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CHARACTERIZATION, ANTIMICROBIAL PROFILE, AND BIOFILM FORMATION OF *ACINETOBACTER* SPECIES ISOLATED FROM MILK AND HUMAN

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ABSTRACT

One hundred milk samples were obtained from diverse places in Qena City, and fifty urine samples were gathered from patients with Urinary Tract Infections (UTIs) attending various laboratories in Qena City. All the samples were examined for the presence of *Acinetobacter* species on Leeds Acinetobacter Medium (LAM). The suspected colonies were identified through Gram staining and biochemical tests. The *recA* gene was identified using polymerase chain reaction (PCR), which was used for the molecular identification of the *Acinetobacter* species, and the identified species were further characterized by sequencing this gene. Phylogenetic analysis of the *Acinetobacter* species was then conducted. The identified *Acinetobacter* species were tested for antibiotic susceptibility and their ability to produce biofilm. The results of this study showed that 20 out of 150 samples were positive for *Acinetobacter* species using conventional isolation methods. Using PCR techniques, 18 samples were positive for *Acinetobacter*. Sequencing of the *recA* gene for the 18 isolates revealed 7 *Acinetobacter* species, which were identified as 3 *A. baumannii*, 1 *A. nasocomialis*, 1 *A. urisingii*, and 2 *A. johnsonii*. The antibiotic susceptibility testing showed that most *Acinetobacter* species were multi-antibiotic resistant (MAR) and harbored antibiotic resistance genes. The results of biofilm formation testing, using both the Congo red and microtiter plate methods, revealed that most *Acinetobacter* isolates were strong biofilm producers. In conclusion, the presence of *Acinetobacter* species which are highly resistant to antibiotics especially *A. baumannii* in milk and urine samples may threaten public health.

Keywords: *Acinetobacter*, milk, *rec* A, PCR, DNA sequencing

INTRODUCTION

Gram-negative coccobacilli and *Acinetobacter* species are strictly aerobic, non-motile, non-sporulating, non-fastidious, non-fermentative, catalase positive, oxidase negative, and have a DNA G + C content of 39–47% (Peleg *et al*., 2008). This genus has 65 species. (Carvalheira *et al*., 2021).

Acinetobacter species are abundantly dispersed throughout nature. In addition to being present in soil, water, sewage, food, and

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milk, it has long been known to be part of the typical bacterial flora on the skin of both humans and animals (Wani *et al*., 2006).

Acinetobacter species are mostly associated with hospital-acquired infections; nevertheless, these organisms are also responsible for community-acquired illnesses and produce significantly high fatality rates. These bacteria's severe antibiotic resistance and the rise of multidrug-resistant forms make them significant for public health (Carvalheira *et al.,* 2021).

A. baumannii is one of the most prominent members. It emerged as one of the most harmful members of the ESKAPEE group due to its long-term survival in the environment and capacity to acquire multiple antibiotic-resistant mechanisms. The World Health Organization (WHO) classified it as a life-threatening bacterium since it is resistant to most current therapeutic measures, if not all. (Pompilio *et al*., 2021).

Acinetobacter species cause several illnesses in humans, such as endocarditis, pneumonia, bacteremia, meningitis, skin infections, and urinary tract infections, especially in immunocompromised patients. It is characterized by significant morbidity and mortality rates (Carvalheira *et al.*, 2021).

Food is a major reservoir of Gram-negative bacteria that have been isolated from a range of products, such as milk, meat, fish, cheese, fruit, vegetables, and drinking water. These bacteria include *Escherichia coli, Klebsiella* spp., and *Acinetobacter* spp. This indicates that foods may be a key source of *Acinetobacter* dispersion between the community and clinical environments, and it stresses the need for greater research on the potential health concerns connected with *Acinetobacter* spp. as foodborne pathogens (Peleg *et al.,* 2008).

One of the primary issues posed by this bacterium is the development of widespread antibiotic resistance. One of the bacterium's resistance mechanisms is the use of efflux pumps. Using this method, antibiotics are directed outward and cannot harm the bacteria (Tashkan *et al.,* 2020).

Acinetobacter species can produce biofilm, which allows them to live in the hospital environment and cling to various surfaces such as vascular catheters, Foleys catheters, and cerebrospinal fluid shunts (Hong Yang *et al*., 2019).

A statistical correlation exists between biofilm generation and multidrug resistance in clinical isolates. *A. baumannii* has a strong propensity to produce biofilms and is related to various antibiotic resistance (Rao *et al.,* 2008).

So, the current study's goal is to identify *Acinetobacter* species in milk and human urine samples, as well as to assess their antibiotic resistance profiles, some antibiotic resistance genes, and *Acinetobacter* isolates biofilm development.

MATERIALS AND METHODS

Sample collection:

A total of 100 raw milk samples were obtained from various locations in Qena City, Egypt. Samples were sent directly to the Microbiology Laboratory at South Valley University's Faculty of Veterinary Medicine to be tested for *Acinetobacter* species (Saad *et al.,* 2018), and 50 urine samples were collected from individuals with UTIs who visited several medical laboratories in Qena, Egypt. The samples were transferred to the lab in sterile containers. The samples were processed shortly after the delivery using aseptic procedures (Nag *et al*., 2021).

Isolation and identification of *Acinetobacter* species*:*

One milliliter of fully mixed milk and urine samples was added to the nutritional broth and incubated at 37°C for 24-48 hours (Jawad *et al.,* 1994). A loopful of broth cultures was placed on LAM plates and incubated at 37°C

for 24-48 hours. The colonies of *Acinetobacter* species were pink with a mauve backdrop. These distinctive colonies were purified by subculturing onto tryptic soya agar plates and cultured aerobically at 37°C for 18-24 hours. The suspected isolates were then subjected to bacterial identification (Jawad *et al.*, 1994), by Gram staining as was described earlier (Quinn *et al*., 2002), and biochemically through catalase, oxidase, sugar fermentation, T.S.I, and citrate utilization tests (Leboffe and Pierce, 2011; Brown and Smith, 2015).

Molecular identification of *Acinetobacter* species by PCR:

PCR technique was used for the identification of the suspected *Acinetobacter* species through the detection of the *rec*A gene and the used primer (Chiang *et al.,* 2011) *rec*A F CCTGAATCTTCTGGTAAAAC and *recA* R GTTTCTGGGCTGCCAAACATTAC with product size 425 bp.

The QIAamp DNA Mini Kit instructions were followed for DNA extraction. 1µl of forward primers and 1µl of reverse primers for each gene independently were included in a 25µl uniplex reaction mixture together with 12.5µl of Emerald Amp GT PCR master mix (2x premix), 6µl of template DNA, and 4.5µl of PCR grade water. The condition of the cycling was as follows: For 35 cycles, there are five stages of denaturation: primary for 5 min at 94˚C, secondary for 30 sec at 94˚C, annealing for 40 sec at 50˚C, extension for 45 sec at 72˚C, and final extension for 10 min at 72˚C. The products that were amplified underwent agarose gel electrophoresis (1.5%) with 0.5 mg of ethidium bromide per milliliter (Sigma). The results were observed and captured on camera using ultraviolet light. By comparing the product amplicon sizes to a 100 bp DNA ladder (Fermentas. cat. no. SM0243). The sizes of the products were approximated.

Sequencing of *rec*A Gene:

A purified PCR product was sequenced in both the forward and reverse directions using an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA), Cat. No. 4336817.

Phylogenetic analysis of *Acinetobacter* species:

Phylogenetic analysis was done on our isolates. It uses the *recA* gene nucleotide sequence to determine the identification of these isolates with one another and with reference strains recorded with GenBank. To conduct a comparative analysis of the sequences, we used the CLUSTAL W multiple sequence alignment program and the MegAlign module of Lasergene DNA Star software Pairwise (Madison, Wisconsin, USA) version 12.1. (Thompson *et al*., 1994) and phylogenetic studies were done using maximum likelihood, neighbor-joining, and maximum parsimony in MEGA7 (Tamura *et al*., 2013).

Antimicrobial susceptibility testing:

Using the disc diffusion method, antimicrobial susceptibility tests were conducted according to the Clinical and Laboratory Standard Institute standards (CLSI, 2021). Sensitivity pattern of the isolates was determined against the following antibiotic discs: Tetracycline (30 mcg), Ampicillin- Sulbactam (10/10 mcg), Doxycycline (30 mcg), Amikacin (30 mcg), Piperacillin (100 mcg), Gentamycin (10 mcg), Ciprofloxacin (5 mcg), Meropenem (10 mcg) and Cefotaxime (30 mcg). Antimicrobial susceptibility testing findings were reported as sensitive, intermediate, and resistant according to zone diameter interpretation guidelines (CLSI, 2021). The Multi-Antibiotic Resistance (MAR) index is calculated by dividing the number of resistant antibiotics in an isolate (a) by the total number of antibiotics used in the research (b). (Ayandele *et al*., 2020).

Detection of some antibiotic-resistant genes:

The antibiotic genes detected in this study were the β-lactamases gene *blaOXA* and

Metallo-β-lactamases (MBL) genes *blaIMP, blaVIM,* and *blaNDM*. The uniplex PCR method was used to amplify genes. Using a thermal cycler (MJ Research, Inc. Watertown), a final volume of 25 µL was used for the amplification. It contained 12.5 µL of PCR Master mix (Emerald Amp GT), 1 µL for each primer (Table 1), 1.5 µL of the DNA template, and 9 µL of PCR-grade water. The optimal cycling conditions are shown in (table. 2). The bands were detected on 1.5% agarose gel electrophoresis.

Detection of biofilm production by *Acinetobacter* species:

Biofilm production ability was detected by microtiter plate methods (quantitative method) (Mathur *et al.,* 2006) as follows: Tryptic soya broth with 0.25% glucose was used to dilute the overnight cultures 1:200. 200µl of each dilution was then planted per well in a sterile 96-well polystyrene microtiter plate, and the plate was cultured at 37°C for eighteen hours. The adherent biofilms were dyed with 1% crystal violet for one minute and the wells were dried for one hour at 60°C following three washes in phosphate buffer saline (PBS) (PH 7.2). The adhering biofilm's absorbance was measured at 620 nm in a microplate reader after being rinsed three times with distilled water and dried at room temperature. The interpretation of biofilm production was computed (Ansari *et al*., 2014): Since all tests were performed in triplicate and repeated three times, the average OD values for all tested isolates and the negative control should be determined. The average OD values of all tested isolates were compared to the optical density cut-off value (ODc).

ODc = the average OD of the negative control plus three times the negative control's standard deviation (SD). Biofilm production was determined as follows: OD ≤ ODc indicates no biofilm production, ODc < OD ≤ 2×ODc indicates weak biofilm production, 2×ODc <OD ≤ 4×ODc indicates moderate biofilm production, and 4×ODc <OD indicates high biofilm production. In addition, the Congo red agar method (qualitative method) was used to determine biofilm generation (Alamrie *et al*., 2020). As slime, all isolates' production was investigated by cultivating on Congo Red Agar (CRA) plates, which were produced by adding 0.8g of Congo red dye and 36g of sucrose to 1L of BHI (Oxoid) and then incubated at 37°C for 24 hours. Slime-generating strains produced black colonies with a dry crystalline quality after incubation, while non-slime-producing strains produced red smooth colonies.

Statistical analysis:

Statistics analysis was done by using the Statistical Package for the Social Sciences (SPSS) v28, and Graph Pad Prism v 9.5.1 (733). $P < 0.05$ was considered statistically significant for all tests. (Qi *et al*., 2016).

RESULTS

Results of isolation and identification of *Acinetobacter* species:

In the present study, 9 of 100 milk samples and 11 of 50 urine samples tested positive for *Acinetobacter* species. Their cultural characteristics were determined through LAM; Gram staining, and biochemical identification. They have pink colonies on LAM, microscopically, they appear as gram-negative bacilli; and biochemically, they were catalase positive and oxidase negative and showed variability in TSI, citrate utilization, and sugar fermentation.

Results of molecular identification of *Acinetobacter* species:

Molecular identification of *Acinetobacter* species by PCR:

All 20 suspected isolates from milk and human urine samples were identified by PCR using *recA* gene specific for *Acinetobacter* species, about 7 isolates were positive for *Acinetobacter* from milk samples and 11 isolates were positive for *Acinetobacter* from urine samples (Figure- 1).

Molecular identification of *Acinetobacter* species by sequencing:

Through sequencing the PCR products of *recA* gene of the 18 isolates, there were 7 isolates identified as typical *Acinetobacter* and isolated species were three *A. baumanii*, one *A. nosocomialis*, one *A. ursingii* and two *A. johnsonii*. The identified species were recorded in the Gene Bank and their accession number is shown in (Table 3).

Phylogenetic analysis of *Acinetobacter* species:

The phylogenetic analysis from partial *recA* gene sequences of the seven *Acinetobacter* species isolated show minor diversity in branching in the reconstructed tree as shown in (Figure 2).

The Phylogenetic tree showed the degree of identity between isolated *Acinetobacter* species and the *Acinetobacter* species available in Gene Bank, Gene Bank accession numbers are indicated at the end. Statistical support for internal branches of the tree was evaluated by bootstrapping.

The isolated *Acinetobacter nosocomialis* is identical with *Acinetobacter nosocomialis* CP040105 with identity percent 100%, all *Acinetobacter baumanii* isolates were identical to each other and grouped with *Acinetobacter baumanii* CP050385 in Gene Bank and the percent of identity were 100%. And the isolated *Acinetobacter ursingii* is similar to *Acinetobacter ursingii* CP089051 with identity percent 100%. And the two isolated Acinetobacter *johnsonii* species were similar to each other with an identity of 100% and grouped with *Acinetobacter johnsonii* CP090180 in the Gene bank database.

Results of antibiotic susceptibility testing of isolated *Acinetobacter* species:

Antibiotic sensitivity for the *Acinetobacter* species (n=7) was tested against 9 antibiotics through the disc diffusion method (table 4).

Some *Acinetobacter* species show resistance to some antibiotics such as Piperacillin (100%), Cefotaxime (85.7%), Tetracycline and Gentamycin (57.1% for each), Ampicillin/ Sulbactam and Ciprofloxacin (42.8% for each). And some species show susceptibility to other antibiotics as Amikacin (85.7%) and Meropenem (71.4%).

Most *Acinetobacter* species show distinctive resistance to several antibiotics with high MAR index as shown in (table 5). *Acinetobacter johnsonii (II)* exhibit resistance to 8 antibiotics (Piperacillin, Cefotaxime, Gentamycin, Ampicillin/ Sulbactam, Tetracycline, Amikacin, Ciprofloxacin and Meropenem) with high MAR index 0.8. *Acinetobacter* johnsonii (I) develop resistance to 7 antibiotics (Piperacillin, Cefotaxime, Gentamycin, Ampicillin/ Sulbactam, Doxycycline, Ciprofloxacin and Tetracycline) with MAR index 0.7, *Acinetobacter baumanii* (III) showed resistance to 5 antibiotics (Piperacillin, Ampicillin/Sulbactam, Tetracycline, Cefotaxime and Meropenem with MAR 0.5. While *Acinetobacter baumanii* (I) showed resistance to 4 antibiotics (Piperacillin, Gentamycin, Ciprofloxacin and Cefotaxime) with MAR 0.4, *Acinetobacter ursingii* developed resistance to 3 antibiotics (Piperacillin, Tetracycline and Cefotaxime) with MAR 0.3. And both *Acinetobacter baumanii* (II) and *Acinetobacter nosocomialis* showed resistance to 2 antibiotics (Piperacillin and Cefotaxime) and (Piperacillin and Gentamycin), respectively with MAR index 0.2 for both.

PCR results for the occurrence of carbapenem resistance genes among *Acinetobacter* species:

The PCR results showed that among the 7 *Acinetobacter* species detected, there were 5 (71 %) species that have *bla*OXA and *bla*VIM genes. And 4 (57 %) species have *bla*IMP and *bla*NDM genes as shown in Figure 3.

Results of biofilm production of isolated *Acinetobacter* species:

Among the isolated *Acinetobacter* species, four isolates (57.1%) produced black colonies on Congo red agar while three (42.8%)

isolates produced red colonies. While by microtitre plate method all the isolated species were biofilm producers (four strong, two moderate, and one weak) (Figure 4).

Correlation between antibiotic resistance and biofilm production of isolated *Acinetobacter* species:

A strong significant correlation was noticed between biofilm formation and antibiotic resistance (r=0.689, and p= 0.043) (Figure 5). The biofilm production ability and the antibiotic resistance for each isolate were described in the heat map (Figure 6)

Correlation between antibiotic resistance and antibiotic resistance genes of isolated Acinetobacter species:

No- significant correlation was noticed between Antibiotic resistance and Antibiotic resistance gene (chi-square = 0.237 and p-value = 0.609), the p-value is significant at ≤ 0.05 Table (6)

Table 1: Oligonucleotide primers sequences for Metallo-β-lactamases genes.

Gene	Sequence	Amplified product	Reference
blaIMP	CATGGTTTGGTGGTTCTTGT	488 bp	**Xia *et al*., 2012**
	ATAATTTGGCGGACTTTGGC		
blaVIM	AGTGGTGAGTATCCGACA	280 bp	
	ATGAAAGTGCGTGGAGAC		
blaNDM	AAC GGT TTGGCG ATC TGG TTT TC	627 bp	**Hatrongjit *et al.*, 2023**
	GGC GGA ATG GCT CAT CAC GAT C		
BlaOXA	TCAACTTTCAAGATCGCA	609 bp	**Colom *et al*., 2003**
	GTGTGTTTAGAATGGTGA		

Table 2: cycling condition of the primers during PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
***bla*IMP**	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
***bla*VIM**	94˚C 5 min.	94˚C 30 sec.	53˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
***bla*NDM**	94˚C 5 min.	94˚C 30 sec.	62.5˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
***bla*OXA**	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

Table 3: The Accession number of the isolated *Acinetobacter* species

Identified species	Accession number
Acinetobacter nosocomialis	OR103122
Acinetobacter baumanii	OR103123
Acinetobacter baumanii	OR103124
Acinetobacter johnsonii	OR103125
Acinetobacter johnsonii	OR103126
Acinetobacter baumanii	OR103121
Acinetobacter ursingii	OR103120

Table 4: Results of sensitivity of different *Acinetobacter* isolates to different antibiotic discs.

Antibiotic disc	Sensitive		Intermediate		Resistant	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Piperacillin (PRL)	-	-	-	-	7	100
Ampicillin/Sulbctam (A/S)	2	28.5	2	28.5	3	42.8
Tetracycline (TE)	3	42.8	-	-	4	57.1
Doxycycline (DOX)	3	42.8	3	42.8	1	14.2
Gentamycin (GEN)	3	42.8	-	-	4	57.1
Amikacin (AK)	6	85.7	-	-	1	14.2
Cefotaxime (CTX)	1	14.2	-	-	6	85.7
Ciprofloxacine (CIP)	3	42.8	1	14.2	3	42.8
Meropenem (MEM)	5	71.4	-	-	2	28.5

Table 5: Antibiotic resistance pattern for isolated *Acinetobacter* species.

Acinetobacter species	Antibiotic resistance patterns	MAR index
Acinetobacter johnsonii (II)	Piperacillin, Cefotaxime, Gentamycin, Ampicillin/Sulbactam, Tetracycline, Amikacin, Ciprofloxacin and Meropenem	0.8
Acinetobacter johnsonii (I)	Piperacillin, Cefotaxime, Gentamycin, Ampicillin/Sulbactam, Doxycycline, Ciprofloxacin and Tetracycline	0.7
Acinetobacter baumanii (III)	Piperacillin, Ampicillin/Sulbactam, Tetracycline, Cefotaxime and Meropenem	0.5
Acinetobacter baumanii (I)	Piperacillin, Gentamycin, Ciprofloxacin and Cefotaxime	0.4
Acinetobacter ursingii	Piperacillin, Tetracycline and Cefotaxime	0.3
***Acinetobacter baumanii* (II)**	Piperacillin and Cefotaxime	0.2
Acinetobacter nosocomialis	Piperacillin and Gentamycin	0.2

Table 6: Correlation between antibiotic resistance and antibiotic resistance genes of isolated Acinetobacter species

		Antibiotic resistance	Antibiotic resistance gene
Antibiotic resistance	Pearson Correlation	1	0.237
	Sig. (2-tailed)		0.609
	N (Isolates)	7	7
Antibiotic resistance gene	Pearson Correlation	0.237	1
	Sig. (2-tailed)	0.609	
	N (Isolates)	7	7

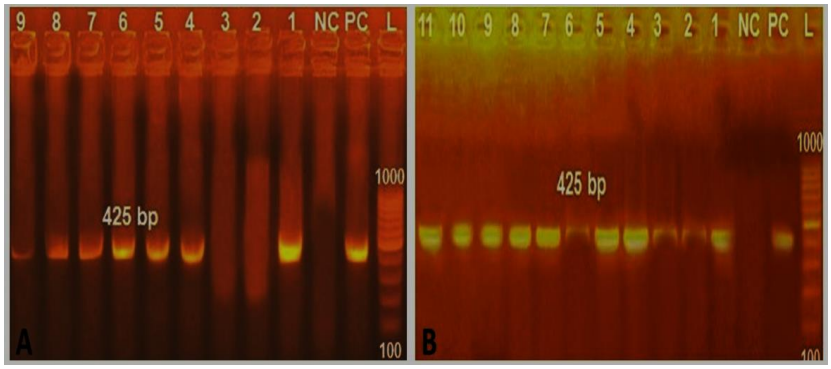
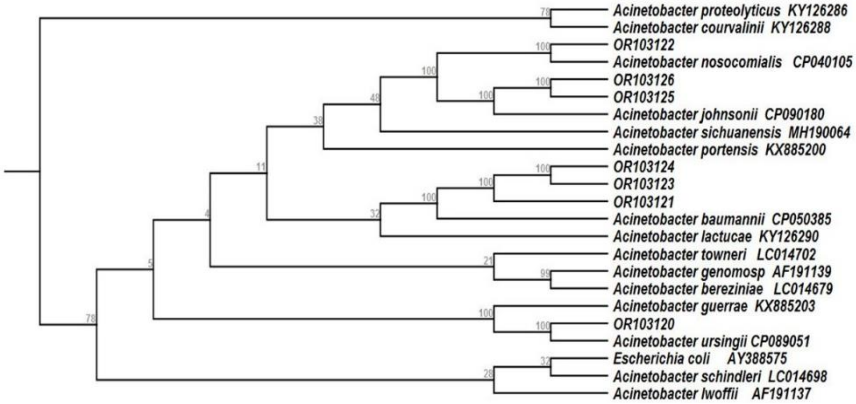


Figure 1: PCR products of *Acinetobacter* isolates from milk samples (A) and human urine samples (B) using primers targeting 425bp of *recA* gene. The products were electrophoresed in 1.5% agarose gel and stained by 0.5μg/ml ethidium bromide. PC: positive control is *Acinetobacter baumannii* ATCC 19606, NC: negative control is *Escherichia coli* (0157:H7) ATCC® 43888, L: 100bp DNA ladder, for the milk samples (A): Lanes 1,4-9 positive *Acinetobacter* isolates, Lanes 2,3 negative isolates. And for the human urine samples: lanes 1-11 in (B) are positive for *Acinetobacter* isolates.



F**igure 2:** Phylogenetic tree showing the position of the isolated *Acinetobacter* species compared to other *Acinetobacter* species available in the Gene Bank database.

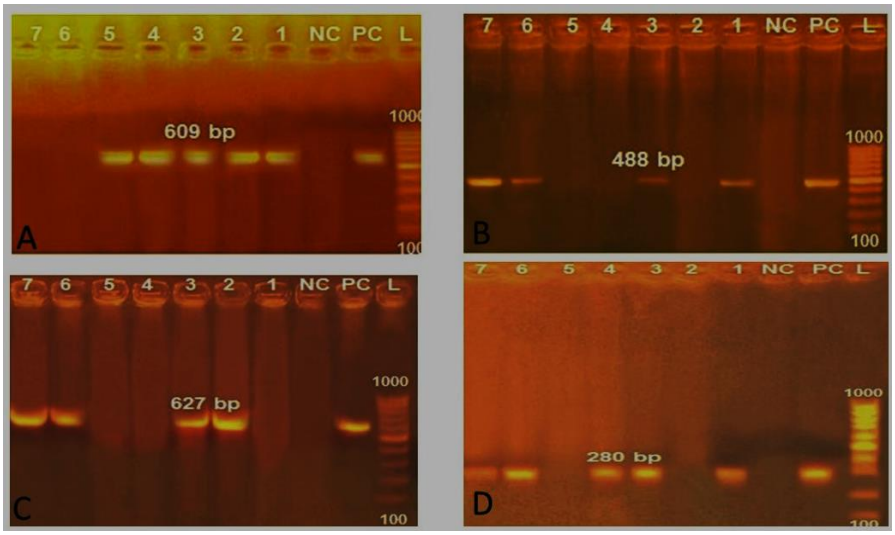


Figure 3: PCR Products of *Acinetobacter* isolates tested for the presence of Carbapenem resistance genes using primers targeting 609 bp of *blaOXA* gene (A), 488 bp of *blaIMP* gene (B), 627 bp of *blaNDM* gene (C) and 280 bp of *blaVIM* gene (D). The products were electrophoresed in 1.5% agarose gel and stained by 0.5 μg/ml ethidium bromide. L: 100bp DNA ladder, PC: positive control, NC: negative control, Lanes 1-5 (+*blaOXA*), Lanes 1,3,6,7 (+*blaIMP*), Lanes 2,3,6,7 (+*blaNDM*) and Lanes 1,3,4,6,7 (+*blaVIM*).

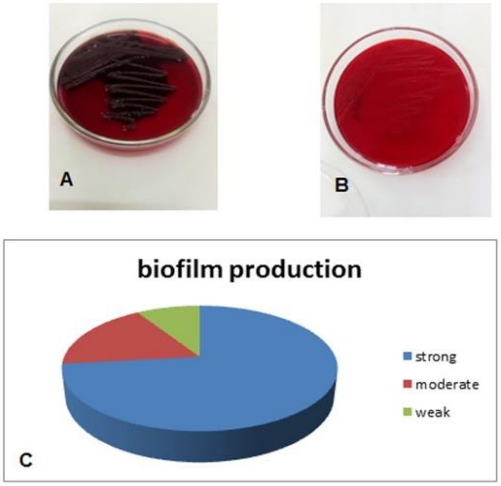


Figure 4: biofilm production of *Acinetobacter* isolates by Congo red agar (A: black colonies, B: red colonies) and a pie chart of biofilm formation by the isolates using microtiter plate (C).

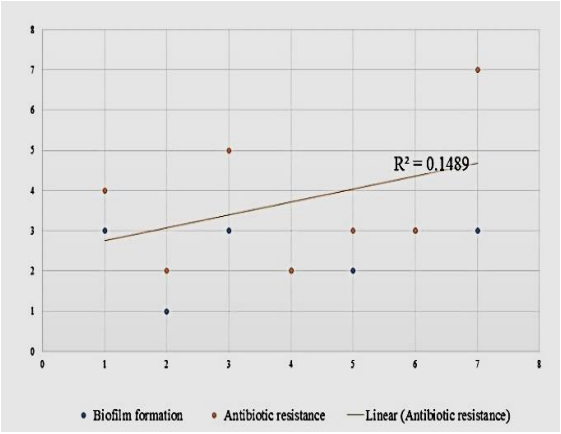


Figure 5: Antibiotic resistance vs biofilm formation.

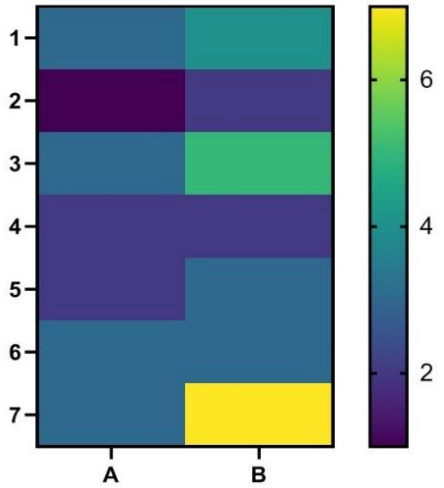


Figure 6: Heat map for the 7 isolates Biofilm formation (A), Antibiotic resistance (B).

DISCUSSION

Acinetobacter species are considered a serious threat to the health of humans because of their extended environmental survival and quick development of resistance to a broad spectrum of antibiotics. Furthermore, they can spread by direct contact between people, contaminated water, and contaminated food (Doughari *et al.*, 2011).

Acinetobacter species have recently become a widespread cause of nosocomial infections. It is recognized as a ubiquitous organism. However, there is little data available on the incidence of these species in food products and drinking water. Multidrug-resistant *Acinetobacter* spp. was isolated from food products (Carvalheira *et al*., 2021).

The results showed that 9% of milk samples were suspected to have *Acinetobacter* on Leeds Acinetobacter Agar. This result is in the same line with (Nam *et al.*, 2009) who isolated *Acinetobacter* spp. in a percentage of 8.2%. Some authors reported the isolation of *Acinetobacter* in a higher incidence (Güneri, 2023), who isolated *Acinetobacter* in a percentage of 21.6% of tank milk samples, (Saad *et al.*, 2018) reported that *Acinetobacter* species were detected in 13.3% of dairy shops' raw milk samples, and (Mohamed *et al*., 2022) who reported that 13% of collected milk samples were suspected to have *Acinetobacter* spp. On the other hand, Rafei *et al*. (2015) isolated *Acinetobacter* in a lower incidence as he reported that *A. baumannii* was detected in 2.7% of milk samples. The difference in the isolation percentage of *Acinetobacter* species from milk may refer to the geographical difference and the type of milk samples collected.

The present study showed that 20% of human urine samples were suspected to have *Acinetobacter* on Leeds Acinetobacter Media. Other study and (Sheck *et al*., 2023) reported that the *Acinetobacter non*

baumanii (Anb isolates) were most often isolated from clinical samples of hospitalized patients with urinary tract infections 27.8% and (Colquhoun and Rather, 2020) reported that 17.1% of *A. baumannii* isolated from clinical studies over the last two decades were obtained from urinary samples which nearly agree with our results. Lower incidence was reported by (Mwanamoonga *et al*., 2023) who found that out of 40 *Acinetobacter* isolates identified from different clinical patient samples, only one isolate was isolated from patients with urinary tract infection and) Taha *et al.,* 2023) who reported that *Acinetobacter baumanii* was recovered from 3 of 39 (4.4%) urine samples taken from patients. These differences may be due to the difference in the infection control measures applied in hospitals and the hygienic measures in the community.

Leeds Acinetobacter Media (LAM) is a differential medium and supports the growth of *Acinetobacter* species selectively (Benoit *et al*., 2020). It contains cefsulodin and cephradine to inhibit the growth of Gram-negative bacteria and vancomycin to prevent Gram-positive growth. LAM also contains fructose and sucrose, which are not fermented by *Acinetobacter* species, resulting in pink coloration of the medium upon growth of *Acinetobacter* species (McConnell *et al*., 2011).

The original description of LAM by (Jawad *et al.,* 1994) reported that other bacteria as *Stenotrophomonas, Burkholderia, Citrobacter*, and *Serratia* species could grow on LAM. and (McConnell *et al*., 2011) found that LAM permits the growth of *Klebsiella pneumoniae* in addition to *A. baumannii*. These findings are in accordance with our results in isolation of other species other than *Acinetobacter* species on Leeds Acinetobacter Agar as Lactococcus *lactis, Proteus terrae* and *Enterococcus faecium.*

The suspected isolates were subjected to PCR through detection of *rec*A gene, it showed that 7 isolates were detected from milk samples and 11 isolates from human urine samples this referred to that PCR technique is a highly sensitive technique.

*Rec*A gene is employed as response control in PCR assays because it is highly conserved among *Acinetobacter* species (Krawczyk *et al.,* 2002). These results were consistent with those of prior investigations as (Chen *et al.,* 2014; Chen *et al*., 2007) who found that all *Acinetobacter* species had the *rec*A gene. For a significant number of *Acinetobacter* isolates, the *rec*A genotypic method is a good first screening technique (Krawczyk *et al*, 2002) and other molecular techniques as sequencing, should be used as the final confirming step.

So, in the present study, the PCR products of the *rec*A gene for the 18 isolates (All isolates) were sequenced in the forward and reverse directions. It showed that 7 Acinetobacter isolates were detected, and the isolated species were *A. baumanii, A. nosocomialis, A. johnsonii* and *A. ursingi*. As the RecA protein is both ubiquitous and well conserved among a range of prokaryotes. This result agrees with (Güneri, 2023) who isolated *A. baumannii, A. ursingii* from tank milk samples, (Rafei *et al.,* 2015) who isolated *A. baumannii* in raw milk samples and (Ramos and Nascimento 2019) who identified *A. ursingii* from milk samples. The results are also in the same line with (Sheck *et al*., 2023) who isolated *A.johnsonii* from clinical samples and reported that the *Acinetobacter non baumanii* (*Anb* isolates) were most often isolated from clinical samples of hospitalized patients with urinary tract infections (UTIs) , (Taha *et al.,* 2023) who reported that *A. baumanii* was recovered from 3 of 39 (4.4%) urine samples taken from patients and (Pour *et al*., 2011) who isolated *A. baumannii* and *A. lwoffii* from

urinary tract infections and urinary catheter samples.

The Phylogenetic analysis of *rec* A sequences showed that the isolated species in the present study are highly identical to each other as all of them originated from the same family (Kuhnert *et al.,* 2011). The isolated *A. nosocomiali*s is similar to the *A. nosocomialis* CP040105 which was earlier reported by (Carruthers *et al.,* 2013) and isolated in 1996 from a hip infection of a patient at Cleveland Metro Health Systems (Cleveland, OH) in the USA, The *A. baumanii* isolates are identical to each other and are identical with *A. baumanii* CP050385 which isolated by (Veeraraghavan *et al*., 2020) from blood samples of patients with bloodstream infection in India, the *A. ursingii* isolate is similar to *A. ursingii* CP089051 which was isolated by (endrickx *et al.,* 2022) from Homo sapiens in Netherlands and *A. johnsonii* isolates are identical and similar to *A. johnsonii* CP090180 which were isolated by (liu *et al*., 2021) from patients in China. These results indicate the origin of our isolates.

Antibiotic resistance is the main problem of *Acinetobacter* bacterium. We detected a significant level of antibiotic resistance brought on by this bacterium as a result of people using broad-spectrum antibiotics carelessly. Numerous antibiotic resistance genes are proliferating in this bacterium, which is linked to its high level of antibiotic resistance. According to several studies, *A. baumannii* is becoming more resistant to aminoglycoside antibiotics and is resistant to most beta-lactam antibiotics and quinolones (Vázquez-López *et al*., 2020, Kyriakidis *et al*., 2021).

In this study, the isolates were tested for antimicrobial susceptibility through disc diffusion method. It showed the highest resistance to Piperacillin (100%), Cefotaxime (85.7%), Tetracycline (57.1) and Gentamycin (57.1) and it showed low resistance to Amikacin (14.2%), Doxycycline (14.2%), Meropenem (28.5%), Ciprofloxacin (42.8%) and Ampicillin/Sulbactam (42.8%). The highest resistance to Piperacillin (100%) is agreement with (Basatian-Tashkan *et al*., 2020) and (Noori *et al*., 2019) who found that all isolates were resistant to Piperacillin (100%). Other studies include (Abdar *et al*. 2019) who found that the resistance to Meropenem and Ceftazidime was 71% and 93%, respectively. Another study by (Ranjbar *et al*., 2019) showed that Ceftazidime, Ciprofloxacin, Piperacillin, Gentamicin, Amikacin, and Ampicillin/ Sulbactam antibiotic resistance percentages were, in order, 97.5%, 96.3%, 95.1%, 92.1%, 87.2%, and 76.1%. Also (Boral *et al*., 2019) reported that antibiotic resistance was 100%, 99.4%, 99.4%, and 91.8% for Ceftazidime, Amikacin, Ampicillin/ Sulbactam, and Ciprofloxacin, respectively. When compared to the current study, these studies demonstrate an increase in antibiotic resistance. The variation in antibiotic resistance between prior studies and ours could be attributed to the different geographic locations of samples collected.

Class D beta-lactamases, also known as carbapenem-hydrolyzing class D β-lactamases (CHDLs) or oxacillinases (OXA), are the primary mechanism of carbapenem resistance and can inactivate all beta-lactams, primarily those in the OXA-10 family. (Antunes and Fisher, 2014). Both chromosomes and plasmids include the genes (*bla*OXA) that code for these enzymes (Wang *et al.,* 2021).

In the present study the antibiotic-resistant gene *bla*OXA, was amplified in (71 %) of the isolated species and this percent includes all *A. baumannii* isolated in the study. These findings were identical to previous studies as (Mohamed *et al*., 2022, Almasaudi 2018 and Patrice *et al*., 2011) who reported that 100% of *A. baumannii* had *bla*OXA-51-like genes and (Turton *et al*., 2006) who found that the

blaOXA51-like genes were crucial in helping to distinguish *A. bummannii* from other *Acinetobacter* species.

The spread of *Acinetobacter s*pp. producing metallo-beta-lactamase (MBL) has become a major global public health concern (Papp-Wallace *et al.,* 2011). The MBLs genes detected in the present study were *bla*IMP, *bla*VIM, *bla*NDM. The results of our study showed that (57 %) of the isolated species had *blaIMP* and *bla*NDM. Additionally, (71%) of the isolated species had *bla*VIM.

The results of the present study were nearly similar to the results of (Silago *et al*., 2022) who reported that the *bla*IMP was present in 45.5% of the isolated species and similar to the study of (Bansal *et al.,* 2020) who found that the MBL gene (*bla VIM*) present in 71% of the isolates by PCR analysis. Previous studies as (Goudarzi *et al.,* 2019, Milillo *et al.,* 2013) found that although the genes of *blaIMP* and *blaVIM* were detected in the clinical samples, there was no amplification of *blaNDM* in clinical isolates of *Acinetobacter*, and (Mohamed *et al*., 2022) who found that the *blaIMP* and *blaNDM* genes were amplified in one and two isolates, respectively, but not the *blaVIM* gene. These researchers did not agree with the current study. These discrepancies could be due to changes in antibiotic use patterns, the type of collected sample, the number of samples investigated, the sampling method, environmental factors, or the geographical distribution of these genes.

Biofilm is a community of microbial cells encased in a polysaccharide matrix; this structure provides the ideal condition for the exchange of genetic material amongst the various microorganisms (Donlan, 2002). Biofilm production has been linked to the survival of harmful bacteria in the hospital environment, as well as illnesses associated with indwelling medical equipment. Also, it protects the bacterial communities from environmental hazards and protects the bacteria itself from the action of disinfectants and detergents (Branda *et al.,* 2005).

In this study, biofilm production was assessed using the CRA and microtiter plate methods. The CRA method identified 57.1% of isolates as biofilm producers and 42.8% as non-biofilm producers, whereas the microtiter plate method indicated that all the isolates were biofilm producers (four strong, two moderate, and one weak). This referred to that the microtiter plate method is a more sensitive technique than the Congo red agar method for detecting biofilm formation which agrees with (Melo *et al*., 2013), who noted that the microtiter plate test had a 100% sensitivity while the Congo red agar test had an 88.9% sensitivity. The present study's outcome agrees with that of (Malta *et al*., 2020) who found that all A. *baumannii* strains isolated from goat milk can produce biofilm. Other authors as (Rao *et al*., 2008) and (Rodroguez *et al.* 2008) reported that the *Acinetobacter* isolates which produce biofilm were only 62% and 63%, respectively which were not in agreement with our study.

Statistical analysis of the data shows a strong association between the ability to form biofilms and antibiotic resistance. As all the bacteria in this study that were capable of forming biofilms were multidrug-resistant, this outcome is consistent with the study conducted earlier (Hassan *et al.,* 2011). Additionally, we observed that bacteria with low biofilm-forming powers exhibited antibiotic resistance and this is like the results of other studies (de Breij *et al*., 2009; Freeman *et al.,* 1989; Abdi-Ali *et al*., 2014). It referred that antibiotic resistance may result from several pathways, such as the presence of β-lactamases, modifying enzymes, permeability defects, alteration of target sites, and multidrug efflux pumps as suggested by (Abdi *et al.*,2020)

CONCLUSION

In conclusion, molecular approaches, such as DNA sequencing, are ideal for identifying *Acinetobacter* species because they are more sensitive than traditional methods and can accurately distinguish between closely related species. The identification of multiple drug-resistant *Acinetobacter* species from foods such as milk highlights the importance of food supply in the potential spread of this species in both community and clinical settings. Further research is necessary to investigate antibiotic resistance in *Acinetobacter* species.

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توصيف وخصائص مضادات الميكروبات وتكوين الأغشية الحيوية لأنواع *Acinetobacter* المعزولة من الحليب والإنسان

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تم جمع ١٠٠ عينة حليب من مواقع مختلفة بمدينة قنا، كما تم جمع ٥٠ عينة بول من مرضى التهابات المسالك البولية المترددين على المعامل المختلفة بمدينة قنا. تم فحص جميع العينات للتأكد من وجود أنواع *Acinetobacter* على وسط ليدز .Acinetobacter تم التعرف على المستعمرات المشتبه بها من خلال صبغه الجرام والاختبارات البيوكيميائية. وتم إجراء التعرف الجزيئي لأنواع *Acinetobacter* باستخدام تفاعل البوليميراز المتسلسل (PCR) للكشف عن جين*recA* ، وتم تمييز الأنواع المحددة أيضًا عن طريق تسلسل هذا الجين. ثم تم إجراء التحليل الوراثي لأنواع *Acinetobacter*. تم اختبار أنواع *Acinetobacter* التي تم تحديدها للتأكد من حساسيتها للمضادات الحيوية وقدرتها على إنتاج الأغشية الحيوية. أظهرت نتائج هذه الدراسة أن ٢٠ من أصل ١٥٠ عينة كانت إيجابية لأنواع *Acinetobacter* باستخدام طرق العزل التقليدية. وباستخدام تقنياتPCR ، كانت ١٨ عينة إيجابية لبكتيريا Acinetobacter. كشف تسلسل جين *recA* للعزلات الـ ١٨ عن ٧ أنواع من بكتيريا*Acinetobacter* ، والتي تم تحديدها على أنها ٣*A. baumannii* ، ١ *A. nasocomialis*، ١ *A. urisingii*، و٢ *A. johnsonii*. أظهر اختبار الحساسية للمضادات الحيوية أن معظم أنواع *Acinetobacter* كانت مقاومة للمضادات الحيوية المتعددة (MAR) وتحتوي على جينات مقاومة للمضادات الحيوية. كشفت نتائج اختبار تكوين الأغشية الحيوية، باستخدام كل من طريقتي لوحة الكونغو الحمراء والميكرو تيتر ، أن معظم عزلات *Acinetobacter* كانت منتجة للأغشية الحيوية القوية. نستنتج من ذلك أن وجود بكتيريا *Acinetobacter* ذات المقاومة العالية للمضادات الحيوية وخاصة *A. baumannii*في عينات الحليب والبول قد يهدد الصحة العامة.