

## Subclinical mastitis in dairy cattle and buffaloes among small holders in Egypt: Prevalence and evidence of virulence of *Escherichia coli* causative agent

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**Abstract.** This study aimed first to estimate the prevalence of subclinical mastitis (SCM) among dairy cows and buffaloes of smallholders who sell milk directly to consumers through bulk tanks of milk distributors. The second aim is to estimate the prevalence of *Escherichia coli* in milk of SCM cases and identify its virulence genes and to emphasize the public health risk from drinking this milk. A total of 227 and 174 dairy cows and buffaloes, respectively from Kafrelsheikh Governorate, Egypt were examined with California Mastitis Test (CMT) to estimate the prevalence of SCM. Samples were also screened for *E. coli* using classical bacteriological and molecular methods. The prevalence of CMT-positive cows and buffaloes samples examined was 47.4% (49.9% and 44.3% for cows and buffaloes, respectively). Cows were found to be at a higher risk of getting high CMT score than buffaloes. *E. coli* was detected bacteriologically in 16.4% and in 27.2% of the CMT-positive cows and buffalo samples, respectively. A total of 83.1% and 75.6% of isolates were confirmed as *E. coli* using PCR technique. A multiplex PCR assay was used to identify five virulence genes in the *E. coli* isolates; the *eae* gene for enteropathogenic *E. coli*, *stx* for Shiga toxin-producing *E. coli*, *elt* and *est* for enterotoxigenic *E. coli*, and the *hlyA* of the enterohemolysin gene for enterohemorrhagic *E. coli*. Only one *E. coli* strain identified carried two virulence genes (*eae* and *est*). The high prevalence of SCM among dairy cows and buffaloes in the study area indicated that there is high risk to consumers who consume milk of these animals. Also, control of SCM is a prerequisite among smallholders in Egypt in order to minimize its deleterious effects such as microbial antibiotic resistance and public health hazards. To our knowledge, this is the first study that highlights the ecology of virulence by *E. coli* causing SCM in Kafrelsheikh governorate, Egypt. This study offers the basis for further phenotypic and molecular characterization of *E. coli* found in raw milk in order to guarantee safe consumption of raw milk and milk products.

### INTRODUCTION

Subclinical mastitis (SCM) is a problem of dairy animals that cause enormous losses for owners and has a negative impact on the national income especially in developing countries. The quality of milk will determine its market value and severe losses in milk

yield are one of the major consequences of SCM (Losinger, 2005). Milk obtained from animals with SCM may have certain zoonotic pathogens which can cause serious public health and food-borne diseases (Cursons *et al.*, 2005; Abdel-Rady & Sayed, 2009).

In developing countries, livestock constitutes a major source of national income

(Aidaros, 2005). Egypt is one of the countries where production of smallholders are predominant. Cattle and buffaloes in Egypt are reared as domestic animals and account for 80-85% of the livestock population (Galal *et al.*, 1996; Aidaros, 2005). Milk from Egyptian villages are collected by milk collectors and is considered as the main source of dairy products offered to the people in Egypt. Milk collectors usually sell fresh unpasteurized milk either to i) local consumers in the same village, ii) food shops in other villages or nearby-cities which sell it to consumers as fresh unpasteurized milk, iii) small and/or large dairy processing plants which in turn either sell it as fresh milk, cream or butter without heat treatment or as pasteurized milk (Hatem & Metwally, 2002). A larger number of animals with subclinical mastitis has resulted in a major negative effect on the quality and safety of the milk offered to consumers (Swai & Schoonman, 2011; Ntuli *et al.*, 2016).

Several *E. coli* pathotypes are responsible for SCM in dairy animals. These pathogens are responsible for serious fatalities and milk borne disease outbreaks around the world (EFSA, 2015). The great heterogeneity of *E. coli* genome classified these bacteria as either commensal or pathogenic; causing intestinal or extra-intestinal human infections. The ability of *E. coli* to acquire mobile genetic elements carrying different virulence factors (VFs) are responsible for the pathogenicity of the virulent strains (Lawrence, 2005; Croxen & Finlay 2010). Recent serious foodborne outbreaks of hemorrhagic colitis or hemolytic-uremic syndrome (HUS) or acute renal failure caused by Shiga toxin-producing *E. coli* (STEC) were recorded worldwide (Rhoades *et al.*, 2009; Etcheverría & Padola, 2013). The virulent genes of STEC *E. coli* include (*stx1* and *stx2*) which inhibits protein synthesis of host cells, and the intimin gene (*eae*) which mediates colonization (Ntuli *et al.*, 2016). Another virulent factor of STEC is Enterohaemolysin (*hly*) which is encoded by the *hlyA* gene (Surendraraj Alagarsamy *et al.*, 2010). The virulent genes of *E. coli* also include *elt*, *est* of STEC and enterotoxigenic *E. coli* (ETEC). Antimicrobial resistance

among some commensal *E. coli* strains isolated from cattle can be associated with the presence of virulence factors and this is of a major public health risk with the introduction of these bacteria to the food chain (Martínez & Baquero, 2002; Newell *et al.*, 2010). Currently little information is available regarding the molecular characterization of *E. coli* isolated from milk of SCM cases in Kafrelsheikh governorate, Egypt.

The aim of this study is to determine the frequency of SCM among dairy cows and buffaloes in Kafrelsheikh governorate, Egypt and also, to determine the distribution of five virulence genes (*eae*, *stx*, *est*, *elt* and *hlyA*) of *E. coli* isolated from milk of SCM cases in study area.

## MATERIALS AND METHODS

This cross sectional study was carried out between January to July 2015 to examine 401 lactating animals (227 mixed breed cows and 174 native Egyptian buffaloes) that showed no clinical signs in Kafrelsheikh governorate, Egypt to SCM. Cows and buffaloes udder consists of four mammary glands (udder quarters). Each quarter is considered as independent unit, from a mastitis point of view. As a rule, mastitis does not affect the whole udder but rather single quarters (Eriksson, L. 2013). One milk sample was obtained from each functioning quarter of examined animals. There are some non-functioning quarters in the examined animals due to chronic inflammation called blind quarters. A total of 1,547 milk samples (865 and 682 from cows and buffaloes, respectively) were collected from apparently healthy lactating animals and were examined with CMT for detection of SCM. Positive milk samples for CMT were used for isolation and identification of *E. coli* using traditional bacteriological examination and PCR. The detection of *E. coli* selected virulence genes was carried out for the identified strains.

### California Mastitis Test and Prevalence Estimation

Quarter milk samples, were screened in the field using the California Mastitis Test (CMT)

(Schalm *et al.*, 1971). After excluding the first streams of milk, 5 ml of milk sample was collected from the animals examined in CMT paddle and an equivalent volume of the test reagent was added. Samples were scored as negative or trace or 1 plus (weak positive) or 2 plus (positive) or 3 plus signs (strongly positive).

The prevalence of SCM among dairy buffaloes and cows was estimated by dividing the number of positive quarters to CMT by the number of quarters examined and then multiply the result by 100. CMT positive milk samples were collected under aseptic conditions in labelled sterile screw capped bottles.

### Isolation of *E. coli*

Ten milliliters of the milk samples were centrifuged. The sediment was suspended with equal volume of sterile distilled water. Loopfull of prepared milk samples were streaked onto the MacConkey's agar and incubated at 37°C for 24 to 48 h. Colonies were identified biochemically and by molecular means.

### Biochemical Identification of *E.coli*

Suspected colonies were identified by their morphological characteristic appearance and hemolytic activity, followed by Gram staining and motility tests before it is transferred into semisolid or slope agar for further identification according to Quinn *et al.* (2011). Biochemical tests, specifically indole production, methyl red test, urease production, citrate utilization, triple sugar iron agar (TSI) and sorbitol were performed as required.

### Molecular Identification of *E. coli* Isolates and its Virulence Genes

DNA extraction for the isolated and identified colonies of *E. coli* was performed according

to the instructions of the kits for bacterial DNA genomic extraction obtained from Jena bioscience co. Amplification of DNA to determine presence of *E. coli* DNA was carried out according to Riffon *et al.* (2001) using primer and target gene and size as shown in Table 1. The working concentration of primers: 20 pmol/µl was prepared with sterile filtered distilled water; aliquoted and stored at -20°C that were ready to use in each cycle. PCR was performed in Eppendorff PCR System in a final volume of 25 µl of each of the primer pair (Metabion international AG, Germany), 5 µl of master mix 5X, 12.5 µl of double deionized water (Jena Bioscience, Germany) and 5 µl of the DNA template. Initial denaturation was done at 95°C for 2 min before applying 35 cycles, each cycle with denaturation at 95°C for 45 s, annealing 50°C for 1 min, and extension at 72°C for 30 sec. After the final cycle, one cycle of extension at 72°C for 5 min was carried out to complete the reaction. Initially PCR assay was performed for each pathogen using known standard DNA and with 5 pmol each of the specific primer pair. Subsequently PCR was performed with mixture of standard DNAs with various combinations of templates and primers so as to rule out no specific amplification or internal primer-primer hybridization. PCR products were then electrophoresed in a 2.5% agarose gel, stained with ethidium bromide, and visualized by UV trans-illumination.

Amplification of the DNA to determine presence of selected virulence gene in isolated *E. coli*, that achieved by amplification of DNA of the selected *E. coli* to the target genes (*cae*, *stx*, *elt*, *est*) and (*hlyA*) using primer and target genes and size as in Table 2 according to Paton and James (1998) and Toma *et al.* (2003). The primers were reconstituted in double deionized water to a final concentration of 1000 pmol/µl and

Table 1. primer and target gene and size for amplification of the DNA to determine presence of *E. coli* in milk of subclinical cases of dairy animals in Egypt

Target of <i>E. coli</i>	Oligonucleotide	Sequence (5'-3')	PCR product size (bp)
ECO-F	( <i>tra T</i> ) gene	TCT GCG GGA GTC TCA GGG ATG GCT G	313 bp
ECO-R	( <i>tra T</i> ) gene	GTA TTT ATG CTG GTT ACC TGT TT	

Table 2. Primer sequences used for amplification of virulence genes in isolated *E. coli* in milk of subclinical cases of dairy animals in Egypt and expected target size

Target of <i>E. coli</i>	Oligonucleotide	Sequence (5'-3')	PCR product size (bp)
SK1 F	( <i>eae</i> ) gene	CCCGAATTCGGCACAAGCATAAGC	881 bp
SK1 R	( <i>eae</i> ) gene	CCCGGATCCGTCTCGCCAGTATTCG	
VTcom u, d F	( <i>stx</i> ) gene	GAGCGAAATAATTTATATGTG	518 bp
VTcom u, dR	( <i>Stx</i> ) gene	TGATGATGGCAATTCAGTAT	
AL 65, 125 F	( <i>est</i> ) gene	TTAATAGCACCCGGTACAAGCAGG	147 bp
AL 65, 125 R	( <i>est</i> ) gene	CCTGACTCTTCAAAAGAGAAAATTAC	
LT L, R F	( <i>Elt</i> ) gene	TCTCTATGTGCATACGGAGC	322 bp
LT L, R R	( <i>Elt</i> ) gene	CCATACTGATTGCCGCAAT	
hlyA-F (O111) F	( <i>hlyA</i> ) gene	GCATCATCAAGCGTACGTTCC	534 bp
hlyA-F (O111) R	( <i>hlyA</i> ) gene	AATGAGCCAAGCTGGTTAAGCT	

stored at -80°C as stock solution. The working concentration of 20 pmol/µl was prepared with sterile filtered distilled water; aliquoted and stored at -20°C ready to use each cycle. PCR was performed in Eppendorff PCR System in a final volume of 25µl. Consisting of 5 pmol each of the primer pair, 5µl of Master mix 5X (Jena Bioscience). The condition of the PCR program was 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, for 30 cycles, and 72°C for 10 min, as in Table 4. PCR products were then electrophoresed on a 2.5% agarose gel, stained with ethidium bromide and visualized with an UV transillumination device.

### Statistical analysis

The statistical significant difference at  $P < 0.05$  of the prevalence of SCM and its intensity between cows and buffaloes was calculated using chi square. Also the same test was used to find the statistical significance difference at  $P < 0.05$  between the number of isolated *E. coli* among cows and buffaloes.

## RESULTS

The prevalence of SCM using CMT among examined quarters was 47.4% where 734 out of 1, 547 quarters reacted positive with variable degrees. The degree was related to

the CMT score as represented in table (3). It was found that the prevalence of quarters positive to CMT in cows 432/865 (49.9%) was significant higher than the prevalence in buffaloes 302/682 (44.3%),  $P < 05$ , Table 3. Regarding the intensity of CMT, buffaloes have a significant higher prevalence, 46.4% of weak positive quarters than cows, 20.1%  $P < 05$  (Table 3). On the other hand, cows have a significant higher prevalence, 43.1% of strong positive quarters than buffaloes, 11.6%  $P < 05$  (Table 3).

### Results of Isolation and Identification of *E. coli* and its Virulence Genes

Based on biochemical identification, out of 734 CMT positive milk samples, *E. coli* was isolated from 16.4% and 27.2% of cows and buffaloes samples, respectively (Table 4). Molecular identification of the former biochemically identified *E. coli* isolates confirmed that 83.1% (59/71) and 75.6% (62/82) of isolates of cows and buffaloes samples, respectively were *E. coli* using PCR technique (Table 4).

The distribution of virulence genes detected in the *E. coli* isolates is shown in Table 5. All isolates lacked the following genes: *stx*, *elt*, and *hlyA*. While *eae* and *est* virulence genes were detected in one strain which gave a characteristic band at 881 bp and 147 bp, respectively.

Table 3. The prevalence of subclinical mastitis among dairy cows and buffaloes in Egypt using California mastitis test and the intensity of infection

Animals	No. of animals	Blind Quarters	Examined Quarters	Total positive CMT		CMT categories					
						CMT 1		CMT 2		CMT 3	
				No.	%	No.	%	No.	%	No.	%
Cow	227	43	865	432	49.9*	87	20.1	159	36.8	186	43.1*
Buffaloes	174	14	682	302	44.3	140	46.4*	127	42.1	35	11.6
Total	401	57	1547	734	47.4	227	30.9	286	39.0	221	30.1

\* indicates significant difference at  $P < 0.05$ .  
CMT is California mastitis test.

Table 4. Prevalence of *E. coli* isolated from CMT positive milk samples based on culturing on MacConkey's agar and biochemical tests and its molecular identification among dairy animals in Egypt

Animals	Total quarters positive to CMT No. (%)	Isolated <i>E. coli</i> (Microbiological Examination Based on Biochemical Tests) No. (%)	Molecular identification of <i>E. coli</i> isolates No. (%)
Cow	432 (49.9%)	71 (16.4%)	59** (83.1%)
Buffaloess	302 (44.3%)	82* (27.2%)	62 (75.6%)
Total	734 (47.4%)	153 (20.8%)	121 (79.1%)

\* indicates significant difference at  $P < 0.05$ .  
\*\* indicates no significant difference at  $P < 0.05$ .

Table 5. Molecular identification of virulence genes in *the E. coli* isolates in milk of subclinical cases of dairy animals in Egypt

Target of <i>E. coli</i>	Oligonucleotide	PCR product size	PCR type	Cows <i>E. coli</i> strains	Buffaloes <i>E. coli</i> strains
SK1	( <i>eae</i> ) gene	881 bp	Multiplex	1	0
VTcom u, d	( <i>stx</i> ) gene	518 bp	Multiplex	0	0
AL 65, 125	( <i>est</i> ) gene	147 bp	Multiplex	1	0
LT L, R	( <i>elt</i> ) gene	322 bp	Multiplex	0	0
hlyA-F (O111)	( <i>hlyA</i> ) gene	534 bp	Single	0	0

n.b. the positive 2 genes were in one *E. coli* strain.

## DISCUSSION

Subclinical mastitis is an annoying problem in dairy farms, it is responsible almost for 70% of economic losses of mastitis cases and its prevalence is about 15 to 40 times the prevalence of clinical mastitis (El-Attar *et al.*, 2002; Losinger, 2005; El-Awady & Oudah 2011). Consequently, early detection of SCM cases is a prerequisite to minimize its deleterious outcomes. California mastitis

test is a very useful diagnostic aid for the early detection of SCM. Also, it provides an effective approach for the detection of the udder health status of a herd, identifying infection trends, detecting herd risk factors and monitoring herd performance and management interventions (Pyorala, 2003).

California mastitis test used in the current cross-sectional study to estimate the prevalence of SCM among dairy cows and buffaloes in Egypt revealed a high

prevalence of SCM among examined animals. These results agreed with the findings obtained by Ahmed *et al.* (2015), who observed similar results, while different figures for SCM prevalence estimates were recorded by Abdel-Rady & Sayed (2009), Lamey *et al.* (2013) and Enany *et al.* (2012).

The high prevalence of SCM in the studied area may be attributed to many reasons such as the keeping of a large number of old cows and buffaloes with low udder immunity by the farmers for milk production. Also, farmers do not look for a genetic selection of dairy cows and buffaloes with udder confirmation suitable for prevention of infection. Furthermore, the poor conditions of animals' management practiced by smallholders of cows and buffaloes' in rural areas such as keeping animals with pendulous udders, blind teat and chronic infection in one quarter for breeding for long period without culling may anticipate an increased percentage of SCM cases. Finally, the inappropriate way of antibiotics usage by the farmers in Egypt – without a prior prescription and incomplete course of application – lead to the emergence of an antibiotic-resistant pathogen and this may be another reason of the high prevalence of SCM obtained.

The prevalence of SCM was significantly higher among cows than buffaloes. This may be attributed to the low susceptibility of buffaloes to mastitis (Uppal *et al.*, 1994) and/or the high milk production of cows compared to buffaloes.

This high prevalence of SCM in the study area may forecast the microbiological quality of milk offered to consumers from the dairy cows and buffaloes in the study area. The hot weather nature of the country and the poor management of milk tanks increase the magnitude of poor milk quality. The poor quality of milk may reduce its shelf life and also it can be a reservoir of pathogenic bacteria that potentially pose public health risks. One of the most important public health pathogens which can be transmitted to humans via milk of SCM cases is *E. coli*. *E. coli* is a commensal organism which can act as opportunistic pathogens, especially in elderly and immune-compromised patients

who have received multiple courses of antibiotics (Kaipainen *et al.*, 2002).

In this study, the prevalence of *E. coli* isolates from SCM cases and their virulence genes were identified. The results showed a high prevalence of isolation of *E. coli* from the milk of SCM cases in the study area. This agreed to some extent with what found by Ahmed *et al.* (2008) and El-Khodery & Osman (2008) who reported a high percentage of *E. coli* isolation from milk samples of Egyptian buffalo-cows suffering from SCM. On the other hand, a higher percentage of *E. coli* isolation than what we have in this study was recorded by Abd El-Razik *et al.* (2011), while a lower percentage was reported by Enany *et al.* (2012).

Multiplex PCR was used for identification of five virulence genes in the isolated *E. coli* strains. The distribution of virulence genes identified in this study showed that all *E. coli* isolates are lacking *stx*, *elt*, and *hlyA* genes while *eae* and *est* virulence genes were detected in one strain. These results are consistent with those of Cursons *et al.* (2005) and Moussa *et al.* (2006) who showed positive amplification of *eaeA* gene in *E. coli* isolated from milk of SCM cases by using same primers used in the current study. Also, Lamey *et al.* (2013) identified *stx2* in all *E. coli* isolates revealed from the milk of mastitis cases, while *eaeA* gene was found only in three *E. coli* isolates.

Horizontal transfer of genes is a typical characteristic for *E. coli* which may result in the evolution of new pathogenic clones. The detection of *eae* gene is a major marker of virulence sufficient to define EPEC in the milk of examined animals and it is responsible for attachment of *E. coli*. This gene may help *E. coli* to persist in the udder for a long period or to adhere and invade mammary epithelial cells and so enhancing *E. coli* capacity to provoke mastitis. Enteropathogenic *E. coli* is an emerging category of pathogens involved recently in many foodborne outbreaks (Huss *et al.*, 2003). On the other hand, *est* detected in this study is suggestive for ETEC (Toma *et al.*, 2003). Enteropathogenic *E. coli* and ETEC are the most important group of *E. coli* in terms of human diarrhoeal outbreaks worldwide

(Nataro and Kaper 1998). The presence of these virulence genes in pathogens isolated from milk of dairy animals is considered a public health threat for the population consuming milk and dairy products of these animals.

Our findings highlight the importance of following control measures against SCM. These measures should consider first the reduction of risk factors responsible for the presence and spread of contagious pathogens on the farm and secondly to minimize the usage of antibiotics to prevent the mutation of microbes such as *E. coli*. The success of these control measures can be justified through regular monitoring of the individual animal's somatic cell counts.

Finally, this study revealed that assessment of the potential risk to public health by the milk-borne pathogens in developing countries is a much-required exercise through quantitative microbial risk assessment. This could offer potentially effective mitigation efforts to enhanced food security through reduction of the prevalence of milk-borne diseases (Ntuli *et al.*, 2016).

## CONCLUSIONS

The prevalence SCM is very high among dairy cows and buffaloes of small holders in Egypt and its control strategies should focus on risk factors associated with management practices followed by the farmers. Also, milk presented to the population from these animals may act as a potential reservoir of virulent pathogens such as *E. coli* which may constitute a public health hazard.

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