Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Characterization of some virulence properties of subclinical mastitis-associated *Escherichia coli* in Egyptian cows and buffalos

Ibrahim N. Alkhouly[1,2], Abdelmoneim M. Moustafa[1], Nahla A. Abou El Roos2, and Sahar A. Kandeel1[,*]

[2]*Department of Animal Medicine (Infectious Diseases), Faculty of Veterinary Medicine, Benha University, Kalyobiya, 13637, Egypt*

[1]*Animal Health Research Institute - Shibin El Koom branch, Agriculture Research Center, Egypt*

ARTICLE INFO

Keywords

Virulence factors

PCR

Serological identification

VITEK-2 compact system

Received 27/05/2023
Accepted 23/06/2023
Available On-Line 01/07/2023

ABSTRACT

Bovine mastitis is a common worldwide infection caused by different types of pathogens including coliform bacteria. Understanding mastitis pathogens and their dominance, as well as risk factors, is required to facilitate disease prevention and control, and to enhance udder health within the herd. Our objectives were to investigate the prevalence of different mastitis-causing pathogens and describe some genetic traits which outline mastitis-associated *E.coli* (MAEC). Four-hundred twenty-eight quarter foremilk samples were collected from 107 apparently healthy lactating cows and buffaloes from El-Menofia Governorate between 2020-2022. The California Mastitis Test (CMT) was used to estimate the quarter somatic cell count (SCC), with subclinical mastitis (SCM) defined as non-negative CMT score. The bacterial culture of milk was used as a reference method to identify SCM based on the isolation of the causative pathogens. VITEK-2 compact system was used for isolates identification. Serological identification of *E.coli* serotypes and molecular identification of some virulence genes using PCR were also performed. The prevalence of SCM was 60.7% with *E.coli* being the most commonly isolated organism. The enteropathogenic serotypes of *E.coli* isolated from the examined milk samples showed; O_{26}:H_{11} (EHEC) 21.70%, O_{15}:H_2 (ETEC) 13.04%, O_{127}:H_6 (ETEC) 4.34%, O_{121}:H_7 (EHEC) 13.04%, O_{117}:H_4 (EHEC) 17.39%, O_{146}:H_{21} (EPEC) 17.39% and O_{103}:H_2 (EHEC) 13.04%. The molecular identification of the most common virulence genes of *E.coli* revealed the presence of *sfa*, *pap*C, and *tra*T genes in 100% of the examined samples while *cnf*1 gene was present in only 30% of the samples.

1. INTRODUCTION

Bovine mastitis is one of the highly prevalent and costly diseases worldwide. It causes economic losses due to decreased milk quality and output, impaired conception, early culling, and greater usage of medications and veterinary services (Gonçalves et al., 2018).

Mastitis is caused by a variety of pathogens including coliform bacteria (*Escherichia coli, Enterobacter aerogenes, Klebsiella oxytoca, and Klebsiella pneumonia*). Traditionally, mastitis-linked *E.coli* is considered a significant opportunistic environmental pathogen that is linked to potential enteric risk (Dogan et al., 2012; Urseler, et al., 2022). It is a major and significant cause of acute mastitis with a rapid recovery rate, frequently observed in early lactation and highly producing cows with low somatic cell count (SCC) (Hogan and Smith, 2003). However, it can occasionally result in serious systemic clinical signs such as sepsis along with fever (Shpigel et al., 2008). Occasionally, infection with *E.coli* leads to persistent and subclinical disease (Zadoks et al., 2011). Accordingly, the severity and consequence of *E.coli* mastitis are primarily credited to environmental factors, the virulence of invading strain rather than cow's immune response (Burvenich et al., 2003).

Escherichia coli is a highly diverse species, commonly found in the intestines and feces of animals where the bovine gastrointestinal tract is a natural reservoir for both commensal and pathogenic *E.coli* with considerable phylogenetic and genotypic variety, as well as the potential to induce mastitis (Houser et al., 2008). However, studies have suggested that various *E.coli* genotypes with characteristics phenotypes are better adapted to trigger mastitis than others (Blum and Leitner, 2013).

E.coli encompasses a wide range of commensal and pathogenic strains, with the ability to colonize, grow, and survive in both humans and animals, as well as abiotic habitats (Tenaillon et al., 2010). The *E.coli* population is mostly clonal and can be classified into six main phylogenetic groups, each with a varying frequency in different animal and human communities, with no strains exclusively associated with a particular host (Tenaillon et al., 2010). Pathogenic *E.coli* strains are categorized into several pathotypes based on the site of the infection, clinical features, and virulence factors. However, a significant portion of these virulence-associated factors are found in commensal strains as well, which enable the establishment of infection and initial colonization. Only up to 60% of each genome, the core genome, is shared by all strains (Kaas et al., 2012). The remaining flexible genome displays notable variation across different strains. It

* Correspondence to: saharkandeel@fvtm.bu.edu.eg

contains genes that enable adaptations for particular habitats or environmental conditions and is responsible for the *E.coli* phenotypic variations (Touchon et al., 2009).

Smallholder production is the predominant type of production in Egypt where cows and buffaloes are the predominant livestock population. The quality and safety of milk supplied to consumers have been significantly compromised due to a higher prevalence of SCM among animals (Ntuli et al., 2016). Numerous types of *E.coli* are accountable for SCM in dairy animals, and these microorganisms caused severe fatalities and outbreaks of milk-borne diseases worldwide, according to EFSA's 2015 report. Previous literature studied the prevalence of *E.coli* isolates from SCM cases in Egypt. In 2017, Abdel-Tawab et al. reported a 14.9% incidence of *E.coli* mastitis in different Egyptian governorates. In another Egyptian study by Ahmed et al., (2018) the prevalence of *E.coli* isolated from CMT-positive milk samples was 20.8% where *E.coli* was detected bacteriologically in 16.4% and in 27.2% of the CMT-positive cows and buffalo samples, respectively. A more recent study by Abdel-Fattah et al. (2023) stated an 18% incidence of *E.coli* from mastitic milk samples, and the detected *E.coli* serotypes were O_{26}, O_{44}, O_{55}:K_{99}, O_{111}, O_{119}, and O_{157}:H_7.

Despite extensive studies proposing the mammary pathogenic *E.coli* pathotype, so far, no common genetic features or virulence-associated variables have been identified among isolates of *E.coli* causing mastitis (Blum and Leitner, 2013). Recently, several studies analyzed the genome of *E.coli* strains isolated from intramammary infections (IMI) to better understand the bacteria causing mastitis. These studies identified various MAEC genome regions and genes that are associated with different levels of specificity and significance. Therefore, our main objective was to describe some genetic traits which define MAEC and to investigate the prevalence of different mastitis-causing pathogens.

2. MATERIAL AND METHODS

2.1 Animals, housing, feeding, and milking:

This study was conducted using convenient randomly selected milk samples of 107 apparently healthy dairy cows and buffaloes of different age groups, parties, and different stages of lactation. All methods were evaluated and approved by Benha University Institutional Animal Care and Use Committee Research Ethical Board (BUFVTM10-02-23). The study was performed on cows and buffaloes in moderate and small animal aggregations in the El-Menofia governorate in Egypt during the period from 2020 to 2022. Most animals under study were in tie-stall barn houses and hand milked twice daily and most hand milkers have no routine for udder disinfection before and/or after milking.

2.2 Sample collection and analysis for SCC

Each animal was examined physically to detect any systemic disorders. Udder and milk were inspected and palpated for detecting any abnormalities. A total of 428 quarter foremilk samples were collected separately. The teat end was cleaned and disinfected with alcohol 70%. Sterile milk samples were collected separately in screw-capped tubes by hand stripping (NMC, 1999). Milk SCC was determined cow-side using CMT according to Schalm and Noorlander (1957) and the change in viscosity was visually scored. The milk samples were stored in an ice box and transported to the laboratory at Animal Health Research Institute in El-Menofia Governorate for performing other tests.

2.3. Cultural method

Milk culturing was carried out according to NMC (1999) recommendations. Each milk sample was streaked on blood agar (Remel®) and MacConkey plates (Remel®) separately in a manner allowing the growth of separate bacterial colonies, then incubated at 37 °C for 24-48 hrs. The microbiological results were reported as CFU/ mL of milk.

2.4. Identification of the bacterial isolates

The microbial growth was identified using colonial morphology, hemolysis pattern, metabolic activity, biochemical tests, Gram-staining reaction, and cell morphology according to NMC (1999) recommendations before being transferred onto slope agar for further identification using VITEK-2 compact system.

Bacterial isolates characterization was performed with the VITEK-2 compact system in accordance with the manufacturer's instructions (Biomeriux, 2006). The turbidity was adjusted to the equivalent of 0.5-0.6 McFarland turbidity. Analysis was done using Gram-negative and Gram-positive bacteria identification cards. Cards were automatically read every 15 min. Data was analyzed using the VITEK-2 software (Version 9.02) according to the manufacturer's instructions.

2.4 Serological identification of E. coli

The *E.coli* isolates were serologically identified in accordance with Kok et al. (1996) using a rapid diagnostic *E.coli* antisera sets (Denka Seiken Co., Japan) for enteropathogenic type identification.

2.5. Molecular analysis of E.coli isolates

Oligonucleotide primers to detect the studied *E.coli* genes were synthesized by Biobasic (Canada) but designed and evaluated by previous literature (Table 1). The DNA was extracted from a single *E.coli* colony obtained from an overnight MC agar pure culture according to QIAamp DNA mini kit instructions. The Master Mix was prepared according to Emerald Amp GT-PCR master mix (RR310A kit). The examined virulence genes (*sfa*, *pap*C, *Trat*T, and *Cnf*1) were detected by PCR (Biometra, Germany) with amplified products at 410, 501, 307, and 620 bp, respectively as described previously (Sambrook et al., 1989). A 100 bp DNA ladder (Cat.NO. SM0243) supplied by Fermentas (USA) was used.

Table 1 Oligonucleotide primers sequences for detection of *E.coli's* four virulence genes.

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
Sfa	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410 bp	Yazdi *et al.* (2018)
*pap*C	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	501 bp	Wen-jie *et al.* (2008)
*Tra*T	GATGGCTGAACCGTGGTTATG CACACGGGTCTGGTATTTATGC	307 bp	Kaipainen *et al.* (2002)
*Cnf*1	TATATAGTCGTCAAGATGGA CACTAAGCTTTACAATATTGAC	620 bp	Kadhum *et al.* (2008)

2.6 Statistical analysis

The study's data were analyzed using ANOVA and Turkey-Kramer HSD post hoc test using SPSS statistical software. $P<0.05$ was considered significant.

3. RESULTS

3.1 The prevalence of mastitis

The quarter prevalence of SCM using CMT is shown in Table (2). The non-negative CMT reaction was the optimal cut-point used. The results showed SCM prevalence of 65.5% using CMT with the HR quarter showing the highest prevalence (112/280, 40,0%).

The prevalence of SCM based on milk culture on a quarterly basis was 60.7% (Table 2). The frequency of isolated microorganisms in relation to positive samples was shown in Fig (1). Organisms were isolated in 260 of 428 (60.7%) quarter samples with *E.coli*, and *Klebsiella Spp.* being the most commonly isolated organisms (28.4% and 23.1%, respectively). Contagious pathogens, primarily *S. aureus*, were identified in 40 samples (15.4%) while environmental pathogens were identified in 220 samples (84.6%) from the isolated pathogens with *E.coli* being the most isolated environmental pathogens (33.6%).

Table 2 The prevalence of subclinical mastitis in 428 quarter milk samples obtained from 107 dairy cows and buffalo.

Test	Quarter side	Positive (%)	Significance value
California mastitis test (CMT)	HR	112 (40%)[a]	P ≤ 0. 01
	HL	85 (30.5%)[b]	
	FR	47 (16.8%)[c]	
	FL	36 (12.7%)[c]	
	Total	280 (65.5%)	
Cultural method	HR	101(38.8%)[a]	P ≤ 0. 01
	HL	80 (30.8%)[b]	
	FR	40 (15.4%)[c]	
	FL	39 (15%)[c]	
	Total	260 (60.7%)	

* Different letters indicate significantly different

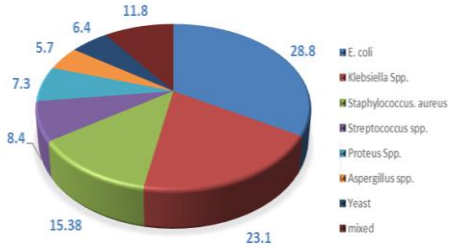

Fig 1 The prevalence of different isolated mastitis pathogens obtained from 107 dairy cattle and buffalo in relation to positive samples.

3.2. Serotyping of enteropathogenic E.coli

Table (3) declared the presence of different enteropathogenic serotypes of *E.coli* isolated from the examined milk samples.

Table 3 Serotyping of E.coli strains isolated from 23 subclinical mastitis cases.

***E. coli* strains**	**Type**	**Percent (%)**
O_{26}:H_{11}	EHEC	21.70%
O_{15}:H_2	ETEC	13.04%
O_{127}:H_6	ETEC	4.34%
O_{121}:H_7	EHEC	13.04%
O_{117}:H_4	EHEC	17.39%
O_{146}:H_{21}	EPEC	17.39%
O_{103}:H_2	EHEC	13.04%

EHEC: enterohemorrhagic *E.coli;* EPEC: enteropathogenic *E.coli;* *ETEC:* enterotoxigenic *E.coli*

3.3. Detection of virulence genes of E.coli

The molecular identification of four common virulent *E.coli* genes revealed the presence of *sfa*, *papC*, and *tra*T genes in all examined samples (n=10) while the *cnf*1 gene is present in 30% (3/10) of the examined samples (Table 4, Fig. 2-5).

Table 4 Occurrence of four virulence genes in ten *E.coli* strains isolated from subclinical mastitis cases.

E. coli serotype	Virulence factor			
	Sfa	*pap*C	*Tra*T	*Cnf*1
O_{103}:H_2	+	+	+	-
O_{127}:H_6	+	+	+	+
O_{26}:H_{11}	+	+	+	-
O_{26}:H_{11}	+	+	+	-
- O_{146}:H_{21}	+	+	+	+
O_{146}:H_{21}	+	+	+	+
O_{15}:H_2	+	+	+	-
O_{117}:H_4	+	+	+	-
O_{117}:H_4	+	+	+	-
O_{121}:H_7	+	+	+	-

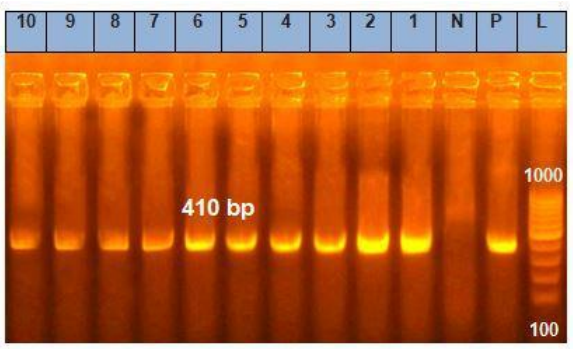

Fig. 2 Agarose gel electrophoresis of *sfa* virulent gene (410 bp) identified by PCR from *E.coli* strains isolated from subclinical mastitis cases. Lane L: 100 bp ladder as molecular size DNA marker. Lane P+: Control positive *sfa* (410 bp) virulent gene characterization of *E.coli* strains. Lane N: Control negative. Lane 1-10: positive for *sfa* (410 bp) virulent gene characterization of *E.coli* strains.

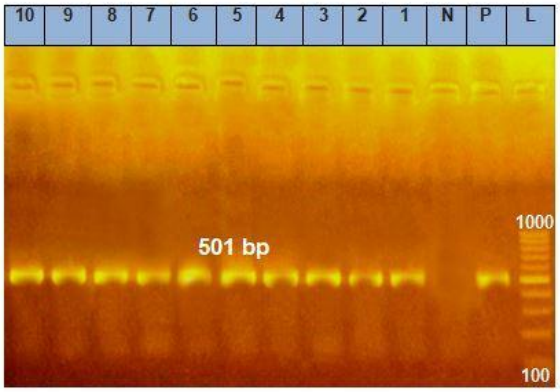

Fig. 3 Agarose gel electrophoresis of *pap*C virulent gene (501 bp) identified by PCR from *E.coli* strains isolated from subclinical mastitis cases. Lane L: 100 bp ladder as molecular size DNA marker. Lane P+: Control positive *pap*C (501 bp) virulent gene characterization of *E.coli* strains. Lane N: Control negative. Lane 1-10: positive for *pap*C (501 bp) virulent gene characterization of *E.coli*.

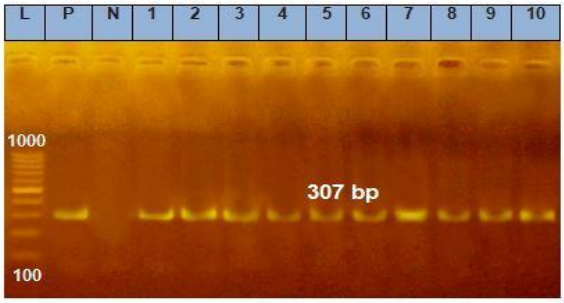

Fig. 4 Agarose gel electrophoresis of *Tra*T virulent gene (307 bp) identified by PCR from *E.coli* strains isolated from subclinical mastitis cases. Lane L: 100 bp ladder as molecular size DNA marker. Lane P+: Control positive *Tra*T (307) bp virulent gene characterization of *E.coli* strains. Lane N: Control negative. Lane 1-10: positive for *Tra*T (307 bp) virulent gene characterization of *E.coli*.

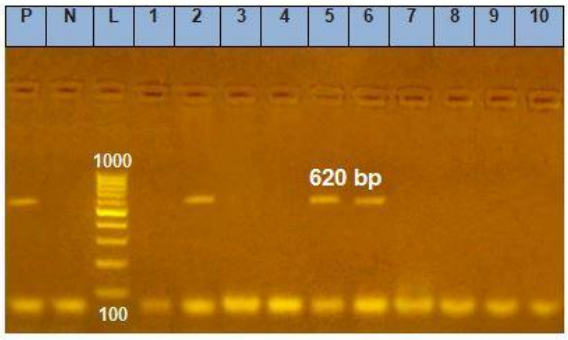

Fig. 5 Agarose gel electrophoresis of *Cnf*1 virulent gene (620 bp) identified by PCR from *E.coli* strains isolated from subclinical mastitis cases. Lane L: 100 bp ladder as molecular size DNA marker. Lane P+: Control positive *Cnf*1 (620 bp) virulent gene characterization of *E.coli* strains. Lane N: Control negative. Lane 2, 5, 6: positive for *Cnf*1 (620 bp) virulent gene characterization of *E.coli* strains.

4. DISCUSSION

Microorganisms isolated from mastitic milk samples can cause economic problems, disturb animal health, and pose a public health hazard. Knowing the etiology of IMI is extremely essential for controlling bovine mastitis and targeted therapy. *E.coli* can cause clinical or subclinical mastitis. Therefore, to better understand the disease, we serologically identified *E.coli* isolated from SCM, and investigated the distribution of four virulence genes among these isolates. Consequently, we can learn more about how the disease develops and how it can be prevented. Our study showed that the prevalence of SCM was 60.7% based on milk culture, and 65.5% based on CMT. A previous study by Mureithi and Njuguna, (2016) reported similar results (64%) using CMT. On the other hand, other studies by Kamal et al. (2014) and Mbindyo et al. (2020) showed a higher prevalence (73.0% and 73.1%, respectively) than we reported here; while Ayano et al. (2013), and El-Kholy et al. (2018) found a lower prevalence of 41.02% and 44.83%, respectively.

Many factors e.g., poor hygienic measures, malfunctioning milking machines, improper milking procedures, and insufficient treatment approaches may contribute to the higher prevalence of SCM among dairy animals. These variations might be related to mastitis nature as a complex disease involving interactions of multiple factors such as management, environment, and animals and pathogens factors (Constable et al., 2017).

Our study found that the RH quarters had the highest prevalence of SCM, while the LF quarters had the lowest prevalence. In earlier investigations by Sudhan et al. (2005), the RH quarter of dairy cows was found to be most commonly affected by mastitis with a prevalence of 38.18% when compared to other quarters. Additionally, Lee and Lee (2007) noted that the somatic cell score of milk samples from the front quarters was lower than that from the rear quarters which suggests the higher susceptibility of the rear quarters to mastitis. Our findings disagreed with another study that found the prevalence of SCM was highest in LF quarters, followed by RF, LH, and RH quarters (Hussein et al., 2022). It was hypothesized that rear quarters are more vulnerable to mastitis compared to front quarters due to higher milk production, greater exposure to environmental effects, and udder's anatomical structure that increase the likelihood of exposure to dirt and trauma due to lower teats (Berry and Meaney, 2006), and the higher possibility of contamination by urine and feces.

The bacteriological examination showed that the most predominant isolated bacteria are *E.coli* followed by *Klebsiella spp*., and *S. aureus*. In a study by Bakr et al. (2019), the most common isolated pathogens were *S. aureus, S. agalactiae,* and *E.coli.* These microorganisms are all known to cause disease in humans, and their presence in milk or animal products can pose a serious public health risk. According to a previous study by Fahim et al. (2019), *E.coli* is responsible for more than 80% of coliform mastitis. Inadequate cleaning, faulty drainage, insufficient udder washing, use of unclean washing towels, and failure to use post-milking teat dipping could all be linked to increased *E.coli* infections (Ayano et al., 2013).

E.coli is found in human and animal intestines; most strains are harmless while others can cause disease. *E.coli* strains are traditionally characterized by serological identification of somatic O, flagellar H, capsular K, and fimbria F antigens (Quin et al., 1998). Pathogenic *E.coli* strains can be distinguished from normal flora strains by identifying the presence of these virulence factors. The most common pathogenic strains are: enteropathogenic *E.coli* (EPEC) which causes disease by attaching and invading target cells, enterohemorrhagic *E.coli* (EHEC) which produces toxins leading to cell damage, and enteroaggregative *E.coli* (EAEC) that causes disease by attaching to host cells and forming aggregates leading to cell damage (Nagy and Fekete, 1999), in addition to the enterotoxigenic *E.coli* (ETEC) which produce special toxins (heat-stable and heat-labile toxin). The results of this study showed that the most common enteropathogenic serotypes of *E.coli* isolated from milk samples were *O26:H11 (EHEC), O15:H2 (ETEC), O127:H6 (ETEC), O121:H7 (EHEC), O117:H4 (EHEC), O146:H21 (EPEC),* and *O103:H2 (EHEC)*. This is a concern because these serotypes are known to cause diarrhea in humans or serious illness and their presence in milk is a potential risk to public health. Therefore, it is important to take some steps to prevent IMI and milk contamination with *E.coli* including maintaining a clean and sanitary milking environment, washing cows' udders before milking, using clean and sanitized milking equipment, and pasteurizing milk before consumption (Scallan et al., 2011).

Pathogenic *E.coli* strains have a variety of virulent factors that are necessary to combat the host's immune response and to colonize, proliferate, and survive in the udder (Kaipainen et al., 2002). These include toxins, adhesins, invasions, capsule production, serum resistance, and iron scavenging. Only isolates with successful combinations of virulence factors will be capable of causing disease (Kaper et al., 2004). The presence of virulent factors facilitates the identification of pathogenic *E.coli* strains. This information can be used to develop new treatments as well as prevent the spread of *E.coli* infections. Considering the losses associated with *E.coli* mastitis, recognition of the *E.coli* ability to adapt to diverse environments, the limited efficacy of treatment, and the potential public health implications; the current study sought to investigate the presence of some virulence factors in *E.coli* isolates from IMI in an attempt to establish a potential correlation between these characteristics and mastitis. The results of our study showed the presence of three virulence genes (*sfa*, *pap*C, and *tra*T*)* in all *E.coli* isolates from SCM cases. These genes are all known to contribute to the pathogenicity of *E.coli*, and their presence in all of the examined samples suggests that they are essential for the development of the disease. The *sfa* gene (S and F1 fimbriae) encodes a surface adhesin that allows *E.coli* to adhere to and colonize host cells, while the *pap*C gene (P fimbriae) encodes a protein that is required for the assembly and function of P fimbriae, which are adhesive structures that allow *E.coli* to attach to, invade, and colonize specific host tissues, and the *traT* gene (serum resistance-associated protein) encodes a protein thought to play a role in protecting *E.coli* from the host's immune system by inhibiting the complement system, that allows *E.coli* to spread from cell to cell (Sarowska et al., 2019). Similar results were obtained by Campos et al. (2022) as *tra*T genes are the most common isolated genes. Guerra et al. (2019) identified *tra*T gene as a common feature among *E.coli* isolates from mastitic samples. Another investigation failed to identify virulence factors, including the *pap* genes, in *E.coli* strains linked to persistent bovine mastitis, in contrast to our study where this gene is detected in all examined isolates (Dogan et al., 2006). We also noticed the presence of *cnf*1 gene (cytotoxic necrotizing factor1), encodes for a protein toxin called CNF1 which damages the host cells by interfering with cellular signaling, in 30% of the examined isolates which suggests that although it is one

of the *E.coli* virulence genes, it is not essential for the development of the disease; however, it may contribute to the severity of the disease. In 2022, a quite different result was reported by Jouini et al. who found that the *cnf*1 gene is a common feature among *E.coli* isolates. These findings have important implications for the prevention and treatment of *E.coli* infections where targeting the virulent genes involved in the disease such as *sfa, pap*C, and *tra*T, can develop more effective treatments and strategies to prevent the transmission of *E.coli* from the environment to cows.

Overall, our study provides insight into the pathogenesis of mastitis caused by *E.coli* infection and has a significant implication for the prevention and treatment of the disease. However, further studies using different virulence factors are necessary to understand the link between these factors and their classification in distinct pathotypes and to investigate the potential involvement of commensal *E.coli* strains as a possible mastitis pathogen.

5. CONCLUSION

Our study provides an overview of the prevalence of SCM and the most common isolated pathogens with a special reference to *E.coli* associated SCM. These findings suggest that enteropathogenic serotypes of *E.coli* isolated from milk samples have a public health hazard, highlighting the need for designing effective prevention and treatment strategies in Egypt.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors didn't receive funding support or product donations anywhere. This report represents a portion of a master thesis submitted by the first author to Faculty of Veterinary Medicine, Benha University.

6. REFERENCES

1. Abd El-Fattah, O.A., Kotb, E.E.Z., Ibrahim, H.S., El-Gohary, A.H., Mohammed, A.A.E. 2023. Detection and Molecular Characterization of Some Virulence Genes of Escherichia Coli Isolated from Milk in Dairy Cow Farms. Journal of Applied Veterinary Sciences, 8(2): 01 – 09. 10.21608/javs.2023.173140.1190
2. Abdel-tawab, A.A., Nabih, A. M., Agag, M.A., and AL-Abbou, M. A., 2017. Bacteriological and molecular studies on Shiga-Toxin producing Escherichia coli causing cattle clinical mastitis. Benha Veterinary Medical Journal, 33(2):17–26. https://www.researchgate.net/publication/320557730
3. Ahmed, H.F., Straubinger, R.K., Hegazy, Y.M., and Ibrahim, S. 2018. Subclinical mastitis in dairy cattle and buffaloes among small holders in Egypt: Prevalence and evidence of virulence of Escherichia coli causative agent. Tropical Biomedicine. 35(2): 321–329. PMID: 33601806. https://pubmed.ncbi.nlm.nih.gov/33601806/
4. Ayano, A.A., Hiriko, F., Simyalew, A.M., Yohannes, A. 2013. prevalence of subclinical mastitis in lactating cows in selected commercial dairy farms of Holeta district. Journal of Veterinary Medicine and Animal Health. 5(3), 67-72. https://doi.org/10.5897/JVMAH12.056
5. Bakr, N., Shaker, E.M., Sayed, M. 2019. Detection of subclinical mastitis in milk of dairy cows in Sohag city, Egypt. Assiut Veterinary Medical Journal. 65(160), 51-58. http://www.aun.edu.eg/journal_files/6...
6. Berry, D.P. and Meaney, W.J. 2006. Interdependence and distribution of subclinical mastitis and intra-mammary infection among udder quarters in dairy cattle. Preventive Veterinary Medicine. (75), 81-91. 10.1016/j.prevetmed. 2006.02.001
7. Biomeriux VITEK-2 Compact ref Manua –Ref-414532. 2006. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7A5. Normal committee for clinical laboratory standards. Wayne, https://clsi.org/media/1928/m07ed11_sample.pdfPa.
8. Blum, S.E. and Leitner, G. 2013. Genotyping and virulence factors assessment of bovine mastitis Escherichia coli. Veterinary Microbiology. 163(3–4), 305–12. https://doi.org/10.1016/j.vetmic.2012.12.037
9. Burvenich, C., Van-Merris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L. 2003. Severity of E.coli mastitis is mainly determined by cow factors. Veterinary Research. 34(5),521–64. 10.1051/vetres:2003023
10. Campos, F.C., Castilho, I.G., Rossi, B.F., Bonsaglia, É.C., Dantas, S.T., Dias, R.C., Rall, V.L. 2022. Genetic and Antimicrobial Resistance Profiles of Mammary Pathogenic E.coli (MPEC) Isolates from Bovine Clinical Mastitis. Pathogens. 11(12), 1435. 10.3390/pathogens11121435
11. Constable, P.D., Hinchcliff, K.W., Done, S.H., Grünberg, W. 2017. Mastitis. Chapter 20, Pages 2113–2208 in Veterinary Medicine: Aa textbook of the diseases of cattle, horses, sheep, pigs, and goats. Elsevier Health Sciences. 11th ed. Published by Saunders Ltd.
12. Dogan, B., Klaessig, S., Rishniw, M., Almeida, R.A., Oliver, S.P., Simpson, K. et al. 2006. Adherent and invasive Escherichia coli are associated with persistent bovine mastitis. Veterinary Microbiology. 116(4),270–82. 10.1016/j.vetmic.2006.04.023
13. Dogan, B., Rishniw, M., Bruant, G, Harel, J., Schukken, Y.H., Simpson, K.W. 2012. Phylogroup and lpfA influence epithelial invasion by mastitis-associated Escherichia coli. Veterinary Microbiology. 159(1–2), 163–70. 10.1016/j.vetmic.2012. 03.033
14. EFSA (European Food Safety Authority). 2015. Scientific opinion on the public health risks related to the consumption of raw drinking milk. EFSA Journal. 13: 3940. https://doi.org/10.2903/j.efsa.2015.3940
15. El-Kholy, A.M., Hassan, G.M., Zeinhom, M.M.A., El-Klawy, M.A. 2018. Detection of subclinical mastitis in a dairy farm in Beni Suef city, Egypt. Assiut Veterinary Medical Journal. 64(157), 18-24. https://www.cabdirect.org/cabdirect/abstract/20183301170
16. Fahim, K.M., Ismael, E., Khalefa, H.S., Farag, H.S., Hamza D.A. 2019. Isolation and characterization of E. coli strains causing intra-mammary infections from dairy animals and wild birds. International journal of veterinary science and medicine. 7(1), 61-70. 10.1080/23144599.2019.1691378
17. Gonçalves, J.L., Kamphuis, C., Martins, C.M.M.R., Barreiro, J.R., Tomazi, T., Gameiro, A.H., Hogeveen, H., dos-Santos, M.V. 2018. Bovine subclinical mastitis reduces milk yield and economic return. Livestock Science. 210, 25–32. https://doi.org/10.1016/j.livsci.2018.01.016
18. Guerra, S.T., Dalanezi, F.M., Paula, C.L., Hernandes, R.T., Pantoja, J.C.F., Listoni, F.J.P., Langoni, H., Ribeiro, M.G. 2019. Putative virulence factors of extra-intestinal Escherichia coli isolated from bovine mastitis with different clinical scores. Letters in Applied Microbiology. 68, 403–408. 10.1111/lam. 13113
19. Hogan, J., Smith, K.L. 2003: Coliform mastitis. Veterinary Research. 34, 507-519. doi.org/10.1051/vetres:2003022
20. Houser, B.A., Donaldson, S.C., Padte, R., Sawant, A.A., DebRoy, C., Jayarao, B.M. 2008. Assessment of phenotypic and genotypic diversity of Escherichia coli shed by healthy lactating dairy cattle. Foodborne Pathogens and Disease. 5(1),41–51. 10.1089/fpd.2007.0036
21. Hussein, O.H., Abdel-Hameed, K.G., El-Malt, L.M. 2022. Prevalence and public health hazards of subclinical mastitis in dairy cows. SVU-International Journal of Veterinary Sciences. 328 5(3), 52-64. 10.21608/svu.2022.131652.1189
22. Jouini, A., Klibi, A., Kmiha, S., Hamrouni, S., Ghram, A., Maaroufi, A. 2022. Lineages, Virulence Gene Associated and

Integrons among Extended Spectrum β-Lactamase (ESBL) and CMY-2 Producing Enterobacteriaceae from Bovine Mastitis, in Tunisia. Pathogens. 11(8), 948. 10.3390/pathogens11080948

23. Kaas, R.S., Friis, C., Ussery, D.W., Aarestrup, F.M. 2012. Estimating variation within the genes and inferring the phylogeny of 186 sequenced diverse Escherichia coli genomes. BMC Genomics. 13:577. https://doi.org/10.1186/1471-2164-13-577
24. Kadhum, H.J., Finlay, D., Rowe, M.T., Wilson, I.G., Ball, H.J. 2008. Occurrence and characteristics of cytotoxic necrotizing factors, cytolethal distending toxins and other virulence factors in Escherichia coli from human blood and faecal samples. Epidemiology and Infection.136, 752–760. 10.1017/ S0950268807009247
25. Kaipainen, T., Pohjanvirta, T., Shpigel, N.Y., Shwimmer, A. Pyörälä, S. Pelkonen, S. 2002. Virulence factors of Escherichia coli isolated from bovine clinical mastitis. Veterinary Microbiology. 85,37–46. 10.1016/s0378-1135(01)00483-7
26. Kamal, R.M., Bayoumi, M.A. and Abd-El-Aal, S.F.A. 2014. Correlation between some direct and indirect tests for screen detection of subclinical mastitis. International Food Research. 21(3), 1249-1254. https://www.cabdirect.org/cabdirect/ abstract/ 20143199569
27. Kaper, J.B., Nataro, J.P., Mobley, H.L. 2004. Pathogenic Escherichia coli. Nature Reviews Microbiology. 2,123–140. https://doi.org/10.1038/nrmicro818
28. Kok, T., Worswich, D., Gowans, E. 1996. Some serological techniques for microbial and viral infections. In: Practical Medical Microbiology, 14th Ed., Edinburgh, Churchill Livingstone, UK.
29. Lee, J.C. and Lee, C.Y. 2007. Study on the relationship between udder and teat characteristics and somatic cell count. Journal of Veterinary Clinics 24, 172-176. https://www.researchgate.net/ publication/286050332_Study_on_the_relationship_between_ udder_and_teat_characteristics_and_somatic_cell_count
30. Mbindyo, C.M., Gitao, G.C., Mulei, C.M. 2020. Prevalence, Etiology, and Risk Factors of Mastitis in Dairy Cattle in Embu and Kajiado Counties, Kenya. Veterinary Medicine International. 2020, 8831172. 10.1155/2020/8831172
31. Mureithi, D.K. and Njuguna, M.N. 2016. Prevalence of subclinical mastitis and associated risk factors in dairy farms in urban and peri-urban areas of Thika Sub County, Kenya. Livestock Research for Rural Development. 28(2), 13. https://www.researchgate.net/publication/292604416
32. Nagy, B. and Fekete, P.Z. 1999. Enterotoxigenic Escherichia coli (ETEC) in farm animals. Veterinary Research. 30, 259-284. https://pubmed.ncbi.nlm.nih.gov/10367358/
33. NMC. 1999. Laboratory Handbook on Bovine Mastitis. Natl. Mastitis Counc. Inc., Madison, WI.
34. Ntuli, V., Njage, P.M.K., Buys, E.M. 2016. Characterization of Escherichia coli and other Enterobacteriaceae in producer-distributor bulk milk. Journal of Dairy Sciences 99: 1-16. https://doi.org/10.3168/jds.2016-11403
35. Quin, M., Lathem, T.L., Smith, H.W. 1998. Serological identification of Escherichia coli. Methods in Molecular Biology. 100, 197-211. Springer
36. Sambrook, J., Fritscgh, E.F. Mentiates. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York.
37. Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G., Choroszy-Krol, I. 2019. Virulence factors, prevalence and potential transmission of extraintestinal pathogenic Escherichia coli isolated from different sources: recent reports. Gut Pathogens. 11, 10. 10.1186/s13099-019-0290-0
38. Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Griffin, P.M. 2011. Foodborne illness acquired in the United States—major pathogens by food category, 1999-2008. Emerging Infectious Diseases. 17(1), 16-22. 10.3201/eid1701.p11101
39. Schalm, O.W., D.O. Noorlander. 1957. Experiments and observations leading to development of the California mastitis test. Journal of the American Veterinary Medical Association. 130,199–204. https://europepmc.org/article/MED/13416088
40. Shpigel, N.Y., Elazar, S., Rosenshine, I. 2008. Mammary pathogenic Escherichia coli. Current Opinion in Microbiology. 1(1),60–5.
41. Sudhan, N.A., Singh, R., Singh, M. Soodan, J.S. 2005. Studies on prevalence, etiology and diagnosis of subclinical mastitis among cross bred cows. Indian Journal of Animal Research 39, 127-130. https://arccjournals.com/journal/indian-journal-of-animal-research/ARCC3313
42. Tenaillon, O., Skurnik, D., Picard, B., Denamur, E. 2010. The population genetics of commensal Escherichia coli. Nature Reviews Microbiology. 8(3),207–17. 10.1038/nrmicro2298
43. Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., et al. 2009. Organised genome dynamics in the Escherichia coli species results in highly diverse adaptive paths. PLOS Genetics. 5(1),e1000344. https://doi.org/10.1371/ journal.pgen.1000344
44. Urseler, N., Bachetti, R., Morgante, V. Agostini, E., Morgante, C. 2022. Groundwater quality and vulnerability in farms from agricultural-dairy basin of the Argentine Pampas. Environmental Science and Pollution Research. 29, 63655–63673. 10.1007/s11356-022-20073-9
45. Wen-jie, J., Zhi-ming, Z., Yong-zhi, Z., Ai-jian, Q., Hong-xia, S., Yue-long, L., Jiao, W., Qian-qian, W. 2008. Distribution of virulence-associated genes of avian pathogenic Escherichia coli isolates in China. Agricultural Sciences in China. 7(12),1511-1515. doi.org/10.1016/S1671-2927(08)60410-1
46. Yazdi, M., Bouzari, M., Ghaemi, E.A. 2018. Detection of fim, pap, sfa and afa Adhesin-Encoding Operons in Escherichia coli Strains Isolated from Urinary Tract Infections. Medical Laboratory Journal.12 (5), 10-15. https://doaj.org/article/ c73d4a69cb7743f9a689da5e7d108c76
47. Zadoks, R.N., Middleton, J.R., McDougall, S., Katholm, J., Schukken, Y.H. 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Mammary Gland Biology Neoplasia. 6(4),357–72. 10.1007/s10911-011-9236-y