Phenotypic screening and molecular characterization of carbapenemase-producing Gram-negative bacilli recovered from febrile neutropenic pediatric cancer patients in Egypt. PLoS ONE, 13(8): e0202119.

[42] Mahato, S.; Mahato, A.; Pokhare, E. and Tamrakar, A. (2019): Detection of extended-spectrum beta-lactamase-producing *E. coli* and *Klebsiella* spp. in effluents of different hospitals sewage in Biratnagar, Nepal. BMC Res Notes, 12(1): 641.

[43] Yang, F.; Deng, B.; Liao, W.; Wang, P.; Chen, P. and Wei, J. (2019): High rate of multiresistant *Klebsiella pneumoniae* from human and animal origin. Infect Drug Resist, 12: 2729–2737.

[44] Nepal, H.P.; Neopane, P.; Shrestha, R.; Gautam, R.; Paudel, R.; Ansari, S.; Shrestha, S. and Thapa, S. (2017): Biofilm formation and antimicrobial resistance in *Klebsiella pneumoniae* isolated from patients visiting a tertiary care center of Nepal. Asian Pac J Trop Dis, 7(6): 347-351.

Figure legends

Figure (2): Agarose gel electrophoresis displaying PCR amplification fragments for 16S-23S ITS gene (130 bp) *K. pneumoniae*. Lane M: 100 bp molecular weight marker, lanes (1-12): positive *K. pneumoniae* isolates Lane P: positive control, lane N: negative control.

Figure (3): Antimicrobial resistance rates of *klebsiella* isolates from human and animal sources.

الملخص العربي

انتشار كليبسيلا نيموني المعزولة من مصادر مختلفة المقاومة لأغلب المضادات الحيوية والمكونة للبيوفيلم

أحمد محمد عمار¹, مروة إبراهيم عبد الحميد¹, نها على جمعة²

مصر ، ، الشرقية ، الزقازيق 44511 الزقازيق، جامعة البيطري، الطب ، كلية الميكروبيولوجيا قسم

² مديرية الطب البيطري، الزقازيق ، الشرقية، مصر

كليبسيلا نيموني هي ميكروب مهم مقاوم لأغلب المضادات الحيوية ومكون للبيوفيلم والذي يسبب إصابات كثيرة في الإنسان والحيوانات لذلك كان الهدف الأساسي من هذا البحث هو تقييم انتشار عزلات بكتيريا كليبسيلا نيموني ومقاومتها للمضادات الحيوية وقدرتها على تكوين البيوفيلم في عينات بلغم وبول مأخوذة من أناس مرضى وعينات لبن مأخوذة من بقر مصابة بالتهاب الضرع في مدينة الزقازيق، محافظة الشرقية، مصر. وقد تم تجميع 100 عينة من الإنسان والحيوان وفحصها بالطرق التقليدية على PCR البكتيريولوجية التقليدية لعزل ميكروب كليبسيلا وتأكد هذه العزلات باستخدام تفاعل البلمرة المتسلسل (كما تم 16S-23S ITS و *pehX* ، وتصنيفها على مستوى الفصيلة باستخدام جينات *gyrA* gene مستوى الجنس باستخدام وقدرتها على تكوين البيوفيلم. وقد أظهرت النتائج أن 10 عزلات اختبار حساسية عزلات الكليبسيلا نيموني للمضادات الحيوية من بين 50 عينة مأخوذة من الإنسان بنسبة (20%) هي عزلات الكليبسيلا. وقد تم عزل 7 عزلات من 25 عينة بلغم بنسبة 28% وعزل 3 عزلات من 25 عينة بول بنسبة 12% كما تم عزل سلالتين كليبسيلا نيموني من 50 عينة لبن من بقر مصاب بالتهاب الضرع بنسبة 4%. كما أن نتائج اختبار حساسية المضادات الحيوية أظهرت أن كليبسيلا نيموني تمتلك أعلى مقاومة للأمبيسليلين والأمبيسيلين/ كلافيولانات بنسبة (100% لكلا منهما) بمؤشر مقاومة لعدد من المضادات الحيوية يبلغ 0.083 ويليه في المقاومة الأزيترومايسين وسيفبيم وترايميثوبريم/سلفاميثوكزazol بنسبة 91.7% لكل منهم ولكن هذه العزلات تمتلك أعلى حساسية للكورامفينيكول بنسبة 83.3% بمؤشر مقاومة لكثير من المضادات الحيوية (0.014). وعلاوة على ذلك ، 91.7% من عزلات كليبسيلا نيموني لهم القدرة على تكوين البيوفيلم ولكن 8.3% كانت غير منتجة للبيوفيلم وأوضحت النتائج أن 6 من 11 عزلة كليبسيلا نيموني بنسبة 54.5% كانوا متوسطين في إنتاج البيوفيلم و 3 من 11 عزلة كليبسيلا نيموني بنسبة 27.3% كانوا أقوى في إنتاج البيوفيلم وعزلتين من 11 عزلة كليبسيلا نيموني كانوا ضعفاء في إنتاج البيوفيلم. ولقد أظهرت نتائج هذا البحث الانتشار المرتفع لكليبسيلا نيموني المقاومة لأغلب المضادات الحيوية والتي لها القدرة على تكوين البيوفيلم في عينات البلغم والبول المأخوذة من أناس مرضى لذلك يجب الانتباه عند استخدام المضادات الحيوية وتوفير طرق جديدة للتخلص من بيوفيلم الكليبسيلا.