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Distribution of Multidrug-Resistant Gram Negative Bacteria Causing Clinical Mastitis in Dairy Cows

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Abstract: This study was carried out to screen and characterize the genetic bases of Multidrug-resistance in Gram-negative bacteria isolated from dairy cows with clinical mastitis in dairy farms at Dakahlia and Damietta governorates, Egypt. A total of 120 milk samples were collected from dairy cows suffered clinical mastitis in 12 dairy farms (10 samples/each farm). Multidrug-resistance phenotypes were found in 56 of 131 (42.7%) Gram-negative bacterial isolates which harbored at least one antimicrobial resistance gene. The most prevalent multidrug-resistant (MDR) species were *Klebsiella pneumoniae* (12 isolates; 9.2%), *Escherichia coli* (11 isolates; 8.4%), *Enterobacter cloacae* (9 isolates; 6.9%), *Proteus vulgaris* (8 isolates; 6.1%), *Klebsiella oxytoca* (6 isolates; 4.6%), *Citrobacter freundii* (6 isolates; 4.6%), *Proteus mirabilis* (3 isolates; 2.3%) and *Serratia marcescens* (1 isolates; 0.8%). Most of these isolates displayed A multidrug-resistance phenotype mainly against amoxicillin-clavulanic acid, ampicillin, aztreonam, cephalothin, ciprofloxacin, cefpodoxime, ceftriaxone, cefotetan, cefotaxime, cefoxitin, gentamicin, kanamycin, nalidixic acid, oxacillin, streptomycin, spectinomycin, trimethoprim/ sulfamethoxazole, chloramphenicol and tetracycline. Class 1 integrons were detected in 36 (27.5%) isolates. The gene cassettes within class 1 integrons included those encoding resistance to trimethoprim (*dfrA1, dfrA5, dfrA7, dfrA12, dfrA17* and *dfrA25*), aminoglycosides (*aadA1, aadA2, aadA5, aadA7, aadA12*, *aadA22* and *aac(3)-Id*), erythromycin (*ereA2*) and rifampicin (*arr-3*). Class 2 integrons were identified in 7 (5.34%) isolates. β-lactamase-encoding genes were identified in 46 (35.1%) isolates, plasmid-mediated quinolone resistance genes were identified in 24 (18.4%) isolates and florphenicol resistance genes, *floR*, was identified in 12 (9.2%) isolates. However, 6 (4.5%) isolates didn't have any of the characterized genes. " In conclusion, in this study we isolated and identified multidrug-resistant strains of Gram-negative bacteria and detected several types of resistance genes in those isolates from dairy cows with clinical mastitis in Dakahlia and Damietta governorates, Egypt.

Key words: Clinical mastitis · Anti-biotic resistance gene · Gram negative bacteria · Dairy cows · Egypt

INTRODUCTION

Bovine mastitis, an inflammation of the bovine mammary glands, is recognized as one of the main illnesses that affect the profitability of dairy farms and have a major influence on dairy cows' welfare and productivity [1, 2]. Bovine mastitis adversely affects animal health, quality of milk and economics of milk production and every country including developed ones suffer huge financial losses [3, 4]. Moreover, it presents a public health risk through the possibility of transmission of pathogenic microorganisms, toxins or antimicrobial residues through the milk [5]. Therefore, estimating economic losses as a result of clinical mastitis become an extremely difficult task because of the many levels of infection and many other factors [4, 6].

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Bovine mastitis is a complex multi-factorial disease occurs depending on variables related to the animal, environment and pathogen [7]. Among the pathogens, bacterial agents are the most common one, the greatest share of which resides widely distributed in the dairy cows environment, hence a common threat to the mammary gland [3]. The main mastitis-causing major pathogens are coliforms (mainly *E. coli* & *Klebsiella pneumoniae*), *Streptococcys* spp. (*Strep. agalactiae, Strep. dysgalactiae, Strep. uberis* & *Strep. bovis*), *Staphylococcus* spp. (*Staph. aureus & Staph. epidermidis*), *Actinomyces pyogenes* and a wide variety of other organisms have been identified as potential mastitis pathogens [8-10].

On dairy farms, antimicrobials such as penicillin, cephalosporins and tetracycline, among others, are used to treat and prevent bovine mastitis caused by Gram-positive and Gram-negative bacteria [11]. However, the efficacy of these antimicrobials can be compromised through the emergence of antimicrobial resistance in the relevant mastitis pathogens. The intensive use of antibiotics in human and veterinary medicine may increase bacterial resistance [12]. Thus, dairy farms and the current management practices employed for milk production might be associated with the dissemination of antibiotic-resistant bacterial strains [13].

Increasing prevalence of resistance has been reported in many pathogens over the years in developing countries [14]. This has been attributed to changing microbial characteristics, intensive use of selective antimicrobial and societal and technological changes enhancing the development and transmission of multidrug-resistant organisms. Although antimicrobial resistance is a natural biological phenomenon, it is often enhanced as a consequence of infectious agents' adaptation to exposure to antimicrobials used in humans or agriculture and the widespread use of disinfectants at the farm and the household levels [15]. Antimicrobial resistance threatens the effective prevention and treatment of an ever-increasing range of infection caused by bacteria, parasites, viruses and fungi [16]. The damaging effects of antimicrobial resistance are already manifesting themselves across the world. Antimicrobial-resistant infection currently claim at least 50,000 lives each year across Europe and the US alone, with many hundreds of thousands more dying in other areas of the world. But, reliable estimates of the true burden are scarce [16].

The spread of multidrug-resistant Gram-negative bacteria causing clinical mastitis needs continuous research and study. Therefore, the present study was conducted to provide preliminary information on multidrug-resistant Gram-negative bacteria associated with bovine mastitis in dairy farms and characterization of antibiotic resistant genes in those isolates in Dakahlia and Damietta governorates, Egypt.

MATERIALS AND METHODS

Animals: A total of 120 dairy cows, exhibiting the signs of clinical mastitis, at 4–8 years of age were studied. Samples were collected from 12 private dairy farms (ten samples were taken from each farm) in Dakahlia and Damietta governorates, Egypt suffering problems of mastitis and decrease in milk production throughout 2014. At the time of visit, a questionnaire was applied consisting of objective questions to herd managers to obtain data regarding general characteristics of the property, animal management, hygiene management practices during milking and the milking workers profile. Which antimicrobial drugs were used in treatment of mastitis and other diseases in the herds under study were also checked. This study was approved by the Animal Welfare and Ethics Committee, Mansoura University, Mansoura, Egypt, on August, 2013.

Clinical Examination: Data concerned with the case history, clinical findings and medical record for each cow were recorded. A detailed physical examination of the animals, including examination of the mammary gland and its secretion was carried out and the clinical findings were recorded [17]. Dairy cows with clinical mastitis had clinical signs of abnormalities of secretion, abnormalities of the size; consistency and temperature of the mammary glands and, frequently, a systemic reaction. There are three categories of clinical mastitis: abnormal milk, abnormal gland and an abnormal cow (systemic disease). Abnormal milk is visibly abnormal (i.e. is not 'drinkable'). An abnormal gland is larger and firmer than other quarters. An abnormal cow is pyrexic, depressed or has decreased appetite or milk production.

Sampling: Milk samples were collected from each cow under investigation immediately before milking. Milk samples were collected after washing the teats with soap and water, drying with paper towel and undertaking antisepsis of the ostium of the teats with alcohol at 70%. The foremilk was discarded and 60 ml of pooled milk was collected (15 ml from each quarter) in labeled sterile screw cap tubes. Milk samples were collected before the cow is treated with antibiotics and stored at 4°C from the time of collection until processing within 3–4 hours.

Isolation and Identification of Bacterial Isolates: All milk samples were centrifuged for 15 min at 3000 rpm and a loopful was taken from the sediment and inoculated into nutrient broth (Oxoid) and incubated at 37 °C for 18 hours. Then, sub-cultured on MacConkey agar (Oxoid) and incubated at 37°C for 24 and 48 hours. All isolates were identified as Gram-negative isolates based on their colony morphology and the biochemical testing. Furthermore, all isolates were confirmed biochemically by using API 20E system (BioMe´ rieux, Marcy-l'E´ toile, France).

Antimicrobial Susceptibility Testing of Bacterial Isolates: The antimicrobial sensitivity phenotypes of bacterial isolates were determined using a Kirby-Bauer disk diffusion assay according to the standards and interpretive criteria described by Clinical and Laboratory Standards Institute [18]. The following antibiotics were used: ampicillin (AMP), 10 µg; amoxicillin-clavulanic acid (AMC), 20/10 µg; oxacillin (OXA), 1 µg; cefoxitin (FOX), 30 µg; cefotetan (CTT), 30 µg; cefotaxime (CTX), 30 µg; cefpodoxime (CPD), 10 µg; ceftriaxone (CRO), 30 µg; aztreonam (ATM), 30 µg; imipenem (IMP), 10 µg; nalidixic acid (NAL), 30µg; ciprofloxacin (CIP), 5 µg; norfloxacin (NOR), 10 µg; chloramphenicol (CHL), 30 µg; gentamicin (GEN), 10 µg; streptomycin (STR), 10 µg; spectinomycin (SPX), 10µg; tetracycline (TET), 30 µg; and sulfamethoxazole-trimethoprim (SXT), 23.75/1.25 µg. The disks (Oxoid Microbiology Products, Thermo scientific, United Kingdom) were purchased from scientific beta office (Mansoura, Egypt) and the results were recorded according to the zone-size interpretative chart supplied by the manufacturer.

Bacterial DNA Preparation for PCR: An overnight bacterial culture (200 µL) was mixed with 800 µL of distilled water and boiled for 10 min. The resulting solution was centrifuged and the supernatant was used as the DNA template [19]. Amplification reactions were carried out with 10 µL of boiled bacterial suspensions, 250 µM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 50 pmol of primers for Gram-negative bacteria and 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA). Distilled water was added to bring the final volume to 50 µL. Following PCR, 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: Primers used for PCR and DNA sequencing

Primer	Sequence (5' to 3')	Target	Reference
Integrons			
5'-CS	GGCATCCAAGCAGCAAG	Class 1 integron	Ahmed *et al*. [19]
3'-CS	AAGCAGACTTGACCTGA		
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron	Ahmed *et al*. [19]
hep51	GATGCCATCGCAAGTACGAG		
β-Lactamases			
TEM-F	ATAAAATTCTTGAAGACGAAA	bla_{TEM}	Ahmed *et al*. [19]
TEM-R	GACAGTTACCAATGCTTAATC		
SHV-F	TT ATCTCCCTGTTAGCCACC	bla_{SHV}	Ahmed *et al*. [19]
SHV-R	GATTTGCTGATTTCGCTCGG		
OXA-F	TCAACTTTCAAGATCGCA	bla_{OXA}	Ahmed *et al*. [19]
OXA-R	GTGTGTTTAGAATGGTGA		
CTX-M-F	CGCTTTGCGATGTGCAG	bla_{CTX-M}	Ahmed *et al*. [19]
CTX-M-R	ACCGCGATATCGTTGGT		
CMY-F	GACAGCCTCTTTCTCCACA	bla_{CMY}	Ahmed *et al*. [19]
CMY-R	TGGAACGAAGGCTACGTA		
Florfenicol			
StCM-L	CACGTTGAGCCTCTATATGG	*floR*	Ahmed *et al*. [20]
StCM-R	ATGCAGAAGTAGAACGCGAC		
Plasmid-mediated quinolone			
qnrA-F	ATTTCTCACGCCAGGATTTG	*qnrA*	Ahmed *et al*. [20]
qnrA-R	GATCGGCAAAGGTTAGGTCA		
qnrB-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	Ahmed *et al*. [20]
qnrB-R	ACGATGCCTGGTAGTTGTCC		
qnrS-F	ACGACATTCGTCAACTGCAA	*qnrS*	Ahmed *et al*. [20]
qnrS-R	TAAATTGGCACCCTGTAGGC		
aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	*aac(6')-Ib-cr*	Ahmed *et al*. [20]
aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		

PCR and DNA Sequencing of the Class 1 and Class 2 Integrons: The class 1 integron primers, 5'-CS and 3'-CS, which amplify the region between the 5'-conserved segment (5'-CS) and 3'-CS of class 1 integrons, were used as previously described [19]. For the detection of class 2 integrons, PCR was performed with the primer pair hep74 and hep51, specific to the conserved regions of class 2 integrons [20]. Both DNA strands of the PCR product were sequenced using an ABI automatic DNA sequencer (Model 373; Perkin–Elmer). Primers for PCR and DNA sequencing are compiled in Table 1.

Screening for Antimicrobial Resistance Genes: The bacterial isolates were tested for the presence of TEM-, SHV-, CTX-M-, OXA- and CMY-β-lactamase-encoding genes by PCR using universal primers for the TEM, SHV, OXA, CTX-M and CMY families, as previously described [20]. The florfenicol resistance gene, *floR*, was detected by using StCM-L and StCM-R primers as previously described [20]. Finally, PCR amplification was used for screening of plasmid-mediated quinolone resistance genes; *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*, as described previously [20]. Primers for PCR and DNA sequencing are compiled in Table 1.

Computer Analysis of the Sequence Data: A similarity search was carried out using the BLAST program available at the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

In the microbiological examination, multidrug-resistance phenotypes were found in 56 of 131 (42.7%) Gram-negative bacterial isolates and harbored at least one antimicrobial resistance gene. The most prevalent multidrug-resistant (MDR) species were *Klebsiella pneumoniae* (12 isolates; 9.2%), *Escherichia coli* (11 isolates; 8.4%), *Enterobacter cloacae* (9 isolates; 6.9%), *Proteus vulgaris* (8 isolates; 6.1%), *Klebsiella oxytoca* (6 isolates; 4.6%), *Citrobacter freundii* (6 isolates; 4.6%), *Proteus mirabilis* (3 isolates; 2.3%) and *Serratia marcescens* (1 isolates; 0.8%) (Table 2). Most of these isolates displayed a multidrug-resistance phenotype mainly against amoxicillin-clavulanic acid, ampicillin, aztreonam, cephalothin, ciprofloxacin, cefpodoxime, ceftriaxone, cefotetan, cefotaxime, cefoxitin, gentamicin, kanamycin, nalidixic acid, oxacillin, streptomycin, spectinomycin, trimethoprim/sulfamethoxazole, chloramphenicol and tetracycline (Table 3). Class 1 integrons were detected in 36 (27.5%) isolates. The gene cassettes within class 1 integrons included those encoding resistance to trimethoprim (*dfrA1, dfrA5, dfrA7, dfrA12, dfrA17* and *dfrA25*), aminoglycosides (*aadA1, aadA2, aadA5, aadA7, aadA12*, *aadA22* and *aac(3)-Id*), erythromycin (*ereA2*)

Table 2: Prevalence of multidrug-resistant (MDR) Gram-negative bacteria isolated from dairy cows with clinical mastitis

	Frequency (n=131)		
Bacteria	Non-MDR	MDR	Overall total
K. pneumoniae	37 (28.2%)	12 (9.2%)	49 (37.4%)
E. coli	21 (16.0%)	11 (8.4%)	32 (24.4%)
E. cloacae	6 (4.6%)	9 (6.9%)	15 (11.5%)
P. vulgaris	5 (3.8%)	8 (6.1%)	13 (9.9%)
K. oxytoca	3 (2.3%)	6 (4.6%)	9 (6.9%)
C. freundii	2 (1.6%)	6 (4.6%)	8 (6.1%)
P. mirabilis	1 (0.8%)	3 (2.3%)	4 (3.0%)
S. marcesens	0 (0.0%)	1 (0.8%)	1 (0.8%)
Total	75 (57.3%)	56 (42.7%)	131 (100%)

Non-MDR: None multidrug resistant; MDR: Multidrug resistant

Table 3: Resistance phenotypes of Gram-negative bacteria isolated from dairy cows with clinical mastitis

Antimicrobials tested[a]	Resistant isolates (n=56)
B-lactams	
AMC	26
AMP	52
ATM	35
CPD	17
CRO	10
CTT	20
CTX	18
FOX	27
OXA	41
Aminoglycosides	
GEN	29
SPX	42
STR	51
Quinolones and fluoroquinolone	
CIP	27
NAL	49
Potentiated sulfonamides	
SXT	50
Phenicols	
CHL	40
Tetracycline	
TET	49

AMC, amoxicillin-clavulanic acid; AMP, ampicillin; ATM, aztreonam; CEF, cephalothin; CHL, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CRO, ceftriaxone; CTT, cefotetan; CTX, cefotaxime; FOX, cefoxitin; GEN, gentamicin KAN, kanamycin; NAL, nalidixic acid; OXA, oxacillin; SPX; spectinomycin STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline

Table 4: Incidence of integrons and resistance genes in Gram-negative bacteria isolated from dairy cows with clinical mastitis

	Integrons		Antimicrobial resistance genes		
Bacteria	Class 1 (n=36)	Class 2 (n=7)	β-Lactamases (n=46)	Plasmid-mediated quinolone (n=24)	Florfenicol (n=12)
K. pneumoniae	8	1	8	6	1
E. coli	7	1	10	4	4
E. cloacae	4	1	6	3	2
P. vulgaris	6	1	7	3	1
K. oxytoca	4	1	6	3	1
C. freundii	4	1	5	3	2
P. mirabilis	2	1	3	2	1
S. marcesens	1	0	1	0	0

Table 5: Resistance phenotype and prevalence of integrons and resistance genes in Gram-negative bacteria isolated from dairy cows with clinical mastitis

No.	Bacteria	Resistance Phenotypes[a]	Integrons	Other genes
1	*K. pneumoniae*	AMC, AMP, ATM, CHL, CIP, CPD, CRO, CTT, CTX, FOX, GEN, IPM, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA1-aadA1*, *dfrA15*)	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{CMY-2} bla_{OXA-1},*qnrS*,*aac(6)-Ib-cr*, *floR*
2	*K. pneumoniae*	AMC, AMP, ATM, CPD, CTT, CTX, FOX, GEN, NAL, OXA, STR, SXT, TET	Class 1 (*aac(3)-Id-aadA7*, *dfrA15*)	bla_{TEM-1}, bla_{SHV-12},*qnrB*
3	*K. pneumoniae*	AMP, ATM, CHL, CIP, CPD, CRO, CTX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1(*aadA1*)	bla_{TEM-1}, $bla_{CTX-M-3}$,*qnrS*
4	*K. pneumoniae*	AMC, ATM, CTT, FOX, NAL, OXA, SPX, STR, SXT, TET	Class 2 (*dfrA1-sat2-aadA1*)	bla_{CMY-2},*aac(6)-Ib-cr*
5	*K. pneumoniae*	AMP, CHL, CIP, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA17-aadA5*)	*qnrB*
6	*K. pneumoniae*	AMP, ATM, SPX, STR, SXT	Class 1 (*arr-3-dfrA7*, *aadA12*)	bla_{TEM-1}
7	*K. pneumoniae*	AMC, AMP, ATM, CHL, CTT, CTX,FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1(*aadA1*)	bla_{OXA-1}
8	*K. pneumoniae*	CHL, GEN, NAL, SPX, STR, SXT, TET	Class 1(*dfrA1*)	-
9	*K. pneumoniae*	AMC, AMP, CHL, CPD, CTX, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA17-aadA5*)	-
10	*K. pneumoniae*	AMC, AMP, CIP, NAL, TET	-	*qnrS*
11	*K. pneumoniae*	AMP, ATM, CHL, SXT, TET		bla_{TEM-1}
12	*K. pneumoniae*	AMP, CHL, CIP, CPD, CRO, CTX, GEN, NAL, OXA, SPX, STR, SXT, TET	-	bla_{OXA-1}
13	*E. coli*	AMP, ATM, CHL, CIP, CPD, CRO, CTT, CTX, FOX, GEN, NAL, OXA, STR, SXT, TET	Class 1 (*dfrA12-orfF-aadA2*)	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{CMY-2},*qnrS*,*aac(6)-Ib-cr*, *floR*
14	*E. coli*	AMP, CHL, CIP, CTT, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA5-ereA2-aadA1*)	bla_{TEM-1}, bla_{SHV-12},*qnrB*
15	*E. coli*	AMC, AMP, ATM, CHL, CIP, CTT, CTX, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA12-orfF-aadA2*, *aadA22*)	bla_{TEM-1}, bla_{SHV-12}, *qnrS1*
16	*E. coli*	AMC, AMP, ATM, CHL, CRO, CTX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*aadA1*)	bla_{TEM-1}, $bla_{CTX-M-3}$,*floR*
17	*E. coli*	AMP, ATM, CHL, CIP, CTT, CTX, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA12-orf-aadA2*)	bla_{SHV-12}
18	*E. coli*	AMC, AMP, CHL, CIP, FOX, GEN, NAL, OXA, SPX, STR	Class 2 (*dfrA1-sat2-aadA1*)	bla_{TEM-1},*qnrB*
19	*E. coli*	AMC, AMP, CHL,NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA12-orfF-aadA2*)	bla_{TEM-1},*floR*
20	*E. coli*	AMC, AMP, CHL, OXA, SPX, STR, SXT	Class 1 (*aac(3)-Id-aadA7*)	bla_{OXA-1}

Table 5: Continued

21	*E. coli*	AMP, ATM, CHL, CIP, CPD, GEN, NAL, OXA, STR, SXT, TET		bla_{TEM-1}
22	*E. coli*	AMC, AMP, ATM, NAL, OXA		bla_{TEM-1}
23	*E. coli*	CHL, NAL, SPX, STR, SXT, TET		*floR*
24	*E. cloacae*	AMC, AMP, ATM, CHL, CIP, CPD, CRO, CTT, CTX, FOX, GEN, IPM, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA17-aadA5*)	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-12},*qnrB*,*aac(6)-Ib-cr*, *floR*
25	*E. cloacae*	AMC, AMP, ATM, CHL, CIP, CTT, FOX, GEN, NAL, OXA, STR, SXT, TET	Class 1 (*dfrA12-orfF-aadA2*, *dfrA25*)	bla_{TEM-1}, bla_{SHV-12}
26	*E. cloacae*	AMP, CHL, NAL, SPX, STR, SXT, TET	Class 2 (*dfrA1-sat2-aadA1*)	-
27	*E. cloacae*	AMP, CIP, NAL, TET		*qnrB*
28	*E. cloacae*	AMP, ATM, NAL, STR, SXT, TET		bla_{TEM-1}, *floR*
29	*E. cloacae*	AMP, CHL, SPX, SXT, STR, TET	Class 1 (*dfrA17-aadA5*),	bla_{TEM-1}
30	*E. cloacae*	AMP, ATM, CTX, TET	-	bla_{SHV-2}
31	*E. cloacae*	AMP, SPX, STR SXT, TET	Class 1 (*dfrA17-aadA5*),	bla_{TEM-1}
32	*E. cloacae*	AMC, AMP, CHL, CIP, NAL, NOR, STR, SXT, TET	-	*qnrS*
33	*P. vulgaris*	AMC, AMP, ATM, CHL, CIP, CPD, CRO, CTT, CTX, FOX, GEN, NAL, OXA, SPX, STR,SXT, TET	Class 1(*dfrA1-aadA1*, *dfrA15*)	bla_{TEM-1}, $bla_{CTX-M-15}$,*qnrS*,*aac(6)-Ib-cr*, *floR*
34	*P. vulgaris*	AMC, AMP, ATM, CPD, CTT, CTX, FOX, GEN, NAL, OXA, STR, SXT, TET	Class 1 (*aac(3)-Id-aadA7*, *dfrA15*)	bla_{TEM-1}, bla_{CMY-2}
35	*P. vulgaris*	AMP, ATM, CHL, CIP, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1(*aadA1*)	bla_{TEM-1}
36	*P. vulgaris*	AMP, FOX, NAL, OXA, SPX, STR, SXT, TET	Class 1(*aac(3)-Id-aadA7*, *dfrA15*)	bla_{OXA-30}
37	*P. vulgaris*	AMP, CHL, CIP, NAL, OXA, SPX, STR, SXT, TET	Class 2 (*estX-sat2-aadA1*)	*qnrB*
38	*P. vulgaris*	AMP, ATM, CTT, CTT, CTX, FOX, SPX, STR, SXT	Class 1 (*aadA2*, *dfrA15*)	bla_{TEM-1}, bla_{CMY-2}
39	*P. vulgaris*	AMP, ATM, CHL, CTT, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1(*aadA1*)	bla_{TEM-1}, *aac(6)-Ib-cr*,
40	*P. vulgaris*	AMP, CHL, CPD, CIP, CTT, FOX, GEN, NAL, OXA, TET	-	bla_{TEM-1}
41	*K. oxytoca*	AMC, AMP, ATM, CHL, CPD, CTT, CTX, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA17-aadA5*)	$bla_{TEM-1,}$ bla_{OXA-30},*qnrS1*,*aac(6)-Ib-cr*, *floR*
42	*K. oxytoca*	AMC, AMP, ATM, CHL, CIP, CTT, FOX, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*arr-3-dfrA7*, *aadA12*)	bla_{TEM-1}, bla_{CMY-2},bla_{SHV-12},*qnrA*, *floR*
43	*K. oxytoca*	AMP, ATM, FOX, NAL, OXA, SPX, STR, SXT, TET	Class 2 (*estX-sat2-aadA1*)	bla_{TEM-1}
44	*K. oxytoca*	AMC, AMP, ATM, CHL, CIP, CPD, CRO, CTX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA17-aadA5*)	bla_{TEM-1}, $bla_{CTX-M-3}$, *qnrS1*,*aac(6)-Ib-cr*
45	*K. oxytoca*	AMP, ATM, CHL, CIP, FOX, GEN, NAL, OXA, STR, SXT, TET	Class 1 (*dfrA15-aadA2*, *dfrA15*)	bla_{TEM-1},*floR*
46	*K. oxytoca*	AMC, AMP, ATM, FOX, NAL, OXA, SPX, STR, SXT, TET	Class 1(*aadA1, dfrA15*)	bla_{OXA-30}
47	*C. freundii*	AMC, AMP, ATM, CHL, CIP, CPD, CRO, CTX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*aadA22*)	bla_{TEM-1},$bla_{CTX-M-15}$, bla_{SHV-12},*qnrS*,*floR*
48	*C. freundii*	AMC, AMP, CHL, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*aadA1*)	bla_{TEM-1},*qnrA*

Table 5: Continued

49	*C. freundii*	AMP, ATM, CHL, CIP, CTT,CTX, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA12-orf-aadA2*)	bla_{SHV-12},
50	*C. freundii*	AMC, AMP, ATM, CHL, CIP, NAL, OXA, SPX, STR	Class 2 (*dfrA1-sat2*)	bla_{TEM-1},*floR*
51	*C. freundii*	AMC, AMP, CIP, CPD, FOX, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA12-orfF-aadA2*)	bla_{TEM-1},*qnrB*
52	*C. freundii*	AMP, CHL, NAL, SPX, STR, SXT	Class 1(*dfrA7*)	
53	*P. mirabilis*	AMP, ATM, CHL, CIP, CPD, CRO, CTT, FOX, GEN, IPM, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*aac(3)-Id-aadA7*)	bla_{TEM-1}, bla_{CMY-2}, bla_{SHV-12},*qnrB*,*floR*
54	*P. mirabilis*	AMP, ATM, NAL, OXA, SPX, STR, SXT, TET	Class 2 (*dfrA1-sat2-aadA1*)	bla_{TEM-1}
55	*P. mirabilis*	AMP, CIP, FOX, GEN, NAL, OXA, SPX, STR, SXT, TET	Class 1 (*dfrA12-orfF-aadA2*)	*qnrS*
56	*S. marcesens*	AMC, AMP, ATM, CHL, FOX, NAL, SPX, STR, SXT	Class 1 (*dfrA12-orf-aadA2*)	bla_{TEM-1}

AMC, amoxicillin-clavulanic acid; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CRO, ceftriaxone; CTT, cefotetan; CTX, cefotaxime; FOX, cefoxitin; GEN, gentamicin; IPM,impenam; NAL, nalidixic acid; NOR, norfloxacin; OXA, oxacillin; SPX; spectinomycin STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline

and rifampicin (*arr-3*) (Table 4, 5). Class 2 integrons were identified in 7 (5.34%) isolates. β-lactamase-encoding genes were identified in 46 (35.1%) isolates, plasmid-mediated quinolone resistance genes were identified in 24 (18.4%) isolates and florphenicol resistance genes, *floR*, was identified in 12 (9.2%) isolates (Table 4, 5). However, 6 (4.5%) isolates didn't have any of the characterized genes.

DISCUSSION

The frequencies of clinical mastitis are highly esteemed parameters in evaluation of the health of the bovine mammary gland [21]. In the present study, all of the mammary quarters that showed signs of inflammation and alterations in milk characters were indicative of clinical mastitis. Of the mammary quarters with clinical mastitis, multidrug-resistance phenotypes were found in 56 of 131 (42.7 %) Gram-negative bacterial isolates and harbored at least one antimicrobial resistance gene.

Since clinical mastitis events were based on producer or veterinarian diagnosis, it is possible that the description of clinical mastitis may have differed among farms [22]. Clinical mastitis may manifest as a wide variety of clinical signs, including a sudden onset, moderate to severe inflammation of udder, decreased production and serous milk/fibrin clots. The systemic illness is due to septicemia or toxemia, results in fever, anorexia, depression, decreased rumen motility, dehydration and sometimes death of the cow. Systemic illness often precedes the symptoms manifested in the milk and mammary gland [7, 17 & 23].

Antibiotics are used in food animals to treat clinical disease, to prevent and control common disease events and to enhance animal growth. The different applications of antibiotics in food animals have been described as therapeutic use, prophylactic use and sub-therapeutic use. Antibiotics can be used to treat a single animal with clinical disease or a large group of animals [24]. This broad use of antimicrobials picks out resistant bacteria that may result in animal illness that is less responsive to treatment with conventional antibiotics [25-28]. Hence, antimicrobial-resistant pathogens also pose a severe and costly animal health problem, as they prolong illness and decrease productivity through higher morbidity and mortality rates [29].

Twelve classes of antimicrobials-arsenicals, polypeptides, glycolipids, tetracyclines, elfamycins, macrolides, lincosamides, polyethers, beta-lactams, quinoxalines, streptogramins and sulfonamides may be used at different times in the life cycle of cattle [30]. Consequently, associations between antibiotic use in food animals and the prevalence of antibiotic resistant bacteria isolated from those animals have been detected in observational studies as well as in randomized trials [31].

In the present study, 56 out of 131 (42.7%) isolates of Gram-negative bacteria showed multidrug-resistance phenotypes. Most of these isolates displayed a multidrug-resistance phenotype mainly against amoxicillin-clavulanic acid, ampicillin, aztreonam, cephalothin, ciprofloxacin, cefpodoxime, ceftriaxone, cefotetan, cefotaxime, cefoxitin, gentamicin, kanamycin,

nalidixic acid, oxacillin, streptomycin, spectinomycin, trimethoprim/ sulfamethoxazole, chloramphenicol and tetracycline. Most of these antimicrobial agents are regularly used in veterinary practice [30, 32]. Similar multidrug-resistance phenotypes of Gram-negative bacteria isolated from animals have been reported worldwide [27, 28 & 33]. Similar multidrug-resistance phenotypes are of great clinical significance and can be easily transferred to human pathogens, especially the third-generation cephalosporins which are considered to be frontline therapeutic drugs for treatment of many infection in the hospitals [34].

The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements such as plasmids, transposons and integrons has been the major factor in the development of multiple drug resistance. Integrons are DNA elements that mediate the integration of antibiotic resistance gene cassettes by a site-specific recombination system [35]. Among the different classes of multidrug-resistance integrons that have been identified, integron classes 1 and 2 are the most common in Gram-negative bacteria [35]. The organization of class 2 integrons is similar to that of class 1, but they are associated with transposon Tn7 and are known to carry three classic gene cassettes (dihydrofolate reductase, *dfrA1*; streptothricin acetyltransferase, *sat2*; and aminoglycoside adenyltransferase, *aadA1*), which confer resistance to trimethoprim, streptothricin and streptomycin/ spectinomycin, respectively [36].

β-Lactams belong to a family of antibiotics characterized by a β-lactam ring which is necessary for the activity responsible for inactivation of a set of transpeptidases which catalyze the final cross linking reactions of peptidoglycan synthesis [34]. The integrity of the β-lactam ring resistance to β-lactam antibiotics in Gram-negative bacteria is primarily mediated by β-lactamases, which hydrolyze the β-lactam ring and thus inactivate the antibiotic [34]. Many different β-lactamases have been described, but TEM-, SHV-, OXA-, CMY- and CTX-Mb- lactamases are the most predominant in Gram-negative bacteria [34].

In the current study, ciprofloxacin-modifying aminoglycoside acetyltransferase gene, *aac(60)-Ib-cr*, was identified in Gram-negative isolates from dairy cow with clinical mastitis. The *aac(60)-Ib-cr* encodes a new variant of a common aminoglycoside acetyltransferase, *aac(60)-Ib*, with two amino acid changes, Trp102Arg and Asp179Tyr. This modified enzyme reduces the activity of ciprofloxacin by N-acetylation at the amino nitrogen on its piperazinyl substituent [37].

Quinolone resistance gene was identified in Gram-negative isolates from dairy cows with clinical mastitis. The gene responsible for quinolone resistance, *qnr* including three main types, *qnrA*, *qnrB* and *qnrS*, encodes a protein of the pentapeptide repeat family, which has been shown to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV [38]. Plasmid-mediated quinolone resistance is of great concern since these resistance determinants are potentially spread among bacteria due to plasmid mobility [39].

Chloramphenicol (CHL) including Florfenicol (FFC), a broad-spectrum antibiotic, was used extensively in veterinary practice. The florfenicol resistance gene, *floR*, confers resistance to chloramphenicol and florfenicol. In this study, PCR- and DNA-sequencing screening identified *floR* in Gram-negative isolates from dairy cows with clinical mastitis was carried out. *floR* was previously identified in Gram-negative bacteria isolated from cattle in France [40, 41].

In conclusion, in this study we isolated and identified multidrug-resistant strains of Gram-negative bacteria and detected several types of resistance genes in those isolates from dairy cows with clinical mastitis in Dakahlia and Damietta governorates Egypt.

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