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Multidrug Resistance in Gram Negative Bacteria Isolated from Cases of Mastitis in Buffaloes

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ABSTRACT:

In Egypt, buffaloes are considered as the second largest source of milk. Mastitis is a prime hindrance for milk production with severs economic losses, but scarce data are available about subclinical type in Egyptian buffaloes. The target of this study was to (a) Estimation of some biochemical alteration in mastitic whey milk and its role in milk quality and quantity. Mastitis caused an increase in total whey protein, immunoglobulin and albumin in cows' milk samples with sub clinical mastitis, while α–lactalbumin, and β-lactoglobulin showed significant decreased. There was a highly significant decrease in α -casein and κ casein in sub clinical mastitic samples in comparing with the normal milk. In addition, there were a significant decrease in β-casein and non-significant decrease in γ casein milk protein fractions in sub clinical mastitic milk. (b) investigate the major gram-negative bacteria linked with buffalo's mastitis, and (c) assess the antibiotic resistance pattern of the bacteria. To this end, 208 quarter milk samples were collected from 62 domesticated dairy buffaloes suffered from subclinical mastitis. Bacteriological assessment and biochemical methods, illustrated a total of 51 isolates (24.52%) represented different 10 Gram negative bacterial

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species. The most predominant ones were *Klebsiella pneumoniae* (25.49%), followed by *Escherichia coli* and *Proteus mirabilis* (13.73%) for each. The phenotypic antibiogram revealed that 50.9% of the isolated pathogens were resistant to three or more antibiotic groups. The most noticed resistance was versus ampicillin 43 (84.31%) followed by both, tetracycline and trimethoprim–sulfamethoxazole 41(80.39%) for each one. Contrarily, the bacterial isolates were greatly sensitive to norfloxacin (68.6%), enrofloxacin (64.7%), then ciprofloxacin and amikacin (58.8%). On the other hand, the uniplex PCR, illustrated that 100% of the tested 13 *Klebsiella pneumoniae* strains carried *bla ctx-m* gene while 84.6% and 76.9% were positive to *aacC* and *tet* genes respectively., The implementation of high resistant associated Gram negative bacteria especially ESBL *K*. *pneumoniae* in subclinical mastitis considered a potential public health concern and requires good monitoring for achieving the efficient therapy.

INTRODUCTION

In low to middle income countries, milk and dairy products comprise the major protein source for much of the population. Buffaloes contribute to a predominately large proportion of the total milk production worldwide (13 %) **(FAO 2019)**. In Egypt, buffalo achieves 47 % of the total livestock population compared to cattle and also considered one of the important countries that buffalo's milk is highly output and consumed due to its high fat content, color and flavor **(Arefaine and Kashwa, 2015)**. However, in spite of genetic upgrading and novel methods of livestock rearing, decreased productivity which in turn leads to a decrease in consumption **(Ramesh and Divya 2014)**. Subclinical mastitis is one of the most prevalent economic issues of dairy industry globally. It is mostly caused by bacteria, and the infection implemented in inflammation and pathophysiological alterations in the udder tissue, leading to compromised milk quality and reducing the produced quantities **(Viguier *et al*. 2009)**. Gram negative bacteria constitute important sets of bacteria. Some species are commensals and others are important human and animal's pathogen. Gram negative bacterial mastitogens are mostly environmental pathogens, as *Klebsiella pneumonia*, *Escherichia coli* and *Citrobacter* spp. **(Klaas and Zadoks 2018)**.

Milk is an important part of the diet of human beings. It contains a wide range of dietary components of vital importance like water, proteins, lactose, minerals and vitamins. The exact composition of milk varies with the breed, species, feeding regimes, The identification of sub clinically mastitic causing pathogens and their antimicrobial sensitivity testing are important points in the implementation of control programs (Dhakal et al., 2007). Detection and evaluation of proteins in milk during the course of mastitis are important to elucidate the pathologic mechanism of bovine mastitis (Kato et al., 1989). Electro-

phoresis has played an important role in the study of milk proteins and has been an integral part of research on the genetic variants of the major proteins components of milk. Indeed, the designations of caseins are derived from electrophoretic analysis and minor caseins components were discovered by electrophoresis (Kostyra, 1990). Bovine milk contains 3.0-3.53 % (w/v) protein (Cole, 1986). Caseins are 2.4-2.79% of fluid milk and exist in milk as a micelle containing the four casein types: αs1- αs2- ß and k-casein (Kostyra, 1990). Whey proteins are serum albumin, α-lactalbumin, ß Lactoglobulin, and immunoglobulin.

In Egypt, it seems to be high prevalence of resistance against antimicrobial agents among different Gram negative bacteria due to uncontrolled widespread use of antibiotics in veterinary practice and the potential public health concern due to antibiotic residues **(Ameen *et al*. 2019)**. Most of the mastitis researches have been performed in cows; but recently, reports indicated that dairy buffaloes are also affected with parallel frequency **(Ali *et al*. 2011)**. So, the prime aim of this study concerned with survey of common Gram negative bacterial causative agents implemented in mastitic buffaloes in Giza in addition to assessment of multidrug resistance pattern.

Materials and Method:

Sampling

A total of 208 quarter milk samples were gathered under aseptic conditions from 62 subclinical mastitic dairy buffaloes from organized farms (76 samples) and small holder (132 samples) in Giza governorate during 2021-2022. Before taking the samples, the teat was disinfected after washing and dryness of the udder. Nearly fifteen ml of the foremilk was thrown away and the next fifteen ml gathered into sterile screw-capped bottles and transported to the laboratory in ice box and stored at 4°C till examination.

Sample preparation for electrophoresis:

Fresh warm raw milk was obtained from Holstein cows. Within an hour after milking, milk samples were skimmed by centrifugation at 3000 rpm for 15 min to remove their creams and cells. Samples were then treated with 0.1 M, hydrochloric acid at the controlled PH of 4.8 for casein precipitation. Treated samples were recentrifuged and the supernatants (Whey) were collected. The casein precipitate was separated from the whey by filtration. The casein fraction was washed with distilled water three times, maintaining the PH of the water at 4.7 by addition of dilute HCl.

Casein solution was prepared by dissolving 1g casein in 100 ml of 100 mM Tris-Hcl buffer (PH 8.0) and heating in a boiling bath for 20 min. The solution was filtrated without cooling and stored at 4 °C until to be used. Casein solutions must be discarded after 2 days. The total whey protein was determined according to Henry, (1969), application of SDS-polyacrylamide gel electrophoresis according to **Sambrook, et al. (1989).** The protein standard is ranged from (10-250KDa) BioRad, using methods of **Sambrook, et al. (1989).**

Bacteriological examination:

All samples were centrifuged for fifteen minutes at 3000 rpm and a loopful was taken from the sediment and inoculated onto MacConkey's agar plates and incubated at 37°C for 24 then 48 hr. The growing surface colonies were identified by morphological appearance as well as traditional biochemical tests; oxidase, catalase, indole, methyl red, Voges–Proskauer, citrate utilization, urease, H2S production on TSI, nitrate reduction and motility tests **(Quinn *et al*. 2011)**. Moreover, the bacterial isolates were confirmed biochemically using the VITEK-2 compact identification system (BioMerieux, France), following to manufacturer's instruction using Gram –ve cards **(Ling *et al*. 2003).**

Antimicrobial susceptibility testing:

The antibiogram profile of the isolates was analyzed, via disk diffusion method using 12 antimicrobials from six various groups as follows: (i) aminoglycosides (amikacin 30 µg, gentamicin 10 µg), (ii) Beta-lactams and derivatives (ampicillin 10 µg, amoxicillin/ clavulanic acid 30 µg) (iii) cephalosporins (ceftiofur 30 µg, ceftriaxone 30 µg, cephalexin 30 µg), (iv) fluoroquinolones (ciprofloxacin 5 µg, enrofloxacin 5 µg, norfloxacin 10 µg), (v) sulfonamides (trimethoprim-sulfamethoxazole 25 µg), and (vi) tetracyclines (tetracycline 30 µg). The disks were purchased from TM Media, (Titan Biotech Ltd India). The sensitivity/ resistance was relied on the zone of inhibition diameter, following the guidelines of the Clinical Laboratory Standards Institute **(CLSI 2020)**. Isolates that displayed simultaneous resistance to ≥ 3 various groups of antimicrobials were looked multi-resistant **(Rafailidis and Kofteridis 2022)**.

Bacterial DNA extraction for PCR:

Two hundred microliters were obtained from the thirteen *K. pneumoniae*'s overnight cultures, mixed with eight hundreds microliters of distilled water and boiled for ten minutes. The resulting solution was centrifuged and the supernatant used as the DNA template. DNA extraction from samples was carried out using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's guidelines. The DNA extracts were subjected to uniplex PCR using primers specific for detection of *aac*C, *bla* ctx-m and *tet* genes which responsible for resistance against aminoglycoside, ESBL (β- lactmase) and tetracycline respectively. The primers sequences, and cycle conditions listed in Table (1). Amplification was performed using an applied biosystem 2720 thermal cycler. Following PCR performance, the reaction products were subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light.

Table (1) The primers sequences, and cycle conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
aacC	GGCGCGATCAACGAATTTATCCGA CCATTCGATGCCGAA GGAAACGAT	448	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Lynne et al., (2008)**
bla ctx-m	ATGTGCAGCACCAG TAAAGT ACCGCGATATCGTTGGTGG	545	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Mendonça et al., (2007)**
tet	GCYRTVGGSATHGG CYTKRTYATGC ACMGCMCCWGTVGCBC CKGTGAT	293	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 30 sec.	72˚C 10 min.	**Schnabel et al., (1999)**

RESULTS

In the current study, a total of 51 (24.52%) Gram-negative bacterial isolates were obtained from 208 quarter mastitic buffalo's milk samples in Giza, Egypt, Based on cultural colonial morphology and conventional biochemical tests; the bacteria isolated were belonging to *Klebsiella* spp. (16 isolates), *Proteus* spp. (9 isolates), *Escherichia coli* (7 isolates), *Enterobacter* spp. (7 isolates), *Pseudomonas* spp. (5 isolates), *Citrobacter* spp. (5 isolates), and *Aeromonas* spp. (2 isolates). Further advanced biochemical typing illustrated more species identification as shown in table (2).

Table (2): Comparison between traditional and Vitek2 compact identification of bacterial isolates (n≈ 51).

Traditional identification			Vitek2 compact Identification		
Bacterial isolates	No.	%	Species isolates	No.	%
Klebsiella spp.	16	31.37%	*Klebsiella pneumoniae*	13	31.37%
			Klebsiella oxytoca	3	31.37%
Proteus spp.	9	17.64%	*Proteus mirabilis*	7	13.72%
			Proteus vulgaris	2	3.92%
Escherichia coli	7	13.72%	*Escherichia coli*	7	13.72%
Enterobacter spp	7	13.72%	*Enterobacter cloacae*	5	9.80%
			Enterobacter aerogenes	2	3.92%
Pseudomonas spp.	5	9.80%	*Pseudomonas* spp.	5	9.80%
Citrobacter spp.	5	9.80%	*Citrobacter* spp.	5	9.80%
Aeromonas spp.	2		*Aeromonas* spp.	2	3.92%
Total	51	100%	Total	51	100%

The detailed data obtained from in-vitro susceptibility assays were represented in tables 3 (a) and (b); a high percentage of *Klebsiella pneumoniae* isolates were resistant to ampicillin (92.3%) and trimethoprim-sulfamethoxazole (76.9%), while 69.2% of them were resistant to gentamicin and tetracycline. On the other hand, most of the Gram negative isolates were less resistant to norfloxacin (31.4%), enrofloxacin (35.3%), ciprofloxacin and a mikacin (41.2%).

Table 3 (a): Detailed antibiotic resistance profiles of bacterial isolates.

	Klebsiella pneumonia 13	*Klebsiella oxytoca* 3	*Proteus mirabilis* 7	*Proteus vulgaris* 2	*Escherichia coli* 7
Amikacin	6 (46.1%)	1(33.3%)	1(14.3%)	2(38.5%)	2(38.5%)
Gentamicin	9 (69.2%)	1(33.3%)	4(57.1%)	1(50%)	0 (0%)
Ampicillin	12(92.3%)	3 (100%)	6(85.7%)	2(100%)	5(71.4%)
Amoxicillin/clavulanic acid	7 (53.8%)	0 (0%)	3(42.8%)	0 (0%)	3(42.8%)
Ceftiofur	8 (61.5%)	2(66.6%)	6(85.7%)	1(50%)	4(57.1%)
Ceftriaxone	8 (61.5%)	1(33.3%)	5(71.4%)	1(50%)	4(57.1%)
Cephalexin	6 (46.1%)	1(33.3%)	2(28.5%)	1(50%)	3(42.8%)
Ciprofloxacin	4 (30.8%)	1(33.3%)	4(57.1%)	0 (0%)	3(42.8%)
Enrofloxacin	3 (23.1%)	0 (0%)	5(71.4%)	1(50%)	3(42.8%)
Norfloxacin	4 (30.8%)	0 (0%)	5(71.4%)	0 (0%)	3(42.8%)
Trimethoprim-sulfamethoxazole	10(76.9%)	2(66.6%)	7(100%)	1(50%)	5(71.4%)
Tetracycline	9 (69.2%)	2(66.6%)	3(42.8%)	1(50%)	7(100%)

% calculated according to No. of isolates for each species.

Table 3 (b): Detailed antibiotic resistance profiles of bacterial isolates

	Enterobacter cloacae 5	*Enterobacter aerogenes* 2	*Pseudomonas aeruginosa* 5	*Citrobacter freundii* 5	*Aeromonas hydrophila* 2
Amikacin	3(60%)	0 (0%)	3(60%)	2(40%)	1(100%)
Gentamicin	3(60%)	0 (0%)	4(80%)	1(20%)	1(100%)
Ampicillin	5(100%)	2(100%)	5(100%)	3(60%)	0 (0%)
Amoxicillin/clavulanic acid	2(40%)	1(50%)	3(60%)	5(100%)	0 (0%)
Ceftiofur	2(40%)	1(50%)	1(20%)	2(40%)	1(100%)
Ceftriaxone	1(20%)	0 (0%)	2(40%)	1(20%)	1(100%)
Cephalexin	5(100%)	0 (0%)	2(40%)	1(100%)	1(50%)
Ciprofloxacin	1 (20%)	0 (0%)	3(60%)	3(60%)	2(100%)
Enrofloxacin	0 (0%)	0 (0%)	3(60%)	1(20%)	2(100%)
Norfloxacin	0 (0%)	0 (0%)	3(40%)	0 (0%)	1(50%)
Trimethoprim-sulfamethoxazole	5(100%)	1(50%)	5(100%)	4(80%)	1(50%)
Tetracycline	5(100%)	2(100%)	5(100%)	5(100%)	2(100%)

% calculated according to No. of isolates for each species.

Moreover, the antibiotic susceptibility test showed that 26 out of 51 isolates (50.9%) exposed phenotypic multiple resistance to 3 or more antibiotic groups. The most multidrug-resistant species was *K. pneumoniae* (8 isolates; 61.5%) as shown in figure 1. On the other sight, the most noticed resistance to antibiotic types was against ampicillin 43(84.3%) followed by both, tetracycline and trimethoprim–sulfamethoxazole 41(80.39%) for each one.

Table 4(a): Protein electrophoretic pattern of normal and sub-clinical mastitic whey milk

Protein Fraction	Normal milk	Sub clinical Mastatic milk
Total whey protein	15.03 ± 1.22	17.80 ± 2.08 *
Immunoglobulin	14.70 ± 1.01	19.09 ± 1.04 **
albumin	7.63 ± 0.52	15.87 ± 1.02 **
α-lactalbumin	28.22 ± .62	22.25 ± 0.37 **
β- lactoglobulin	54.02 ± 0.61	31 ± 1.02 **

Table 4 (b): Protein electrophoretic pattern of normal and sub-clinical mastitic whey milk

Protein fraction	Normal milk	Sub clinical Mastatic milk
α-casein	10.58 ± .21	6.16 ± 0.22 **
β-casein	8.78 ± 0.20	7.16 ± 0.19 *
K1-casein	16.16 ± .0.09	14.11 ± 0.18 *
γ-casein	22 ±0.21	21.73 ± 0.20

The detailed data of Protein electrophoretic pattern of normal and sub-clinical mastitic way milk were represented in table 4 (a) showed a significant increase in total fraction of protein of sub clinical mastitic milk comparing with normal milk, while four casein types: α, ß, k and γ casein of sub clinical mastitc milk were significantly decreased.

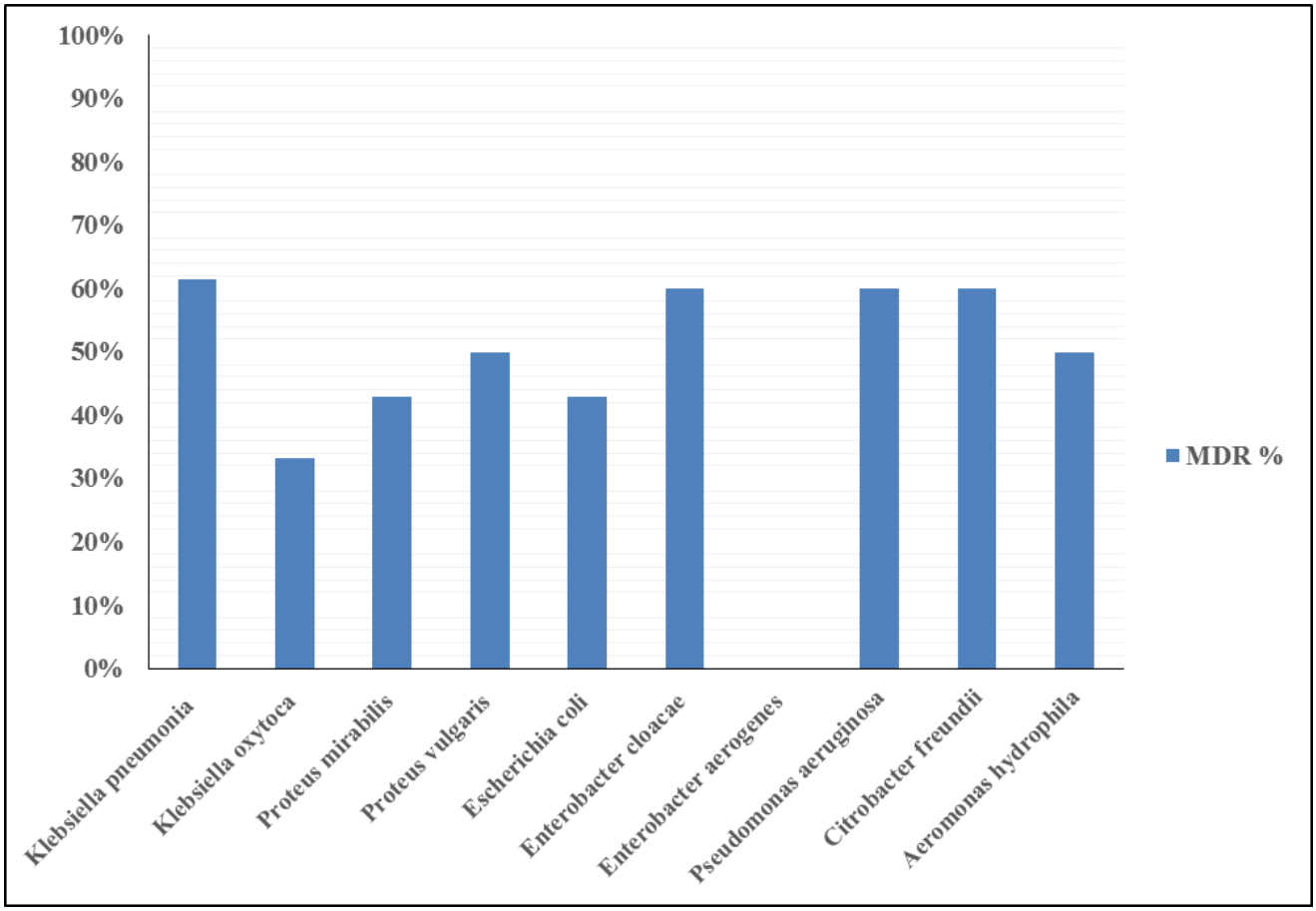

Figure 1: the percentage pattern of Gram negative isolates multidrug resistance.

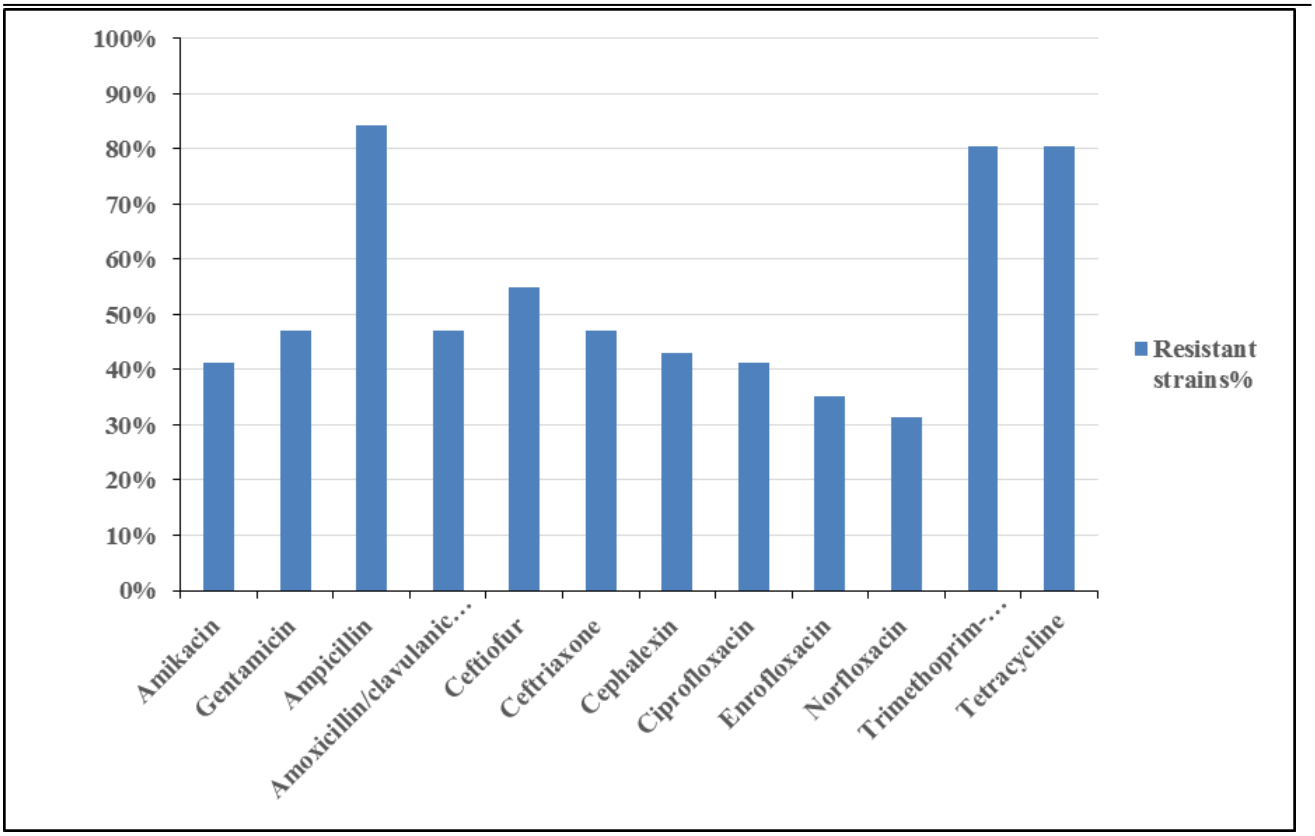

Figure 2: The percentage pattern of antibiotic resistance.

According to the results shown in figures (3-5); the uniplex PCR identified *aac*C, *bla* ctx -m and *tet* genes in 11, 13 and 10 *Klebsiella pneumoniae* isolates (84.6, 100 and 76.9%), respectively.

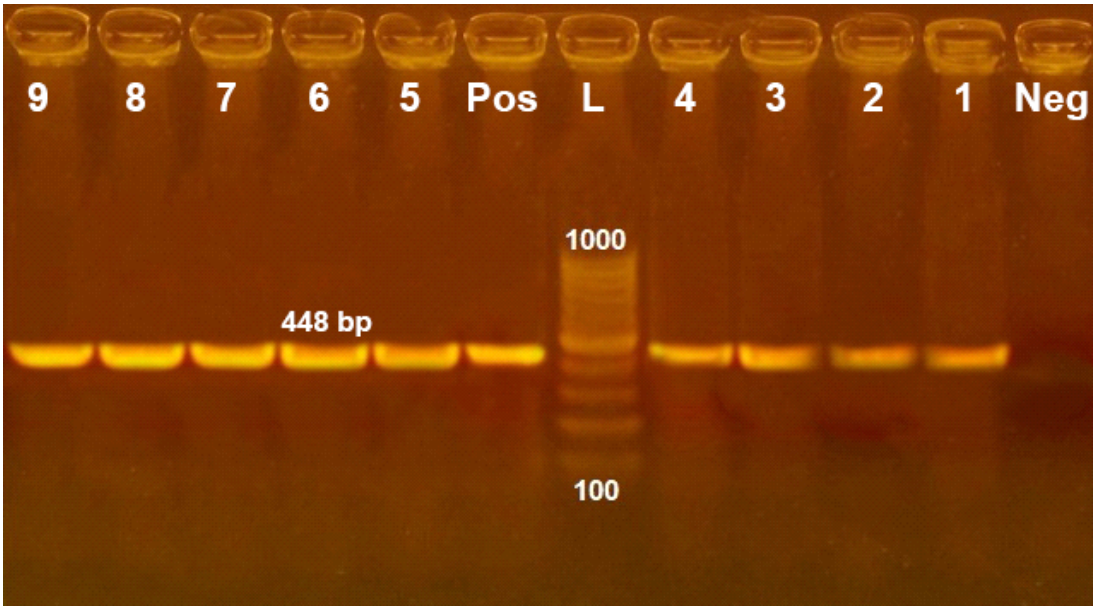

Figure 3: uniplex polymerase chain reaction showing positive amplification of 448 bp represented *aacC* gene in *Klebsiella pneumoniae* isolates. L: 100 bp DNA ladder.

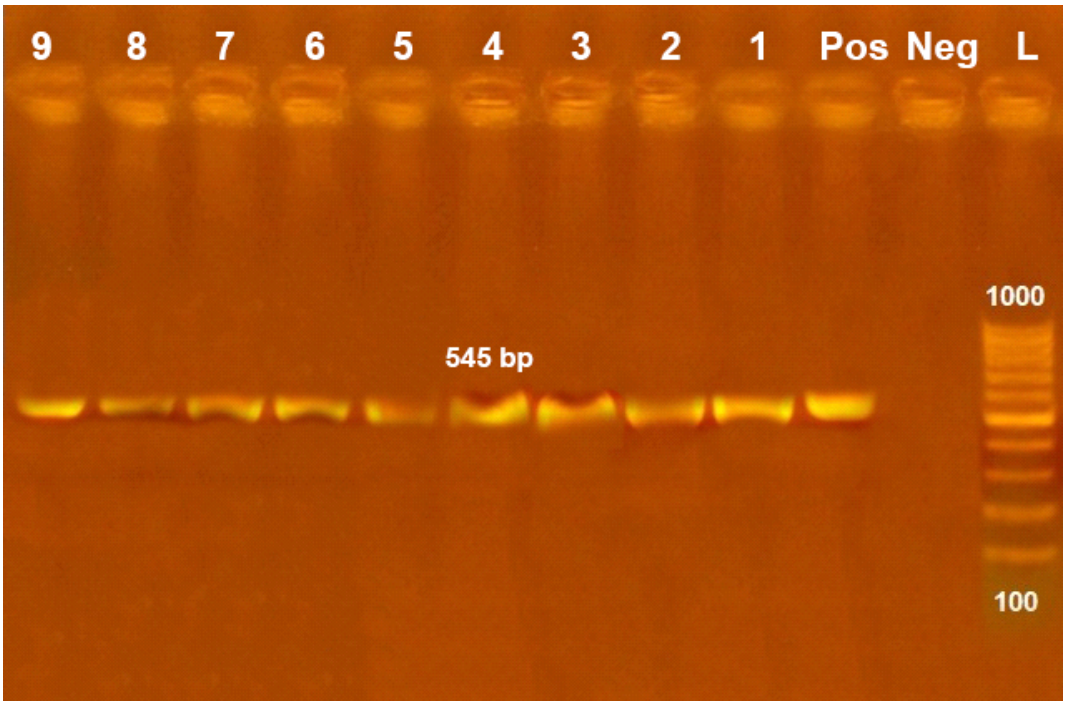

Figure 4: uniplex polymerase chain reaction showing positive amplification of 545bp represented *bla ctx-m* gene in *Klebsiella pneumoniae* isolates. L: 100 bp DNA ladder.

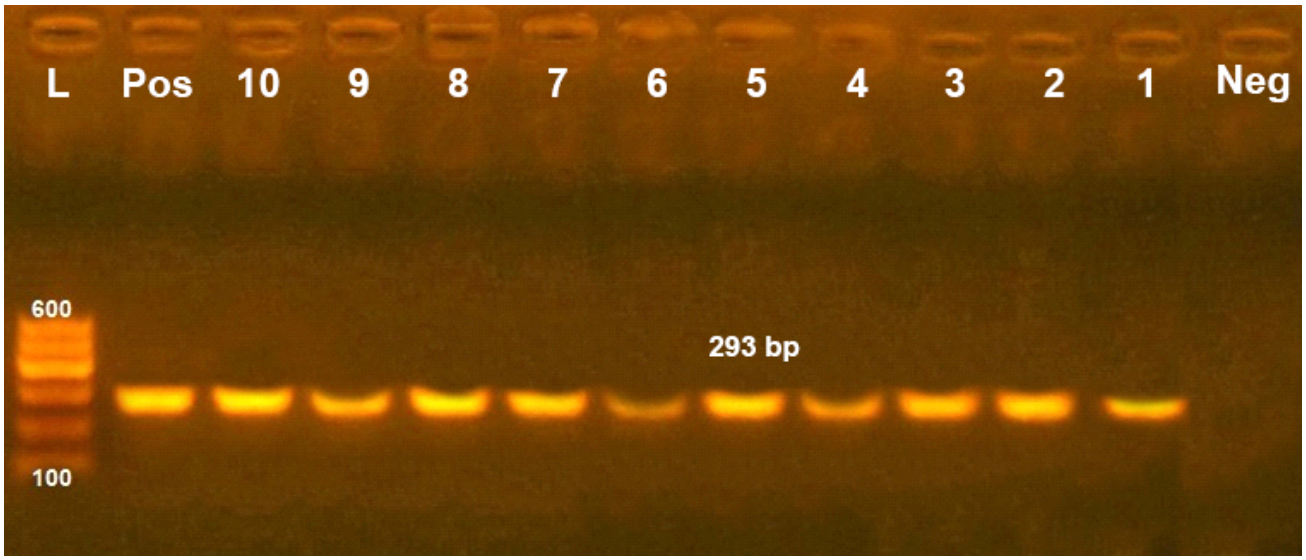

Figure 5: uniplex polymerase chain reaction showing positive amplification of 293bp represented *tet* gene in *Klebsiella pneumoniae* isolates. L: 100 bp DNA ladder.

DISCUSSION

Every time, mastitis is considered one of the most fundamental illnesses affecting the dairy industry, causing great economic losses in Egypt and worldwide (**Azooz *et al*. 2020**). Dairy farmers broadly use antibiotic remedy as an important tool to hinder the intra-mammary infection, particularly before calving. Also antibiotics are used to cure persistent udder infections **(Rowe *et al*. 2021)**. Excessive use of anti-

microbial agents in dairy farms leading to emerging resistance among different bacterial pathogens, and result in a major public health concern **(Olivares-Pérez *et al*. 2015)**. Concerning the data obtained from bacteriological examination (table 2) revealed that out of 208 cultured samples from mastitic buffaloes, a total of 51 Gram negative bacterial isolates were recovered (24.52%). Our result near with that reported by **Sayed *et al*. (2011)** as (33.7%) but much less than that reported by **Kabir et al. (2019)** in which the contribution of Gram-negative bacteria was 68%. In contrast our results were much more than study performed by **Bhutia *et al*. (2019)** as (3.2%). The most frequent isolates recovered were *Klebsiella pneumonia* (13, 25.5%), followed by *E. coli* and *Proteus mirabilis* (7, 13.7%), *Enterobacter cloacae, Citrobacter freundii* and *Pseudomonas aeruginosa* (5, 9.8%), *Klebsiella oxytoca* (3, 5.8%), and lastly *Proteus vulgaris, Enterobacter aerogenes* and *Aeromonas hydrophila* (3, 3.9%). Regarding both *Klebsiella* and *Escherichia coli*; our results were nearly coincided with previous studies **(Sayed, 2014; Ahmed *et al*. 2016; Ahmed *et al*. 2018; Ameen *et al*. 2019)** but much lower than that mentioned by **Nehal, 2019** (33.9%). Cheng et al. (2019) mentioned that *Klebsiella* and *Escherichia coli* are considered from the major common bovine mastitis pathogens.

The data obtained from tables 3 (a) and (b) revealed that *Klebsiella pneumoniae* isolates exposed high resistance pattern against ampicillin (92.3%) and trimethoprim-sulfamethoxazole (76.9%), while 69.2% of them were resistant to gentamicin and tetracycline. These results nearby **Ribeiro *et al*. (2022)**, who reported high resistance pattern of the *Klebsiella* isolates was seen to ampicillin (91.8%), and trimethoprim/sulfamethoxazole (39.7%), and multidrug resistance to ≥ 3 classes of antimicrobials was existed in 20.4% isolates. Also, antibiogram results obtained by **Marashifard *et al*. (2019)** on *E. coli* isolated from bovine mastitis exposed that the highest rate of resistance was noticed versus tetracycline (18.6%), then trimethoprim-sulfamethoxazole (12.9%). Additionally, prevalence of resistance of *Klebsiella* spp. was high to amoxicillin/clavulanate (38%), followed by tetracycline (32%), further, a high proportion (27%) of isolates were multidrug resistant **(Cheng *et al*. 2019)**.**Osman *et al*. (2014)** investigated ten *K. pneumoniae* isolates obtained from mastitic buffaloes in Egypt and reported a multidrug resistance pattern for 40% of the isolates.

Inflammation of the mammary gland leads to a variety of compositional changes in milk either because of local effects or because of serum components entering the milk and the movement of some normal milk components out of the alveolar lumen into the perivascular space **(Harmon, 1994).** As sowed in table 4 (a), Albumin content of milk in sub clinical mastitis was significantly elevated compared to the healthy ones these agree with **Batavani et al., 2007** who reported the increase of albumin content during mastitis in cows. **De Wit, 1998,** said that the main site of albumin synthesis is in the liver, and the albumin enters the milk by leaking through the epithelial tight junction from the blood stream, while **Shamay et al. (2005)** found that the extra hepatic synthesis of albumin has been demonstrated in mammary gland epithelial cells, but in lesser amounts than the liver. The marked significantly increases of albumin in mastitic cow's milk suggest that a major source of this elevation in the content of albumin in milk under inflammatory conditions is the mammary gland itself. Immunoglobulin in mammary secretions is serum-derived or produced in the udder and pass into the milk through the mammary epithelium. The concentrations of immunoglobulin in normal milk are low and depend on the degree of vascular permeability of the udder tissues **(Henry et al., 2007).** During inflammation this permeability barrier is broken, immunoglobulin concentrations increase in secretions from infected mammary glands. The increase in milk immunoglobulins may be effective in reducing severity of mastitis **(Persson, 1992).** The major function of immunoglobulins is opsonization of microorganisms for phagocytosis, and they are believed to prevent bacterial adherence to epithelial membranes, inhibit multiplication and neutralize toxins. The decrease in α –lactalbumin, β-lactoglobulin associated with the sub clinical mastitis agreed with results of previous investigations reported

by **Ishikawa and Shimizu (1982)**.This could be due to both inflammatory damage of the mammary secretary tissues and destruction of the blood milk permeability barriers which restrict and discriminate in transfer of protein from interstitial fluid into milk. Table 4 (b) showed that, The percentages of α casein, β casein, κ casein, and γ casein in mastitic samples were lower than those of healthy normal milk. This result may be explained by that milk from mastitic udders exhibits greatly increased proteolytic activity **(Le Roux et al., 1995).** Plasmin is the most important protease in milk from healthy udders but the non-plasmin proteases become more important with increasing severity of udder inflammation. This proteolysis leads to a decrease in the relative proportion of caseins **(Henry et al., 2007).** On the other hand, our results demonstrated that most of the Gram negative isolates were sensitive to norfloxacin (68.6%), enrofloxacin (64.7%), ciprofloxacin and amikacin (58.8%). Also, **Ribeiro *et al*. (2022)** mentioned that norfloxacin was the most efficient antimicrobial against *Klebsiella* isolates. Regarding the genes responsible for antibiotic resistance, our results revealed that *Klebsiella* isolates harbored *aac*C, *bla* ctx-m and *tet* genes with high proportional (84.6, 100 and 76.9%), respectively. Particularly, the existence of *bla* ctx-m gene which is responsible for resistance against β lactams in the isolates constitutes a significant hazardous pattern to human representing what is termed extended-spectrum β-lactamase (ESBL) pathogens **(Bandyopadhyay *et al*. 2018)**. The authors also detected twelve ESBL producing *Klebsiella pneumoniae* that carried blaCTX-M-15 gene with 63.2% among 19 buffaloes suffered from mastitis. On the other hand, the presence of *aac*C and *tet*A genes also potentially interfere with the mastitis therapy programs **(Nobrega *et al*. 2021)**.

CONCLUSION

Mastitis resides an illness causing great economic losses to the dairy industry, and the success of mastitis remedy is relied on the choice of proper antimicrobial therapies. Therefore, periodic monitoring of the antibiotic sensitivities of pathogenic bacteria isolated from dairy mastitic buffaloes, as well as molecular pattern of this resistance would be an essential tool in detecting emergence and spreading of antimicrobial resistance. High prevalence of resistance rate for antibiotics among Gram negative bacteria isolated from mastitic buffaloes may be indicating significant public health concern. Isolation of multidrug-resistant *Klebsiella pneumoniae*, a typical nosocomial pathogen from sub clinical mastitic buffalo milk, repeats the necessity to keep an eye on farm animals for ESBL producing *Enterobacteriaceae* and asserts on avoiding the indiscriminating usage or misuse of antibiotics in animal husbandry sector. Infected animals should be identified as rapid as possible as it is the potential source of infection to non-infected animals. Identification of the causative agent is an essential step in the application of antimicrobial therapy and herd management. Protein percent of milk serum whey protein, immunoglobulin, and albumin increased during subclinical mastitis. While α -casein, κ casein, and β-casein decreased in sub clinical mastitis. In subclinical mastitis, an increase in relative amount of γ-casein and a decrease in β-casein were noticed. On the other hand, the relative amount of immune globulins increased, but no changes were found in the remaining components of whey-proteins. In the obvious acute case, a relative increase in amount of γ-casein and flattening of theβ-casein peak were observed. Moreover, some abnormalities appeared in the α-casein peak. Of the changes in whey-proteins, the increase in relative amount of immune globulins and serum albumin.

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