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Identification, Serological and Molecular Identification of Coliform Bacteria Recovered from Bovine Mastitis

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ABSTRACT

Aim/Background: Mastitis is one of the serious disease and critical problem in the dairy industry worldwide. This study aimed to determine the prevalence of coliform bacteria, particularly *E. coli,* associated with mastitis in dairy herds. In addition, serological identification and molecular detection for *E. coli* strains from bovine mastitis.

Methods: A total of 400 cows were inspected for clinical and subclinical mastitis, and 100 samples (64 and 36, respectively) from these conditions were collected for bacteriological identification before being subjected to serological and molecular analysis.

Results: Clinical and subclinical mastitis were prevalent; 16% and 9%, respectively. Bacteriological examination revealed that *E. coli* was the most prevalent bacteria 16 isolates (19.51%) followed by *Klebsiella* spp 8 (9.76%), *Citrobacter* 4 (8.16%), and *Enterobacter aerogens* 2 (4.08%). Regarding to the serological results, *E. coli* isolates classified into EHEC which was the most identified serotype followed by EPEC and ETEC serotypes. While, for Klebsiella *spp., Klebsiell* a*oxytoca* and *Klebsiella pneumonia* were serologically identified. The molecular detection some of the virulence genes by PCR approach among *E.coli* isolates reported that all *E. coli* strains carried the *fim*H (100%). Meanwhile, other virulence genes *sxt*1 and *eae*A gene didn't detected in any tested *E. coli* isolates.

Conclusion: Coliform bacteria were identified as major bacterial pathogens involved in bovine clinical and subclinical mastitis.

Keywords: Antibiogram, *E. coli*, Mastitis, Resistance genes and Virulence.

INTRODUCTION

Mastitis is a main economic topic in the global dairy herds especially in developing

countries (Abebe et al., 2016). Furthermore, bovine mastitis is associated mainly with an inflammation of the mammary gland as well as pathological alterations and abnormalities in milk causing major financial losses to the dairy industry. A number of pathogenic *E. coli* serotypes is regarded as a significant pathogen that causes bovine clinical mastitis (Goulart and Mellata, 2022). Moreover, Coliform bacteria are also considered as foremost agents incriminated in bovine mastitis with a devastating effect on dairy milk production (Abegewi et al., 2022). In addition, *E. coli, Staphylococcus aureus*, *Streptococci*, and Coagulase Negative *Staphylococci* were found to be the most common bacterial causes of bovine mastitis in a previous study in Egypt (Abed et al., 2021), with prevalence rates of 49.8%, 44.9%, 44.1%, and 37.1%, respectively.

Although, there are detectable virulence elements genes responsible for adhesion, invasion, biofilm production, fimbria, and toxins had been recognized in E. coli isolated from bovine mastitis (Silva et al., 2014). Of these, adhesin factors such as intimins and fimbria were detected among *E. coli* isolated from bovine mastitis (Marashifard et al., 2019). Also, the fimbriae had role in control of *E. coli* in the host cells (Marashifard et al., 2019), through allowing the phagocytic neutrophils to participate and so the development and progression of the mammary gland inflammatory process (Zhou et al., 2021). Other virulence determinants such as enterobactins and aerobactins were also identified among *E. coli* causing bovine mastitis and its function is to maintain *E. coli* growth in adverse conditions of iron shortage (Ghanbarpour and Oswald, 2010). Toxins produced by *E. coli* such as hemolysins had related to the cytotoxic effect (Blum et al., 2015). Also, the damage of the vascular endothelium is due to the influence of the cytotoxic toxins (Ghanbarpour and Oswald, 2010). The presence of the shiga toxinsis linked to prompt cell apoptosis (Ahmadi et al., 2020). This study aimed to determine the prevalence of coliform bacteria, particularly *E. coli*, associated with mastitis in dairy herds, in addition to serological identification and molecular detection for *E. coli* strains from bovine mastitis.

MATERIAL AND METHODS

Ethics and area of study:

This study was carried out according to ethics commission and current regulation on research and ethical approval of the Faculty of Veterinary Medicine, the University of Sadat City, Egypt. This study was performed in Tala province of the Menoufiya Governorate. The city is located between 30.680108°N 30.943758°E. (Monufia Governorate - Wikipedia, n.d.). The city is considered one of the most critical areas where the government focuses on agriculture and animal husbandry. Therefore, animal breeding constitutes the main occupation for a majority of the inhabitants.

Animal examination and samples collection:

This study work was carried out during the period extend from October 2020 until May 2021. Four-hundred cows raised as individual cases in the field (balady and mixed breed) were examined for detection of clinical mastitis according to (Bartlett et al., 2001) based on the cardinal signs of inflammation on udder and systemic reaction. As well as apparently normal milk was subjected to CMT for detection of subclinical mastitis as described by (Balamurugan and Ranjith, 2018). One hundred mastitis milk samples (64 and 36 from clinical and subclinical) respectively were collected aseptically and transmitted in cold condition according to (Cabral et al., 2015).

Isolation and identification of Coliform bacteria:

Firstly, the samples were cultured into tryptose soy broth (Difco) and incubated at 37°C for 12 hours and then sub-cultured on specific differential medium (MacConkey agar; Difco) for 24-48 h at 37°C. Characteristic pink colonies followed by subculturing into Eosin methylene blue medium (Difco). Appearance of characteristic metallic green colonies was indicative for *E. coli*. All the obtained isolates were subjected to morphological examination and Gram staining. Additionally, typical biochemical tests for identification of coliform bacteria were performed according to (Cowan, 1985) as well as in Congo red medium (Surgalla and Beesley, 1969).

Serological identification of Coliform bacteria:

The serological identification of the *E. coli* and *Klebsiella spp*. was performed using the antisera (Denka and Seiken) ^{R} using the diagnostic "O" sera 51 vials (polyvalent 8 vials and 43 monovalent vials) "DENKA SEIKEN" (product Code 312001, Japan). Group O sera, these are liquid products containing specific somatic (O) antibodies (polyvalent sera: pig and monovalent sera: rabbit) of the organisms and 0.08w/v% sodium azide as preservative and it was used for serotyping.

Molecular detection of E. coli virulence genes:

Extraction of DNA:

The extraction of the DNA from bacterial isolates was carried out by commercial kits (Qiagen, GmbH, Germany) as recommended by the manufacturer's techniques. Three virulence genes (*fimH*, *stx*1 and *eaeA*) of *E. coli* strains were investigated by PCR technique in the PCR thermocycler (Applied Biosystems 2720). Table (1) described the primers sequence, PCR conditions, and target amplified fragment size. All reactions were carried out in a total volume of 25 µl reaction including 12.5 µl of PCR Master Mix, 6 µl of DNA template, 1 µl of each primer of 20-pmol concentration, and 4.5 µl of purified water. The amplified PCR products were then conducted to gel electrophoresis (1.5% agarose) at 1-5 volts/cm for about 30 min and stained with ethidium bromide and visualized through a UV transilluminator. PCR conditions include initial denaturation at 95 ^{0}C for 5 min, followed by 30 cycle composed of denaturation at 94^{0}C for 60sec, annealing at 52-58^{0}C for 60 sec, extension at 72^{0}C for 1 min and the final extension at 72 ^{0}C for 10 min.

Table (2): Sequences of primers used to detect the coliform bacteria isolated from bovine mastitis:

Gene	Primers sequences	Fragments	Reference
***eae*A**	ATATCCGTTTTAATGGCTATCT	425	(Ismail and Abutarbush, 2020)
	AATCTTCTGCGTACTGTGTTCA		
***sxt*1**	ATAAATCGCCATTCGTTGACTACAG	180	
	AACGCCCACTGAGATCATCGGCACT		
***fim*H**	TGCAGAACGGATAAGCCGTGG	508	
	GCAGTCACCTGCCCTCCGGTA		

RESULTS

Prevalence and clinical examination of cattle with clinical and subclinical mastitis:

In the current study, the prevalence of clinical and subclinical mastitis from examination of 400 cows was 64(16%) and 36(9%) respectively. The clinical

examination of clinical cases revealed severe inflammation of udder and clots and fakes, blood in milk with systemic reaction in most cases. While subclinical cases reported different degree of positive precipitation and gel formation in CMT as in fig (1A). The bacteriological examination revealed pink color colonies in MacConkey agar and metallic shin colonies on EMB for the *E. coli* isolates as in fig (1C-1D) respectively. Meanwhile, *Klebsiella spp*. appear as pink to purple colonies in EMB as in fig (1B). The standard biochemical identification includes IMViC tests, Urease, TSI, oxidase as in table (2).

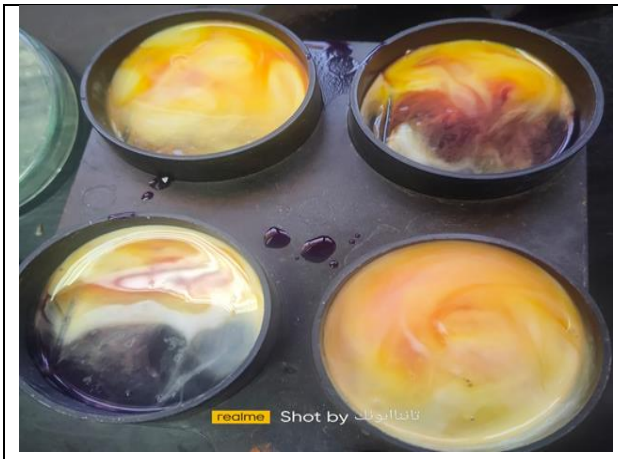

Fig (1A): positive CMT, showed high viscosity of milk in subclinical mastitis and gel formation.

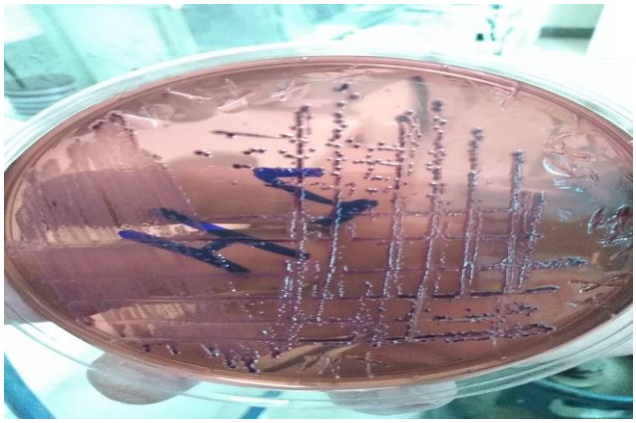

Fig (1B): *Klebsiella* spp. on EMB, showed pink to purple colonies.

Fig (1C): showed *E. coli* colonies in MacConkey agar of pink color.

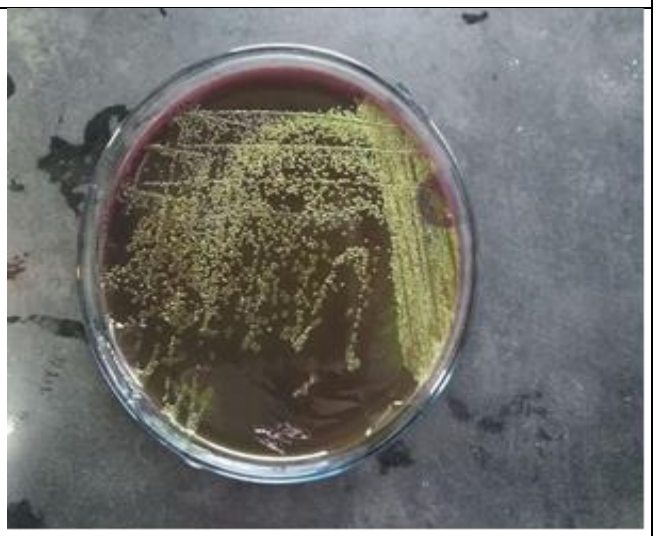

Fig (1D): showed *E. coli* colonies in EMB agar of metallic shin color.

Table (2): Biochemical identification of coliform bacteria isolated from bovine mastitis:

Bacterial culture and IMVIC test	*E. coli*		*Klebsiella oxytoca*		*Citrobacter*		*Enterobacter aerogens*	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
	10	**20.41**	**5**	**10.20**	**4**	**8.16**	**2**	**4.08**

Indole	+	+	+	–
Methyl red	+	–	+	–
Citrate	–	+	+	+
TSI	–	+	+	+
Oxidase	–	–	–	–
Urease	+	+	+	+

Results of serological identification of coliform bacteria isolated from bovine mastitis:

Ten *E. coli* and five *Klebsiella* isolates were selected based on their strong biochemical activity and then subjected for serological identification using specific antisera and the results revealed that EHEC was the most identified 4/10 followed by EPEC and ETEC 3/10 for each. While, *Klebsiella spp.* were serologically identified as *Klebsiella oxytoca* and *Klebsiella pneumonia* in 4/5 and 1/5 isolates respectively as showed in table (3).

Table (3): Results of serological identification of coliform bacteria isolated from bovine mastitis:

No. isolates	**Identified bacterium**	**Serodiagnosis**	**Strain characterization**
3	*E. coli*	O146 : H21	EPEC
4	*E. coli*	O117 : H4	EHEC
3	*E. coli*	O127 : H6	ETEC
4	*Klebsiella oxytoca*	K1	------
1	*Klebsiella pneumoniae*	K1	--------

PCR results of some virulence in E. coli strains:

The *E. coli* isolates were molecularly investigated by PCR assay to identify some virulence genes by using specific primers sets. Based on the molecular screening for virulence genes, the *fimH* gene was found in all examined strains (100%) while the *eaeA*, *sxt1* genes were not detected in any of the samples as in (Fig. 2A-C).

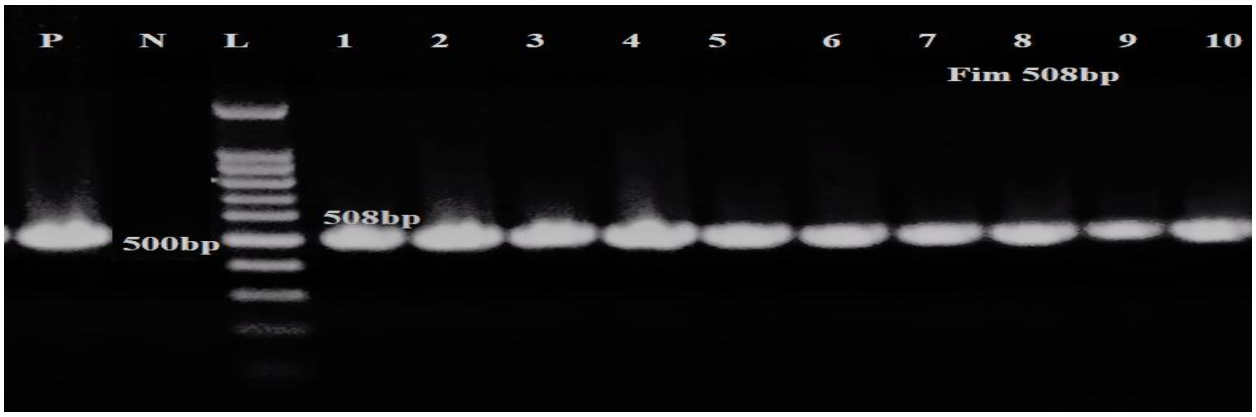

Fig (2-A): Agarose gel electrophoresis 1.5% showed the *fim*H gene of *E.coli* at (**508**) bp: Lane M: 100 bp DNA ladder. +ve.: Positive control (*E. coli*) (KY797673), -ve: Negative control Lanes from (1-10) positive samples.

Fig (2-B): Agarose gel electrophoresis 1.5% showed the *sxt1*gene of *E .coli* at (**180**) bp: Lane M: 100 bp DNA ladder. +ve.: Positive control (*E. coli*) (KY797673), -ve: Negative control Lanes from (1-10) negative samples.

Fig (2-C): Agarose gel electrophoresis 1.5% showed the *eaeA* gene of *E. coli* at (**425**) bp: Lane M: 100 bp DNA ladder. +ve.: Positive control (*E. coli*) (KY797673), -ve: Negative control Lanes from (1-10) negative samples.

DISCUSSION

Mastitis is the most costly illness that affects dairy cattle. It is still a major factor in the wide use of antimicrobials drugs in dairy cattle herds, which contributes to the spread of resistant bacteria strains(Ismail and Abutarbush, 2020). Many predominant species of bacteria such as Coagulase

negative *Staphylococci*, *Enterococci*, *Streptococci*, and *E. coli* are associated with clinical bovine-mastitis with emerging of multidrug resistant strains of bacteria (Kateete et al., 2013). The current study reported that clinical and subclinical mastitis was prevalent with 16% and 9% respectively. Similar studies in Egypt conducted by several authors (ElFaramaway et al., 2019) revealed that clinical mastitis was detected with 30% from 400 cows as well as (Mousa et al. 2015) recorded that prevalence of clinical and subclinical mastitis 20.5% and 32 respectively. Additionally, (Abed et al., 2021) determine higher prevalence of subclinical mastitis by CMT was 46%. Similarly, higher prevalence 30.23% was detected in study of (Hussein et al., 2022) from 440 quarter milk samples from 110 dairy cows. Furthermore, (Ahmed et al. 2018) applied CMT on 227 cows and 174 buffaloes in Kafr elsheikh Governorate with prevalence rate 49.9% and 44.3% respectively. Meanwhile, (Elbably and Asmaa, 2013) revealed the prevalence rate of clinical and subclinical mastitis 9.87% and 33.05% respectively. The variation between our study and other related studies may be attributed to the variance in total examined animals and collected samples, area of study, mangemental factors and climatic changes between different locations and veterinary care offered for control measures and treatment.

The clinical cases revealed severe udder inflammation and clots, fakes, and blood in milk with systemic reaction in most cases, while subclinical cases showed different degree of positive precipitation and gel formation in CMT as well as the bacteriological examination revealed that *E. coli* was the most prevalent bacteria 16 (19.51%) followed by *Klebsiella*spp 8 (9.76%), *Citrobacter* 5(6.1%), *Enterobacter Aerogens* 3(3.66%). Nearly similar findings was observed in study of (Elbably and Asmaa, 2013) who examined 272 milk samples and found that *E. coli* (18.7 %), *K. pneumonae* (3.6 %), *CNS* (37.8%), *S. aureus* (25.8%), *S. agalactiae* (11.8 %), and *S. uberis* (2.8 %). Similarly, (Ahmed et al. 2018) identified *E. coli* in 16.4% in cows with subclinical mastitis and 27.2% in subclinical mastitis in dairy buffaloes. In addition, (Abegewi et al., 2022) isolated *Enterobacter cloacae* (12.6%), *E. coli* with (7%), *K. pneumoniae* (2.4%), *Enterobacter sakazakii* (1.1%), *K. oxytoca* (0.8%) from mastitis. This was higher than reported by (Ismail and Abutarbush, 2020) indicated that prevalence of *E. coli* was 6.5% from 216 bovine mastitis. In another study, (Yu et al., 2020) demonstrated that *E. coli* strains was the common mastitis pathogens in Chinese dairy farms with prevalence rate11.1%. Likewise, (Goulart and Mellata, 2022) recorded that pathogenic *E. coli* is a key bacterial pathogen incriminated in acute bovine clinical mastitis. On the other hand, higher prevalence of *E. coli* (80.5%) and (85.7%) was recorded in subclinical and clinical mastitis respectively from examined 207 milk samples (El-Mohandes et al., 2022). Furthermore, (Xu et al., 2023) screened 156 mastitic milk samples collected from 3 scales farms and found that *E. coli*, *K. pneumoniae* were the most coliform bacteria detected with 26.99%, 23.19% respectively.

Therefore, this study investigates some of the virulence genes among *E. coli* isolates and reported that all *E. coli* strains carried the *fim*H (100%) with no detection of *stx*1, and *eae*A genes in any tested isolates. These were compared with previous investigation as in study of (Campos et al., 2022) who detected *fim*H, *tra*T and *omp*T virulence genes with prevalence 93.6%, 77.3%, and 68.2% respectively.Furthermore, (Memon et al., 2016) identified *Tra*T, *Fim*H, *pap*C, *iuc*D, F4 (K88) and *sfa* virulence gene from

103 *E. coli* isolates from 22 dairy farms in China with no detection of other virulence genes F17A, F41, *stx*1, intimin, CNF1, CNF2, LT and *ST*. The negative detection of *stx*1, and *eae*A genes in our study was in contact with Bag et al. (2021) who also not detect *stx*, *eae* and *cdt* genes in all the *E. coli* isolates.In addition, the negative detection of *sxt*1 gene may be related that the STEC stains from bovine mastitis was varied in their prevalence as it ranged from 0% to 88.9 % (Murinda et al., 2019). In a comparative study in Iran, Aflakian et al. (2022) screened forty-seven *E. coli* isolates from clinical mastitis and the *eae*A, *stx*1 and *F41* genes were most detected virulence genes with 89.3%,72.3% and 2.1% respectively. In comparative study in Brazil, lower prevalence rate of *stx* 1,*stx* 2 and intimin (*eae*) genes among 231 *E. coli* strains with prevalence of 3.5%, 5.2%, 0.8% respectively (Rangel and Marin, 2009).

CONCLUSION

Mastitis is one of the most economically disease in the global dairy herds. In addition, *E. coli* was the most identified coliform bacteria among clinical and subclinical mastitis followed by *Klebsiella* spp. Moreover, EHEC serotype was the most identified E.coli serotype followed by EPEC and ETEC serotypes, while *Klebsiella oxytoca* and *Klebsiella pneumonia* were also identified. The PCR approach was efficient tool for detection of *fim*H virulence gene among *E. coli* isolates reported that all *E. coli* strains.

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