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Research Article

Assessment of Biofilm Formation and Hypermucoviscosity in *Klebsiella oxytoca* from Clinical and Community Sources

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Abstract

Background: *Klebsiella oxytoca* is an emerging pathogen causing hospital- and community-acquired infections. Hypermucoviscous phenotype and biofilm development are considered markers of hypervirulence, causing invasive infections in healthy adults

Objective: The current study aimed to evaluate the ability of *K. oxytoca* isolates to form biofilms and incorporate hypermucoviscous characteristics.

Method: One hundred *K. oxytoca* isolates were collected from hospitals in the Medical City Teaching Hospital Complex, in Baghdad, Iraq. The isolates were identified by conducting manual and biochemical tests. The hypermucoviscous characteristic was analyzed by using the string test. The Congo red agar method was used to detect biofilm formation, while quantitative methods like tube adherence and microtiter plates were used to measure its strength. Statistical analysis was performed by using the SPSS Statistical package (Version 26; SPSS, IBM) and Microsoft Office Excel (2010) for drawing the figures except for the receiver operating characteristic (ROC) curve.

Results: *K. oxytoca* was obtained from 54/100 (54%) male and 46/100 (46%) female specimens. The specimens were from inpatients 62/100 (62%), and the rest, 38/100 (38%), were from outpatients. The isolates were significantly (P = 0.0001) from urine specimens 39/100 (39%), followed by blood specimens 16/100 (16%). The string test identified 14/100 (14%) isolates as hypermucoviscous, with 21.1% being from outpatients. The biofilm was positive in 80/100 isolates. By utilizing the tube method, a strong adhesion was detected in 23/80 (28.75%) isolates. The microtiter plate method (MTP) yielded a strong biofilm in 25/80 (31.25%) isolates. The strongest biofilm was found in isolates from urine specimens (40%).

Conclusion: By forming a strong biofilm and developing hypermucoviscous, the virulence potential of *K. oxytoca* isolates exhibited a risk to the community. Urine was considered to be the main source of the strongest biofilm-forming isolates.

Keywords: Biofilm; Community-acquired infections; Hypermucoviscous; *Klebsiella oxytoca*; String test

Introduction

Klebsiella oxytoca (K. oxytoca) is an important pathogenic Gram-negative bacillus, a non-motile bacteria, commensal in the intestinal tract, in about 2%–10% of the population (1). *K. oxytoca* is rising as a significant opportunistic pathogen, causing nosocomial infections in neonates as well as adults. It is the causative agent of pneumonia and ranks as the second most prevalent nosocomial infection in hospitals with critically ill patients, especially; antibiotic-associated hemorrhagic colitis (AAHC). It is also linked to infections in the newborn's blood, urinary tract, CNS, lungs, skin, and soft tissues. According to reports, it is also linked to patients who *Klebsiella oxytoca* causes a variety of illnesses, from colitis to infective endocarditis (2). Interestingly, it acquired the ability to resist multiple drugs, including the majority of those utilized to treat bacterial infections (3). Because it can exchange plasmid-borne resistance factors with other bacteria in hospitals and other specialized units, it poses a serious threat to nosocomial infections (4).

Klebsiella's pathogenicity is amplified by the formation of biofilms, hypercapsule synthesis, lipopolysaccharide, and siderophore activity. *Klebsiella* spp. are capable of binding to both biotic and abiotic surfaces, and they can also build a dense coating of extracellular biofilm. Patients with bacteremia, urinary tract infections, AAHC, and liver abscesses are found to have biofilm development. They can resist antibiotics, as biofilm and antibiotic resistance are closely linked (5). *Klebsiella pneumoniae* and *Klebsiella oxytoca* are

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known to produce biofilms using a variety of adhesive structures, the most common of which are capsules and type 1 and type 3 fimbria. Biofilms are colonies of surface-attached microbes with unique characteristics. They present serious risks to human health and food security (6).

Biofilms consist of microbial communities that have colonized within an exo-polymeric matrix (7). Proteins like fibrin and polysaccharides like alginate and eDNA are part of the biofilm matrix. Bacteria within biofilms can use a variety of survival strategies to elude the host's defense mechanisms, in addition to the protection provided by the matrix (8).

Bacteria develop biofilms to ensure their survival. Biofilm formation causes a rise in resistance to both antimicrobial agents and external stresses (9). Biofilms increase the tenacity of pathogens on surfaces, which is directly linked to infections. They do this by shielding microorganisms from human immune responses, disinfectants, and antibiotics (10). Certain intrinsic components (such as pili, adhesins, flagella, fimbriae, which help with the initial colonization of the host, and capsular polysaccharides, which protect the organism from phagocytosis) can be crucial for the consistent production of biofilm (11).

K. oxytoca expresses several cell surface adhesins facilitating tissue attachment and biofilm formation, eventually leading to infections such as diarrhea and AAHC (5).

K. pneumoniae with a hypermucoviscosity (HMV) phenotype, known as hypervirulent *K. pneumoniae* (hvKP), is known to cause infections that can spread throughout the body and even cause death. This virulence factor may be confirmed using a simple method: Agar plates are stretched with a loop, and a positive string test is indicated by the formation of viscous threads longer than 5 mm (12). This hypervirulence-associated hypermucoviscous *K. pneumoniae* can infect young, healthy people. Of late, hypermucoviscous *K. oxytoca* has emerged as a serious public health concern; this strain differs from the conventional strains, in that, it produces more capsules than the others (13).

It has been proposed that the establishment and production of biofilms by bacteria is a crucial step in *Klebsiella* pathogenesis (14). Catheter-related infections (CAIS), one of the earliest identified and most researched biofilm disorders, must always be considered when biofilm formation in the urinary tract is suspected. The bacteria are shown to form biofilms on the mucosal surface of the prostate tissue and bladder mucosal tissue, demonstrating that biofilms are linked to UTIs when indwelling devices are not the source. One of the best explanations for persistent and infections is the development of biofilms in the urinary tract (8, 15). With *K. oxytoca*, emerging as a new pathogen in Baghdad hospitals, the current study intends to assess the ability of the isolates to form biofilms and encompass the hypermucoviscous characteristic.

Materials and Methods

Bacterial isolation: A cross-sectional study was conducted during the period from November 2023 to February 2024. A total of 100 different specimens (Urine, Blood, Wound swab, Ascites fluid, Sputum, Stool, Endotracheal tube aspiration, Burn swab, Ear wound swab, Indwelling urinary catheter, Wound pus cell. And CSF) from the patients were collected from hospitals in The Medical City Teaching Hospital Complex in Baghdad, Iraq. The collected specimens from the patients and their personal information remained anonymous. Informed consent was obtained from each participant in the current study. Patients' specimens were divided into two categories: inpatient or hospital infection specimens and outpatient or community infection specimens. A “nosocomial infection” or “health-related infection” occurs in a patient who receives medical care in a hospital or other healthcare facility and was absent at admission (16). Therefore, all specimens in the current study considered to be hospital-acquired infections were approved by physicians who reported that the patients had been admitted to the hospital for another reason and that the recent infection had not been documented at the time of admission.

The isolates with positive *K. oxytoca* were included, while other isolates that revealed other genera or species of bacteria were excluded.

The study was conducted according to the Helsinki Declaration Guidelines and approved by the Ethics Committee at the Department of Microbiology/College of Medicine/University of Baghdad (No. 0234 on 27th Nov. 2023).

Bacterial detection: The specimens were streaked on MacConkey agar and then incubated at 37°C for 24h. To evaluate their phenotypic characteristics, all lactose fermenting and mucoid colonies were selected. Suspected bacterial isolates were further identified by colony morphology, Gram stain, and biochemical test. The utilization of a basic biochemical assay, such as indole, holds significant importance within clinical laboratories to assess the precision of bacterial identification at the species level (17).

To determine the exact genus and species of the bacteria that were isolated, the VITEK-2 Compact System (bioMerieux, Marcy l'Etoile, France) was utilized.

The antibiotics sensitivity test was done by using the Kirby Bauer method for five antibiotics (ciprofloxacin 10µg/disc, impenem 10µg/disc, gentamicin 10µg/disc, tetracycline 10µg/disc, and cefexime 10µg/disc). Multidrug-resistant (MDR) was referred to as having acquired resistance to at least one drug from three or more antibiotic classes (18).

The hypermucoviscous phenotype: All one hundred *K. oxytoca* strains were subjected to a string test in order to identify the hypervirulent (hypermucoviscous) strains. Overnight cultures of the bacteria were grown on MacConkey agar plates.

A positive string test is defined as the development of viscous threads that are > 5 mm long and is achieved by stretching the colony on an agar plate using a bacteriological loop (12).

Detection of biofilm formation by *K.oxytoca*

The Qualitative method

Congo red agar (CRA): Freeman *et al.* have described a simple qualitative method to identify the production of biofilms on Congo Red Agar media (19).

One hundred *K. oxytoca* isolates were cultured overnight on MacConkey agar, streaked onto Congo Red Agar, and then incubated at 37°C for 24 hours to determine their biofilm-forming potential. Bacteria can form biofilms because they can release slim into their environment. Colonies will be red if the isolates are unable to excrete slim, and black if they can. Isolates were classified as biofilm-forming (positive) if they formed nearly black colonies and biofilm-non-forming (negative) if they formed red colonies.

The Quantification methods

The Tube Method: The method was done according to Christensen *et al.*, in which 80 isolates that were found to have positive results in CRA were included. A loopful of bacterial isolate was inoculated in 2 mL of Brain Heart Infusion broth with 2% glucose in test tubes. The tubes were incubated at 37 ºC for 24 hrs. After incubation, tubes were decanted, washed with distal water, and dried.

Later, the emptied tubes were stained with 0.1% crystal violet. To remove any remaining stain, distal water was used. The tubes were put in an upside-down drying oven. The evaluation of the biofilm level as strong, moderate, weak, and non-biofilm producing was achieved according to the density of the line dye that formed in the tubes (20).

The Microtiter Plate Method: The microtiter plate is an inexpensive method that has become the gold standard for testing biofilm formation. A total of 20µl of overnight bacterial culture was used to inoculate flat-bottom microtitre wells containing 180µl of sterile Brain Heart Infusion broth with 2% sucrose (21, 22). Negative control wells contained only 200µl of Brain Heart Infusion broth with the 2% sucrose and incubated for 24 hours at 37ºC. The wells were washed three times with normal saline to eliminate unattached bacterial cells. The microtiter plates were dried at room temperature for 15 minutes and 200µl of crystal violet solution (0.1%) was added and left for 15 minutes. The crystal violet solution was removed carefully and the wells were washed three times with distilled water to remove the unbounded dye and allowed to dry at room temperature. Two hundred microliters of 95% ethanol were added into each well to extract the bound dye. The absorbance was measured at 630 nm using a Microplate Reader. Each test was repeated three times, including the control well. The average of the optical density (OD) value for the control wells was 0.239. Isolates were categorized as follows : (1) non-biofilm producers (OD ≤ ODc); (2) weak biofilm producers (ODc < OD ≤ 2 × ODc); (3) moderate biofilm producers (2 × ODc < OD ≤ 4 ×ODc); (4) Strong biofilm producers (4 × ODc < OD). The optical density cut-off value (ODc) is defined as three standard deviations plus the mean OD of the negative.

The Statistical Analysis: Statistical analysis was performed by using the SPSS Statistical package (Version 26; SPSS, IBM) and Microsoft Office Excel (2010) for drawing the figures except for the receiver operating characteristic (ROC) curve. Normally distributed data were expressed as (Number & %), Pearson chi-square test ($\chi 2$), and binomial Z test for comparisons of qualitative variables between studied groups (i.e., specimens, sex, inpatients or outpatients, string & biofilm).

When the *P*-value was greater than 0.05, it was considered a non-significant difference (NS), when it was less than 0.05, it was considered a significant difference (S), and when it was less than 0.01 it was considered a highly significant difference (HS).

Results

Bacterial identification with hypermucoviscous and MDR criteria: The 100 clinical isolates were diagnosed as *K. oxytoca* by the manual method and the automated VITEK 2 Compact System. The species was confirmed as *K. oxytoca* using the biochemical indole test. About 54 (54%) of *K. oxytoca* isolates were obtained from males and 46 (46%) from females. Among all clinical isolates, 62 (62%) were taken from inpatients and 38 (38%) from outpatient specimens. *K. oxytoca* was significantly isolated from urine specimens 39 (39%), followed by blood specimens 16 (16%), wound swap 9 (9%), and then sputum specimens 8 (8%). The isolates from urethral swabs and cerebral spinal fluid were the less prevalent sources. The prevalence of the isolated *K. oxytoca* was associated significantly with the source of the isolates, as shown in Table 1).

Antibiotic resistance was found to be variable among the isolates. The isolates showed high resistance to βeta-Lactam antibiotics, including Cefotaxime (91/100). It was found that *K. oxytoca* was sensitive to Imipenem (55/100) which belongs to the group of Carbapenems. Different levels of resistance were recorded among the isolates against Gentamicin (46/100), Ciprofloxacin (60/100), and Tetracycline (66/100), respectively. The current findings showed that the level of resistance to antibiotics classified the isolates into MDR with 53 out of 100 isolates (53%).

In addition, out of 100 isolates, only 14 (14%) were identified phenotypically as hypermucoviscous by using the string test. The string test positivity was significantly correlated with the total number of bacteria (Figure 1).

Table 1: Specimens distribution for *Klebsiella oxytoca* isolations

Specimen	NO.	%	*P* - value
Urine	39	39	
Blood	16	16	
Wound swab	9	9	
Ascites fluid	6	6	
Sputum	8	8	
Stool	3	3	
Endotracheal tube aspiration	5	5	*P* = 0.0001 HS
Burn swab	4	4	
Ear wound swab	4	4	
Indwelling urinary catheter	3	3	
Wound pus cell	2	2	
CSF	1	1	
Total	100	100	

Figure 1: The viscous threads that are >5 mm long of the tested *K. oxytoca* isolates.

Detection of biofilm using the Congo red agar

The culture of the isolates on CRA revealed that 80 (80%) isolates out of 100 were classified as biofilm-forming bacteria. Wherein, 74 (74%) out of 80 isolates were positive after 24 hours, and 6 (6%) out of 80 isolates were positive after 72 hours, as illustrated in Figure 2.

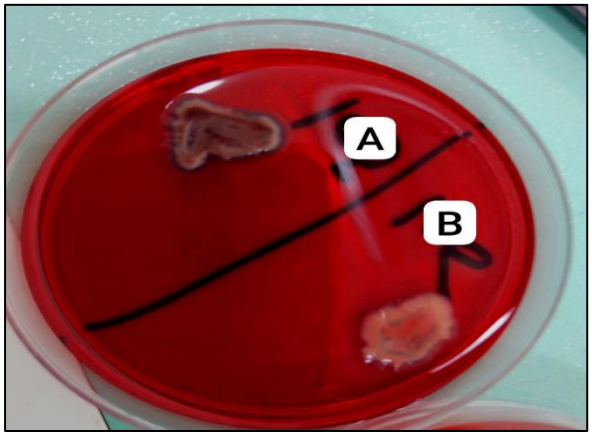

Figure 2: The growth of *K. oxytoca* on Congo red agar with positive biofilm forming black colonies (A) and negative biofilm forming red colonies (B).

Detection of biofilm using the tube method: The quantitative evaluations for the biofilm-forming isolates were carried out on the 80 isolates that were found to be positive on CRA by the two methods, the tube and microtiter methods. The tube quantitative method demonstrated that 23/80 (28.75%) isolates were with strong biofilm which was observed as a line around the tube. While, 22/80

(27.5%) isolates were with moderate biofilm, 27/80 (33.75%) isolates were weak, and 8/80 (10%) isolates were with no biofilm (Figure 3).

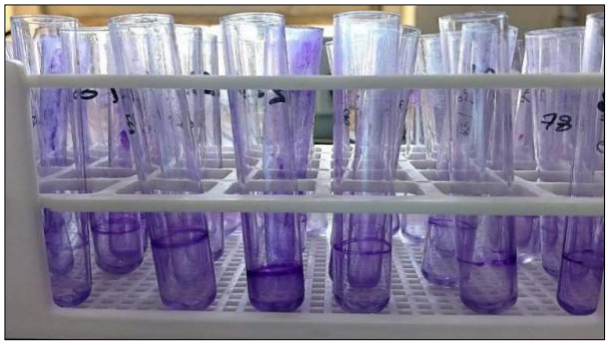

Figure 3: The quantitative tube method

to detect the biofilm formation by *K. oxytoca*. The biofilm level is strong, moderate, weak, and non-biofilm-producing, depending on the density of the line dye that formed in the tubes

Detection of biofilm using the Micro titter plate: According to the most accurate biofilm formation method, among the 80 isolates tested, 25/80 (31.25%) isolates had strong biofilm while 24/80 (30%) isolates were moderate, 18/80 (22.5%) isolates were weak, and 13/80 (16.25%) isolates were with no biofilm as elucidated in (Figure 4).

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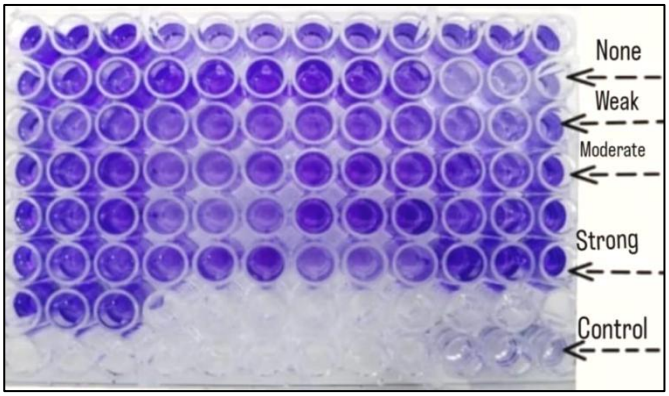

Figure 4: The quantitative microtiter plate method to detect the biofilm formation by *K. oxytoca*. The biofilm level as strong, moderate, weak and none biofilm-producing was recorded depending on the absorbance value of the crystal violet stain.

The Distribution of the biofilm production isolates

The results of the current study showed that there was no significant association between the ability of bacteria to form biofilm and the specimens that were isolated. Otherwise, 85.2% of male specimens had biofilm production capability ($P < 0.05$). Moreover, there was no significant association between the patient's gender and the strength of the biofilm produced by *K. oxytoca* isolates (30.4%) of male specimens were with strong biofilm by the tube method, while 41.2% of female specimens were strong by the MTP method (Table 2).

Table 2: The association of biofilm formation and the gender of patients with positive *K. oxytoca* isolates.

Biofilm			Gender		*P* – value
			Male	Female	
Biofilm	Positive	NO.	46	34	*P* = 0.161 NS
		%	85.2%	73.9%	
	Negative	NO.	8	12	
		%	14.8%	26.1%	
	Total	NO.	54	46	
		%	100%	100%	
Biofilm by MTP	None	NO.	7	6	*P* = 0.337 NS
		%	15.2%	17.6%	
	Weak	NO.	12	6	
		%	26.1%	17.6%	
	Moderate	NO.	16	8	
		%	34.8%	23.5%	
	Strong	NO.	11	14	
		%	23.9%	41.2%	
	Total	NO.	46	34	
		%	100%	100%	
Biofilm by tube method	None	NO.	4	4	*P* = 0.771 NS
		%	8.7%	11.8%	
	Weak	NO.	17	10	
		%	37%	29.4%	
	Moderate	NO.	11	11	
		%	23.9%	32.4%	
	Strong	NO.	14	9	
		%	30.4%	26.5%	
	Total	NO.	46	34	
		%	100%	100%	

The strength of the biofilm demonstrated by both tube and MTP methods was found to be strong among *K. oxytoca* isolated from urine at 60.9% and 40%, respectively. Subsequently, (4.7%) of sputum specimens were able to form strong biofilm by the tube method, and (16%) of blood specimens by the MTP method.

The result showed that from the eighty isolates that were able to form a biofilm, only 44 (55%) were MDR positive ($P < 0.05$). Furthermore, 12/44 (27.3%) and 13/44 (29.5%) of the isolates were positive for MDR with strong biofilm by the tube method and MTP method, respectively.

In addition, there was no significant association between the ability of *K. oxytoca* isolates to form positive string tests with the specimens' sources, if they were from inpatients or outpatients. Hence, it has been found that (21.1%) of the isolates with positive string test were from community-acquired infections (outpatients), whereas 9.7% of positive string test isolates were from hospital-acquired infections (inpatients).

Other than that, there was no significant relation linking the ability of *K. oxytoca* to form biofilm and whether the isolates were from in- or out-patients. However, inpatient isolates with biofilm were found to be as 79%, whereas outpatient were 81.6% while found (29%) and (41.9%) of isolates with strong biofilm by tube method and MTP method, respectively were isolated from outpatient (Table 3).

Table 3: The association of *K. oxytoca* isolates forming the viscous threads and biofilm with the source of the specimens if they were from in- or out-patients

String & Biofilm			Patient		*P* – value
			In	Out	
String	Positive	NO.	6	8	*P* = 0.112 NS
		%	9.7%	21.1%	
	Negative	NO.	56	30	
		%	90.3%	78.9%	
	Total	NO.	62	38	
		%	100%	100%	
Biofilm	Positive	NO.	49	31	*P* = 0.757 NS
		%	79%	81.6%	
	Negative	NO.	13	7	
		%	21%	18.4%	
	Total	NO.	62	38	
		%	100%	100%	
Biofilm by MTP	None	NO.	8	5	*P* = 0.383 NS
		%	16.3%	16.1%	
	Weak	NO.	13	5	
		%	26.5%	16.1%	
	Moderate	NO.	16	8	
		%	32.7%	25.8%	
	Strong	NO.	12	13	
		%	24.5%	41.9%	
	Total	NO.	49	31	
		%	100%	100%	
Biofilm by tube method	None	NO.	7	1	*P* = 0.069 NS
		%	14.3%	3.2%	
	Weak	NO.	19	8	
		%	38.8%	25.8%	
	Moderate	NO.	9	13	
		%	18.4%	41.9%	
	Strong	NO.	14	9	
		%	28.6%	29%	
	Total	NO.	49	31	
		%	100%	100%	

It is important to mention that the statistical analysis found no significant correlation between the ability of *K. oxytoca* to be as hypermucoviscous, as detected by the string test, and its ability to form biofilm. However, (12/14) 85.7% of the isolates were positive for both the string test and biofilm. Furthermore, (16.7%) and (41.7%) of the isolates with positive string test were with strong biofilm by the tube method and MTP method, respectively (Table 4).

Table 4: The association between the ability of *K. oxytoca* isolates to form the viscous threads and biofilm

Biofilm			String		*P* - value
			Positive	Negative	
Biofilm	Positive	NO.	12	68	*P* = 0.564 NS
		%	85.7%	79.1%	
	Negative	NO.	2	18	
		%	14.3%	20.9%	
	Total	NO.	14	86	
		%	100%	100%	
Biofilm by MTP	None	NO.	4	9	*P* = 0.142 NS
		%	33.3%	13.2%	
	Weak	NO.	2	16	
		%	16.7%	23.5%	
	Moderate	NO.	1	23	
		%	8.3%	33.8%	
	Strong	NO.	5	20	
		%	41.7%	29.4%	
	Total	NO.	12	68	
		%	100%	100%	
Biofilm by tube method	None	NO.	1	7	*P* = 0.755 NS
		%	8.3%	10.3%	
	Weak	NO.	5	22	
		%	41.7%	32.4%	
	Moderate	NO.	4	18	
		%	33.3%	26.5%	
	Strong	NO.	2	21	
		%	16.7%	30.9%	
	Total	NO.	12	68	
		%	100%	100%	

Discussion

K.oxytoca is an opportunistic pathogen that has been recently identified as an actual complex (23). In this study, the data showed that the male specimens were more infected with *K. oxytoca* (54%), than specimens isolated from females (46%) (P = 0.424) This result is quite similar to the study by Karimi K., *et al.*, in Iran(25); who revealed that in 57/83 (68%) cases, *K. pneumoniae* was isolated from men.

Klebsiella spp. infections are more common in men than in women. This could be because men are more likely to drink and smoke than women are, both of which lower immune system efficiency. Furthermore, in comparison to men, women usually exhibit more robust immune responses to foreign antigens (17, 24).

The recorded data of the current study found that 62/100 (62%) were from inpatients (hospitalized-acquired infection), while 38/100 (38%) were from outpatients (community-acquired infection), with a significant correlation ($P < 0.05$). These results were also in line with the study by Alvarez et al. (1985), in the United States, who found that 23/44 (52%) were considered nosocomial-acquired infections and 21/44 (48%) isolates were community-acquired (25). Thus, it is reported that *K. oxytoca* primarily causes hospital-acquired infections, most often involving immunocompromised patients or those requiring intensive care (26). Agricultural soil was a contributing factor to *K. oxytoca*'s isolation from the community-acquired infection. The environment contained various types of microorganisms, especially bacteria, including coliform bacteria (such as *Klebsiella*) (27). Among 100 *K. oxytoca* isolates, 14 (14%) isolates were found to be positive in the string test. These results were similar to a study conducted in India, in 2020, that found 9% (4/12) of *K. oxytoca* was positive in the string test (13). The hypermucoviscous phenotype was believed to be a sign of hypervirulence of the bacteria, and related to severe illness (13, 28). Interestingly, there was a significant link between the source from where the specimens were isolated and the positive isolation of *K. oxytoca*. Most of the *K. oxytoca* was recovered from urine, 39%, followed by blood 16%, and wound swabs 9%. A study by Akter et al.(2014), in Bangladesh, and Manhal et al. (2012), reported that urine is the principal source of *Klebsiella* spp. (29).

Biofilms formed on different biotic and abiotic surfaces are responsible for many infections and can lead to significant health problems (6). Among the 100 tested *K. oxytoca*, 80 (80%) were able to form a biofilm; in which, 74/80 isolates were able to form a biofilm after 24 hours and 6/80, after 72 hours. Slime production was examined depending on the colony morphology produced on the Congo red agar (CRA). It is noteworthy to mention that the potential of the current isolates to form a biofilm was significant, as they were able to form a biofilm after 24 hours of incubation. In comparison, a study conducted by Rajivgandhi et al. (2021), in India, recorded that the ability of *K. pneumoniae* to produce black-colored colonies on CRA plates increased after
3, 6, 12, and 24 hours of incubation (30). This indicated that *K. oxytoca* developed biofilms in a time-dependent manner. Furthermore, the current results are consistent with another study in Turkey, in 2020, which reported 14/19 (73.7%) *K. oxytoca* isolates formed a biofilm that was identified by CRA (6). The strength of the biofilm formation was evaluated by two quantitative methods: The tube method, in which out of 80 *K. oxytoca* isolates 28.75%, 27.5% , 33.75%, and 10% were “strong,” “moderate,” “weak,” and “non-biofilm producer”, respectively. This approach had a good correlation with the microtiter plate method (MTP) in detecting the “strong” biofilm producers. However, distinguishing between “moderate,” “weak,” and “non-biofilm” producers by the tube method proved challenging, because of the variability in the results observed by different observers. In other words, the tube method relied on the intensity of the color developed in the tubes, which could reveal individual variations in obtaining positive results. Similarly, a study done by Abebe et al. (2020), in Turkey, found that out of 19 *K. oxytoca* isolates 68% (13/19) and 16% (3/19) were “strong” and “moderate” biofilm producers, when using the tube method, after 24 hours of incubation, respectively (6). Moreover, the current experiment using the MTP method revealed that 31.25% of *K. oxytoca* isolates were “strong” biofilm producers, 30% were “moderate,” 22.5 % were “weak,” and 16.25% were “non-biofilm” producers, after an incubation period of 24 hours. Even though there were non-significant differences between the results of the tube and the MTP methods in the current study, the MTP was considered a more sensitive method than the tube method. This was because it depended on measuring the optical density of the dye, with no individual interaction. These results were quite similar to a study done in Tikrit, which found that (4/8) 50% of *K. oxytoca* had a “strong” and “moderate” biofilm formation, by using the MTP method (31). Another study conducted by Alkhudhairy et al. (2019), in Iran, recorded that all *K. oxytoca* isolates under study (n = 8) produced a “moderate” level of biofilm, by using the MTP method (32). Thus, all the studies had the same outcome, where *K. oxytoca* isolates were converted from “moderate” to “strong” biofilm producers rapidly, which posed a threat to the local health sector. Biofilm production was influenced by various factors, including the availability of nutrients, environmental conditions, geographical origin, specimen types, surface adhesion qualities, and genetic composition of the organism. Therefore, the production of biofilms could be influenced by many growth conditions, including substrates, incubation times, and other relevant factors (32). This could explain the results obtained in the current study, which found that “strong” biofilm-producing isolates detected by the tube method were more from specimens of urine, 60.9%, and endotracheal tube aspiration, 13%.

Likewise, the strongest biofilm-producing isolates obtained from the MTP method were obtained from urine, 40%, followed by blood, 16%. This could be due to the use of catheters for a long period, especially in hospitals (33), as bacteria were able to stick to the indwelling medical devices, such as catheters and ventilators, because biofilm formation facilitates this adhesion. These devices were anticipated to be covered with host cellular components *in situ* (34). Thus, the results underline the fact that biofilm production was influenced by various factors, including the source of the specimens.

Our study found that 85.2% of the isolates were able to form biofilms in the male specimens, while in the female specimens, it was 73.9%. However, the correlation between the patient's sex and the ability of the bacteria to form a biofilm was statistically insignificant. The strongest biofilm obtained by using the MTP method was found in the female specimens (41.2%). Likewise, a study done in 2021 found that 88% and 50% of isolates that form biofilms were from females and males, respectively. Thus, it may be said that being female is one of the risk factors correlated with biofilm growth (35). Cefotaxim, tetracycline, and ciprofloxacin showed the lowest effect on *K. oxytoca* isolates, while imipenem and gentamicin showed the highest effect. Similarly, a recent study in Iraq found that *K. oxytoca* isolates demonstrated a high level of resistance against amoxicillin (98.5%), cefotaxime (92.6%), cefepime (86.7%), and piperacillin (88.2%). While a lower level of resistance (65.4%) and (59.5%) was reported against tetracycline and ciprofloxacin, respectively (36).

Around 53.8% of the isolates acquired hypervirulent criteria, MDR, and the ability to form a biofilm with a prominent formation of strong biofilm.

It is well known that biofilm formation among antibiotic-resistant *K. oxytoca* isolates becomes an obstacle to the eradication of the pathogen. Biofilm production and antibiotic resistance are of great concern in the treatment of disease and infections. Biofilm highly promotes recurrent and persistent infections, which leads to high mortality rates, In addition to prolonging treatment courses with higher costs (37).

Interestingly, the current data analysis concluded that a positive string test was more common among specimens isolated from outpatients (21.1%) than inpatients (9.7%). This highlights the significance of findings that hyper-virulent bacteria are known to be associated with infections in young and healthy people, that is, the outpatient group (38).

Emphasizing the association between the genetic composition of the organism and its ability to form biofilms (39), the current study demonstrated a link between the hypermucoviscous isolates and being "strong" or "moderate" biofilm producers. Out of the total number of isolates, about 14/100 isolates were hypermucoviscous or hyper-virulent *K. oxytoca*. From these isolates, 5 (41.7%) were "strong" biofilm-producing isolates, by using the MTP method. This result indicated that the hyper-virulent *K. oxytoca* is more likely to form biofilms and that biofilm formation could play a role in developing systemic infection. Biofilms frequently occur on medical devices and hospital surfaces, and their effluents pose a high risk to inpatients, outpatients, and even the community (40). The current data uncovered that *K. oxytoca* biofilm producers were more common among outpatient specimens (81.6%) than inpatient specimens (79%). This variation in the biofilm production may be explained by a significant increase in the virulence of the current isolates that demonstrates, in addition to their biofilm production ability, hypermucoviscous characteristics, more among outpatient specimens.

According to the above findings, *K. oxytoca* biofilm producers are more common among outpatient specimens. The results demonstrated that a "strong" biofilm formation by utilizing both the tube and the MTP methods was noticed among outpatient specimens (29%) and (41.9%) more than inpatient specimens (28.6%) and (24.5%), respectively. This indicated an increase in bacterial virulence. It is well- known that biofilms play an important role in generating chronic infections and modulating host immune responses (41). The current findings may attract the attention of the Iraq health facilities and help them to focus and act on the rapid development of the virulence potency of this new emerging pathogen.

Limitation

The need for a larger specimen with positive *K. oxytoca* isolates to be collected from different governments. A larger specimen could give a complete picture of the progress of the virulence potency of the isolates across both the hospital and community areas.

Conclusions

Klebsiella oxytoca is one of the new emerging pathogens with potential virulence in both the community and the health sectors in Iraq. The strong biofilm producers' isolates were detected more among outpatients' specimens with hypermucoviscous phenotype and prominent antibiotic resistance. Thus, it is developing as a risk of community-acquired infections among healthy individuals. Urine was the source with the strongest biofilm. Biofilm production was influenced by various factors and the method used. The quantitative MTP method was more sensitive and accurate in detecting biofilm formation**.**

Authors' declaration

It has been verified that all the tables and figures included in the manuscript are part of the present investigation. In addition, the manuscript includes permission for the re-publication of the figures and images that are not part of the present study. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local

ethical committee in (Department of Microbiology, College of Medicine/University of Baghdad according to the code number (No. 0234) on (27th / Nov / 2023).

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Study conception & design: (Rand R. Hafidh & Mohammed H. Alsodani). Literature search: (Haneen A. Mustaf). Data acquisition: (Haneen A. Mustaf). Data analysis & interpretation: (Haneen A. Mustaf). Manuscript preparation: (Haneen A. Mustaf). Manuscript editing & review: (Rand R. Hafidh).

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تطوير الأغشية الحيوية والكشف عن النمط الظاهري اللزج المفرط في *K. oxytoca* المعزولة من المرضى الراقدين في المستشفى والمرضى غير الراقدين في المستشفى

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الخلاصة:
خلفية البحث: *Klebsiella oxytoca* هو أحد مسببات الأمراض الناشئة التي تسبب العدوى المكتسبة في المستشفيات والمجتمع. يعتبر النمط الظاهري اللزج المفرط وتطور الأغشية الحيوية علامة على فرط الضراوة، مما يسبب التهابات غازية لدى البالغين الأصحاء. **الاهداف** : تهدف الدراسة الحالية إلى تقييم قدرة عزلات *K. oxytoca* على تكوين الأغشية الحيوية ودمج خاصية اللزوجة المفرطة
المواد وطرق العمل: تم جمع 100 عزلة من *K. oxytoca* من مستشفيات مجمع مستشفيات مدينة الطب التعليمية في بغداد، العراق. تم تشخيص العزلات عن طريق الاختبارات اليدوية والكيميائية الحيوية. تم تحليل خاصية فرط اللزوجة باستخدام اختبار السلسلة. تم الكشف عن تكوين الأغشية الحيوية باستخدام طريقة أجار الكونغو الأحمر النوعي. تم استخدام الطرق الكمية باستخدام صفائح العيار الميكروي والتصاق الأنبوب للكشف عن قوة تكوين الأغشية الحيوية.
النتائج: تم الحصول على *K. oxytoca* من 100/57 (57٪) من الذكور و 100/43 (43٪) من الإناث. وكانت معظم العينات من المرضى الخارجيين 100/68 (68٪) و100/32 (32٪) من المرضى الداخليين. كانت العزلات معنوية من عينات البول 100/39 (39%) تليها عينات الدم 100/16 (16%). حدد اختبار السلسلة 100/14 عزلة على أنها شديدة اللزوجة مع (21.1%) من المرضى الخارجيين. كان الغشاء الحيوي إيجابيا في 100/80 عزلة. كان تكوين الأغشية الحيوية أكثر في عينات المرضى الخارجيين (81.6%). تم الكشف عن التصاق قوي بالطريقة الأنبوبية في 80/22 (28.75%) من العزلات. كشفت طريقة لوحة العيار الميكروي (MTP) عن وجود غشاء حيوي قوي في 80/25 (31.25%) من العزلات. تم العثور على أقوى غشاء حيوي في العزلات من عينات البول (40٪). وقد لوحظت العزلات ذات الأغشية الحيوية القوية بواسطة الأنبوب وطريقة MTP بين العينات المأخوذة من العيادات الخارجية (29%) و (41.9%) على التوالي.
الاستنتاجات: ن احتمالية ضراوة عزلات *K. oxytoca* من خلال تشكيل غشاء حيوي قوي وتطوير مادة لزجة مفرطة المخاطية قد أظهرت خطرا على المجتمع. يعتبر الادرار المصدر الرئيسي لأقوى العزلات المكونة للأغشية الحيوية.
الكلمات المفتاحية: *Klebsiella oxytoca*, الغشاء الحيوي , العدوى المكتسبة من المجتمع؛ string test؛ مفرط اللزوجة