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Carbapenem-resistant *Acinetobacter baumannii* in Raw Milk from Egyptian Dairy Farm Animals with Subclinical Mastitis

Sohad M Dorgham[1], Amany A Arafa*[1], Eman S Ibrahim[1] and Abeer M Abdalhamed[2]

[1]Department of Microbiology and Immunology, National Research Centre, Dokki, Egypt
[2]Department of Parasitology and Animal Diseases, National Research Centre, Dokki, Egypt
***Corresponding author:** dr.amanyahmed@yahoo.com

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ABSTRACT

Global concern surrounds *Acinetobacter baumannii* (*A. baumannii*), a dangerous pathogen. The fact that this bacterium can develop resistance to carbapenems used in clinical practice is one of the biggest worries associated with it. Furthermore, it has a strong propensity to rapidly disseminate this resistance, affecting healthcare settings across national borders and socioeconomic levels. Based on our research, out of 70 bovine milk samples were positive for *Acinetobacter spp* with an incidence (17.14%). Phenotypically, all *Acinetobacter spp* were confirmed as *A. baumannii*. Thus, the frequency of *A. baumannii* in milk samples was 17.14% (12/70). The isolates of *A. baumannii* exhibited the highest frequency of resistance to imipenem (10g), gentamicin (10g), and ceftazidime (30g), with an incidence of 100%. The PCR results showed that all *A. baumannii* strains (100%) carried the blaOXA-51, blaOXA-23, blaOXA-24 and blaOXA-58 genes. These results raised the possibility that raw milk consumption could expose humans to a zoonotic risk of carbapenem-resistant *A. baumannii* (CRAB).

Key words: *A. baumannii*; Milk; CHROM agar; Carbapenem-resistant; β-lactamase genes.

INTRODUCTION

Carbapenems are the "last-resort" antibiotics for treating serious infections, they are extremely essential antibiotic agents. On the other hand, carbapenem resistance is spreading globally and is now a serious issue. The United States Centre's for Disease Control and Prevention classify certain bacteria that are resistant to carbapenem as major risks (Huang et al. 2023).

Acinetobacter baumannii (*A. baumannii*) is Gram-negative, aerobic, pleomorphic bacteria connected to animal illnesses (Ewers et al. 2017). *Acinetobacter* species have been found in raw milk and milk products, which are an important source of nutrients for humans. The most frequent sources of *Acinetobacter* in milk include infected udders and teats, incorrect milk transport and storage, residual water in milking machines, milk pipelines, or coolers and poor cleaning of dairy equipment (Malta et al. 2020). These bacteria have the ability to produce heat-stable enzymes such as lipases and proteases that can contaminate milk after pasteurization if stored for a long time (Hoque et al. 2019). Furthermore, *A. baumannii* possesses a number of features that make it a dangerous pathogen, such as the ability to withstand high stress levels, a high degree of adaptability to unfavorable soil conditions (such as the capacity to survive for months in both moist and dry soil), and enhanced expression of efflux pumps that enable high levels of antibiotic resistance (Varnesh 2022). Therefore, it can promote the spread of antibiotic-resistant genes into different environments such as hospitals, by rapidly acquiring many antibiotic-resistant genes from soil and soil-dwelling bacteria (Sharma et al. 2021).

Severe life-threatening illnesses such as sepsis, meningitis and pneumonia are known to be caused by the extremely resistant bacterial pathogen *A. baumannii*. As a leading cause of nosocomial infections globally, *A. baumannii* infection rates have been rising annually in human medicine. Additionally, veterinary nosocomial infections linked to *A. baumannii* have been documented in recent publications. With regard to human and veterinary medicine, carbapenem-resistant *A. baumannii* (CRAB) in particular may be considered as an emerging opportunistic pathogen (Nocera et al. 2021). Based on factors such as clinical impact, availability of effective antibiotics, projections over a ten-year period, transmissibility, incidence rate and barriers to infection prevention, the Centers for Disease Control and the World Health Organization

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have classified *A. baumannii* as the most dangerous threat level in their ranking system for antibiotic-resistant threats (Tacconelli et al. 2018). Right now, CRAB is the bacteria that most seriously endangers human health, demanding the development of new antibiotics (Willyard 2017). Consequently, the discovery of livestock-associated carbapenem-resistant *A. baumannii* (CRAB) has raised concerns about newly emerging infectious pathogens that could spread through livestock products and easily infect humans and animals, this is especially the case when consuming or contacting contaminated milk or other animal products. A better understanding of resistance profiles of *Acinetobacter baumannii* associated with mastitis will help us to understand the treatment of the MDR diseases caused by this pathogen (Lin and Lan 2014; Kyriakidis et al. 2021).

This study's goal was to evaluate the prevalence of carbapenem-*resistant A. baumannii (CRAB)* in milk samples collected from different bovine farms suffering from mastitis in Egypt.

MATERIALS AND METHODS

Ethical approval

This study was approved by the NRC's Medical Research Ethics Committee (permission no. 13050414-1).

Collection of samples

In the Egyptian Giza Governorate, a total of 70 milk samples from dairy cows (native, mixed and foreign bread) with subclinical mastitis, ages of cows ranged from 3 to 6 years old, and 2 to ≥5 parity. Samples were collected from September to November 2023. The hygienic precautions were not taken in those farms to prevent mastitis and other infectious diseases, and there wasn't any antibacterial treatment for 7–10 days. Milking of the cows was carried out in usual manual methods. The California mastitis test (CMT) was carried out which revealed a positive reaction in 70 milk samples of subclinical mastitis. Samples have been tested for the presence of *Acinetobacter spp.*

Isolation and identification of *A. baumannii*

Milk samples

Nutrient broth was inoculated with one milliliter of each milk sample, which was incubated for 24 to 48 hours at 37°C. Plates of CHROM agar Acinetobacter (CHR) media (Oxoid) were streaked with a loopful of the broth cultures (Hrenovic et al. 2016), all plates were incubated at 37°C for 24-48h (Jawad et al. 1994).

Molecular identification of *A. baumannii* using conventional Polymerase Chain Reaction (PCR)

Acinetobacter specific primer sets 16S rRNA (Ac436F - Ac676r) and blaOXA-51 genes (Table 1) were used to identify suspected colonies of *A. baumannii* according to (Gao et al. 2014).

DNA extraction

DNA extraction from bacterial cultures was performed using the GF-1 Bacterial DNA Extraction Kit (Cat No. GF-BA-100, Vivantis Technologies, Malaysia), following the manufacturer's recommendations.

PCR amplification

Using the primer set (Ac436F and Ac676r) specific to *Acinetobacter*, suspected colonies were confirmed and *A. baumannii* identification was further confirmed by blaOXA-51 gene (Table 1).

Table 1: PCR primers employed in the research*

Gene	Sequence (5'–3')	Size bp
Ac436	TTTAAGCGAGGAGGAGG	240
Ac676	ATTCTACCATCCTCTCCC	
OXA-51	TAATGCTTTGATCGGCCTTG	353
	TGGATTGCACTTCATCTTGG	
OXA-23	GATCGGATTGGAGAACCAGA	501
	ATTTCTGACCGCATTTCCAT	
OXA-24	GGTTAGTTGGCCCCCTTAAA	246
	AGTTGAGCGAAAAGGGGATT	
OXA-58	AAGTATTGGGGCTTGTGCTG	599
	CCCCTCTGCGCTCTACATAC	

*Source: Shamsizadeh et al. (2017).

PCR reaction was performed using SimpliAmp™ Thermal Cycler (Cat. No. A24811, Applied Biosysytems, USA) in a final volume of 25µL reaction containing 12.5µL of 2x MyTaq™ Red Mix Master Mix (Cat. BIO-25043, Meridian Bioscience, UK), 0.5µL (10µM) of each primer and 1µL of target DNA with cycling conditions as in Table 2. The PCR products were separated by electrophoresis on 1.5% agarose gel then photographed and analyzed by InGenius3 gel documentation system (Syngene, UK).

Antimicrobial susceptibility testing

According to the Clinical and Laboratory Standards Institute's recommendations (CLSI, 2020), antimicrobial susceptibility testing of the isolates was done using the disc diffusion method on Mueller–Hinton agar with ceftazidime (30g), imipenem (10g) and gentamicin (10g).

Table 2: Cycling conditions for the detection of genes in this study

Gene	Init. Denat.	Denat.	Anneal.	Extension	Final Ext.	Cycles
Ac436	95˚C	95˚C	56˚C	72˚C	72˚C	35
Ac676	2min	20sec	30sec	45sec	7min	
OXA-51	95˚C	95˚C	57˚C	72˚C	72˚C	35
	2min	20sec	30sec	45sec	7min	
OXA-23	95˚C	95˚C	54˚C	72˚C	72˚C	35
	2min	30sec	30sec	45sec	7min	
OXA-24	94˚C	95˚C	54˚C	72˚C	72˚C	35
	2min	20sec	30sec	45sec	7min	
OXA-58	95˚C	95˚C	54˚C	72˚C	72˚C	35
	2min	30sec	30sec	1min	10 min	

Detection of genes encoding carbapenemase of the OXA type

By using certain sets of primers for PCR amplification, isolates of *A. baumannii* were screened for three common OXA-type carbapenemase-encoding genes (blaOXA-23, blaOXA-58, and blaOXA-24) (Table 1) with reaction components and cycling conditions in Table (1 and 2).

RESULTS

Phenotypic detection of *A. baumannii* in milk

Conventionally, 70 bovine milk samples were examined for the presence of *Acinetobacter spp* on (CHROM agar™ *Acinetobacter*). Mucoid smooth pink colonies were collected for further identification. Our findings indicated that 12 out of 70 samples were positive for *Acinetobacter spp* with an incidence (17.14%). Furthermore, all 12 *Acinetobacter spp* are *A. baumannii*, indicating that all isolates are *A. baumannii*. Thus, the frequency of *A. baumannii* in milk samples was 17.14% (12/70).

Antimicrobial susceptibility profile

The results of the antimicrobial susceptibility test for the all *A. baumannii* isolates showed a high-level resistance (100%) to carbapenem-group antibiotics [ceftazidime (30g), imipenem (10g) and gentamicin (10g)].

Molecular identification using conventional Polymerase Chain Reaction (PCR)

Ultimately, *A. baumannii* was identified in all 12 samples using PCR test. PCR using Ac and OXA-51 primers confirmed all the twelve bacterial colonies are *A. baumannii* as shown in Fig. 1 and 2, respectively.

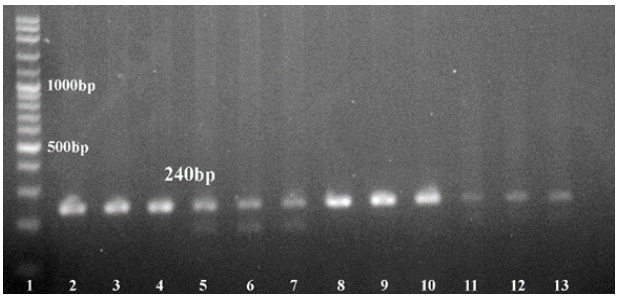

Fig. 1: PCR results of *A. baumannii's* AC gene amplification. Lane 1: 100bp molecular weight marker; Lanes (2 to 13): positive PCR products at 240bp.

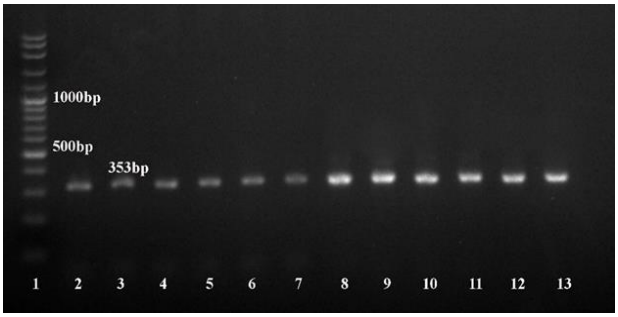

Fig. 2: *A. baumannii's* OXA-51 gene amplification PCR products. Molecular weight marker (100bp) in Lane 1. Lanes (2 to 13): positive PCR products at 353bp.

OXA-type carbapenemase-encoding genes in *A. baumannii* isolates

Fig. 3-5 showed the frequency of carbapenemase-encoding genes in *A. baumannii* isolates. 100% (12/12) strains contained OXA-23, OXA-24 and OXA-58, respectively. Carbapenem resistant *A. baumannii* was found in milk samples, according to the study's findings. Based on the results of PCR analysis of carbapenemase-encoding genes, antibiotic resistance in *A. baumannii* isolates is mostly caused by the three most prevalent OXA-type carbapenemase-encoding genes (blaOXA-23, blaOXA-58, and blaOXA-24).

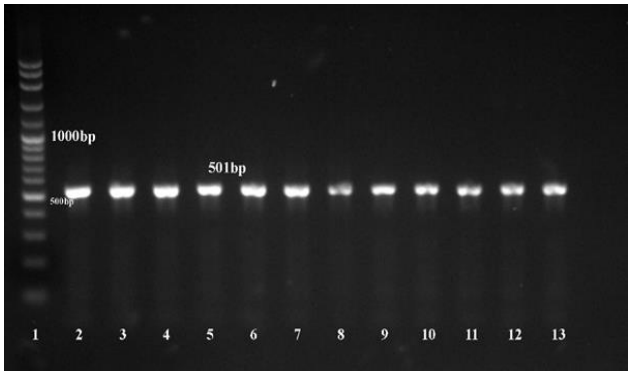

Fig. 3: PCR results of *A. baumannii's* OXA-23 gene amplification. Lane 1: Molecular weight marker (100 bp), Lanes (2 to 13): positive PCR products at 501 bp.

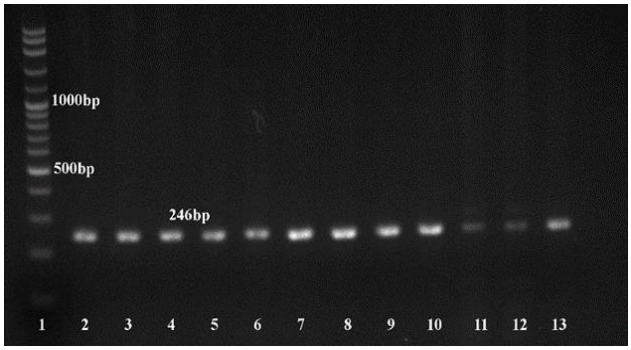

Fig. 4: PCR results of the OXA-24 gene amplification of *A. baumannii*. Lane 1: Molecular weight marker (100 bp), Lanes (2 to 13): positive PCR products at 246 bp.

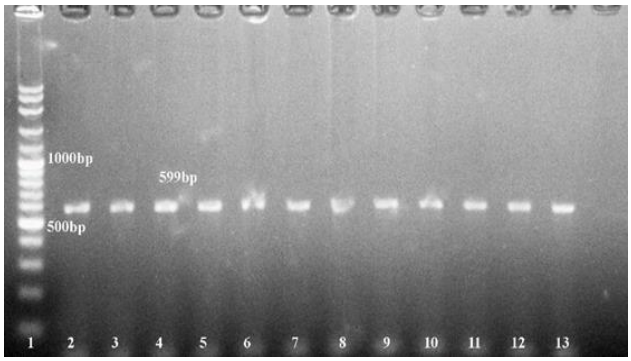

Fig. 5: PCR products of OXA-58 *gene* amplification of *A. baumannii*. Lane 1: Molecular weight marker (100 bp), Lanes (2 to 13): positive PCR products at 599 bp.

DISCUSSION

One of the main concerns for world health is carbapenem resistance (CR). Due to its quick spread and lack of effective treatments, CR is becoming a bigger problem in clinical settings. However, little is understood about the risks to human health posed by CR, its habitat, or how it spreads among animals raised for food. At the present time, there have been few studies conducted to monitor *A. baumannii* in veterinary field settings. Consequently, our work adds significant evidence to support the theory that *A. baumannii*, which has its origins in the veterinary industry, may be a potential human infection source. Consequently, the prevention of *A. baumannii* infection transmission depends heavily on early detection and the application of suitable control measures.

Using CHROM agar media, we detected *Acinetobacter spp*. in 70 milk samples. Because CHROM agar contains chromogenic substrates that *Acinetobacter spp*. enzymes cleave to produce distinctive, red-colored *Acinetobacter* colonies, many researchers have recommended using this medium as a rapid and simple way to detect *Acinetobacter*, this finding supported by (Mohamed et al. 2022).

The current results showed that *A. baumannii* was isolated from milk samples with an incidence of 17.14%. Kim et al. (2017) reported a significant prevalence (27.8%) of *A. baumannii* strains in the raw milk samples, which almost corroborated our results. Besides, *A. baumannii* was found to be highly prevalent in a variety of milk sample types by (Saad et al. 2018), and (Ramos and Nascimento, 2019). In addition, (Gurung et al. 2013) revealed that 176 bulk samples out of 2287 bulk milk samples had *Acinetobacter spp*. identified from them. Likewise, (Ndegwa et al. 2001) discovered that from 21 goat milk samples in Kenya, 10 isolates of *Acinetobacter spp* were isolated. In Egypt, among the 100 milk samples, the authors found in 13 samples *Acinetobacter spp* based on CHROM agar results (Mohamed et al. 2022).

According to our results of the antimicrobial susceptibility test, all *A. baumannii* strains showed the maximum rate of resistance against ceftazidime, imipenem and gentamicin with an incidence of 100%. This may be caused by the overuse of antibiotics as growth promoters and therapies in animal husbandry, which has increased the spread of antibiotic resistance in bacteria associated with livestock. Our results supported by (Santajit et al. 2023) who reported that 172 of the 995 *A. baumannii* isolated from clinical samples were carbapenem-resistant with an incidence (100%). Additionally, (Shamsizadeh et al. 2017) nearly agreed with our findings, 40 isolates of *A. baumannii* had the highest incidences of resistance to ceftazidime, imipenem, and gentamicin, at 92.5, 85 and 80%, respectively. A significant challenge lies in the deficiency of data concerning antimicrobial resistance in distinct pathogens within *Acinetobacter baumannii*, particularly those considered minor or negligible contributors to mastitis (Ali et al. 2023).

As of right now, the most prevalent mechanism of carbapenem resistance in *A. baumannii* appears to be carbapenem-hydrolyzing class D β-lactamases, or so-called CHDLs, also known as oxacillinases (OXAs) due to their impact on oxacillin (Poirel and Nordmann, 2006). There are six subgroups of class D β-lactamases. The OXA-51 intrinsic and acquired β-lactamases that like OXA-23, OXA-58, OXA-24/40, OXA-235, and OXA-143 (Poirel et al. 2010). It was determined that all of the isolates in our research carried OXA-51gene, an oxacillinase, as demonstrated by additional research. Our result is consistent with the findings of (Hassan et al. 2021), who reported that the blaOXA-51-like gene was detected in all isolates, confirming that they were all *A. baumannii*. Fortunately, it is insufficient to impart carbapenem resistance on its own OXA-51gene. It is commonly employed to verify the identity of *A. baumannii* (Evans and Amyes 2014).

PCR screening for carbapenemase-encoding genes (blaOXA-23, blaOXA-58 and blaOXA-24) revealed that three resistance genes were identified in all phenotypically *A. baumannii* isolates. According to previous research, the most prevalent carbapenemase gene was blaOXA-23, which is a member of the Class D β-lactamases family. It was found in 77.7% of the isolates that were examined (Hassan et al. 2021). Ninety percent of 50 isolates of *A. baumannii* that were resistant to carbapenem were found to carry blaOXA-23, according to a study conducted in Zagazig University Hospitals in Egypt (Ramadan et al. 2018). Further study performed in Egypt and Saudi Arabia revealed that isolates of *A. baumannii* resistant to carbapenem had a 100% occurrence rate of blaOXA-23 (Abouelfetouh et al. 2019). These results verified that blaOXA-23 gene may be found on a chromosome or a plasmid. We might draw the conclusion that various genetic structures and plasmids are linked to the present global spread of the blaOXA-23 gene. Controlling the dynamic dissemination of blaOXA-23 will be challenging due to its lack of association with a specific entity (Mugnier et al. 2009). Within this current investigation blaOXA-58 and blaOXA-24 genes were detected with an incidence of 100%. This finding was reinforced by (Tena et al. 2013) who found OXA-24 carbapenemase in one isolate from a serum container's surface and all five isolates from patient samples. On the other hand, research carried out in Italy revealed that *A. baumannii* that produces OXA-58 is frequently isolated (D'Arezzo et al. 2011). However, just two isolates of air samples had OXA-24 and were unable to find any OXA-58 genes (Shamsizadeh et al. 2017). No air isolates in the research by Gao et al. (2014) had OXA-24 or − OXA 58 found in them.

In fact, the resistance to carbapenem seen in our *A. baumannii* isolates is a serious problem since carbapenem is typically employed as a substitute for other β-lactam medications, such as cephalosporins and penicillin. A number of carbapenemase genes caused this resistance. OXA-type genes are intrinsic and can be found on chromosomes and plasmids. They are primarily responsible for *A. baumannii*'s resistance to carbapenem (Meletis 2016). The lack of systematic investigation into carbapenemases in bacteria originating from non-human sources makes these findings extremely concerning. Additionally, there may be a significant danger of acquiring these bacteria from intimate interaction between animal and human populations (Elshafiee et al. 2019).

Milk, which is frequently drunk uncooked or with little preparation may carry carbapenem resistance *A. baumannii*. Furthermore, animals travelling across farms

may have contributed to the persistence and spread of this pathogen. The fact that transmission via hands or equipment should be taken into account could help to explain this. Finally, its ability to form biofilms may also aid in its survival in environments like slaughterhouses and hospitals, raising the possibility of nosocomial infections and epidemics.

Conclusion

The inappropriate use of antibiotics as growth promoters and therapies in animal husbandry, which has increased the spread of antibiotic resistance in livestock-associated bacteria and even led to the formation of new resistances. The need for infection prevention and control strategies for eliminating these pathogens is emphasized by worries about the existence of bacterial pathogens in veterinary settings and their potential influence on public health. These findings indicated serious veterinary public health implications due to the negative impact on bovine mastitis treatment and prognosis. Additionally, milk samples can be pasteurized and boiled before being consumed to lower the chance of developing carbapenem resistance to *A. baumannii* infections. Finally, more research is still needed to understand the antibiotic resistance of *Acinetobacter spp*.

Author's contribution

Sohad M. Dorgham designed the plan of work, performed molecular identification and detection of genes encoding carbapenemase of the OXA type, review and drafting the manuscript. Amany A. Arafa shared bacterial isolation and identification, performed PCR and drafting the manuscript. Eman S. Ibrahim shared in bacterial isolation and identification, performed antibiotic sensitivity test and drafting the manuscript. Abeer M. Abdalhamed collected the samples, shared in bacterial isolation and identification and drafting the manuscript. All authors read and approved the final manuscript.

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