Prevalence of virulent *Yersinia enterocolitica* in subclinical mastitic cow milk in Sharkia Governorate, Egypt

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Abstract: Forty six random samples of cow milk were collected from different localities at Sharkia Governorate, Egypt and transferred to the laboratory to be examined for incidence of mastitis by applying CMT; WST; MSCC and chloride tests as well as bacteriological examination (Isolation and identification of Yersinia spp. and molecular detection of the virulence gene (*virF*) in *Yersinia enterocolitica*). The results of CMT reveal that 13 (28.3%) of the examined cow milk samples were negative and 33 (71.7%) were positive. While, results of WST showed that 12 (26.1%) of examined samples were negative and 34 (73.9%) were positive. The MSCC/ml. was studied and revealed that negative CMT samples contained less than 3.55×10^5 MSCC/ml., while the positive ones contained higher numbers of MSCC exceeded 6.8×10^6 /ml. There was a marked increase in MSCC in cow's udder affected with subclinical mastitis than that in normal milk. The mean value of chloride% of normal cow milk samples (CMT negative samples) was 0.105±0.027, while the CMT positive samples were 0.168±0.002. The comparisons by 2-tailed test compare the mean values for MSCC/ml and chlorides% in normal and mastitis milk samples showed significant difference of mean at 0.01 levels. Yersinia organisms were detected in 52.2% of examined cow milk samples. *Yersinia enterocolitica*, *Y. pseudo tuberculosis, Y. intermedia, Y. kristensenii and Y. frederiksenii* could be isolated from cow milk samples in a percentage of 39.1%, 10.9%, 13.1%, 17.4% and 2.2%, respectively. The presence of *virF* gene in 18 strains of *Y. enterocolitica* was detected by Polymerase chain reaction (PCR). *Y. enterocolitica* strains showing positive for *virF* gene were 10(55.6%). Finally, this study presented out two important findings, the first that the milk is produced under low hygienic measures which increasing the possibilities of mastitis and the second that raw milk may contain very dangerous human health hazard organisms.
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1. Introduction

Subclinical mastitis means that although there are no visible external changes in either infected udder or produced milk, infection is present and inflammation is occurred in the udder where several causative organisms are discharged with milk for long time ***(Blowey and Edmondson, 1995).***

Subclinical mastitis leads to undesirable effect on milk constituents and nutritional value. In addition, subclinical mastitis may cause epizootiological and epidemiological as well as economic hazardous ***(Salem et al., 1993).*** It results in substantial economic losses to dairy producers whereas milk yield generally drop and often never recovered ***(Gröhn et al., 2004)***. Other responses changes are encountered in milk chemical composition which caused by cellular damage and increased permeability in the membranes of the mammary gland ***(Sloth et al., 2003).*** Also, it is orrelated with increased amounts of heat stable protease and lipase in milk resulting in protein and fat degradation during refrigerated storage and production of off flavors as well as reduction in curd firmness ***(Barbano et al., 2006).*** Meanwhile, it leads to involuntary culling of lactating cattle ***(Smith et al., 1985).***

Detection of subclinical mastitis is however, difficult and depends on various test procedures aiming detecting causative agents or products of inflammation in milk ***(IDF, 1987).*** Several methods have been reported for detection of subclinical mastitis as isolation of the causative M.O which is considered the most accurate although, it is expensive and time consuming. So, the need for a simple quite sensitive, rapid and reliable test, sufficient to be applied on large scale of animals, is therefore required ***(Radostitis et al., 2007).*** These tests including: CMT, WST and somatic cell counts evaluation.

In the CMT, the leucocytes of milk are ruptured by the reagent, releasing their deoxyribonucleic acid, which is the active principle in the test ***(Busato et al., 2000).*** While in the WST, nucleic acids of the leucocytes of milk form a sodium salt with sodium hydroxide producing a gelatinous mass to which serum solids and fat globules become absorbed**.** Both tests are widely used in the detection of subclinical mastitis, because they are reliable, rapid, easy to

perform and inexpensive **(*Schalm et al., 1971).***The CMT has been shown to be positively associated with SCC and with the probability of bacterial infection ***(*Contreras et al., 1996).***

Somatic cell counts are accepted as the international standards measurement of milk quality. They are primarily leukocytes or white blood cells, which include phagocytes and lymphocyte. During mastitis, the major increase in SCC is due to the influx of neutrophil to the milk to fight infection ***(Khalil, 2007).***

Regarding public health importance, mastitis is considered of quite vital importance due to its association with many zoonotic diseases in which milk act as a vehicle of infection. Of various conditions of udder, subclinical mastitis is attracting high attention as this condition not only leads to suboptimal milk production, but it also results into transmission of certain disease to human ***(Tijare et al., 2000).***Many infectious agents have been implicated as the cause of subclinical mastitis as Yersinia spp. ***(Magda, 2007).*** Moreover, virulence factors of these bacterial strains can assist in colonization, multiplication and survival of these pathogens as well as antagonizing the host defense mechanisms ***(Wenz et al., 2006).***

Y. enterocolitica is widely distributed through the environment and have been isolated from raw milk, sewage-contaminated water, soil and humans**.** *Y. enterocolitica* is considered as a, food borne pathogen causing symptoms such as fever, diarrhea, nausea and abdominal pain, the diseases of which range from self-limiting gastroenteritis to fatal septicemia. *Y. pseudo tuberculosis* is associated mainly with mesenteric adenitis. Cases of mastitis caused by *Y. pseudo tuberculosis* have been reported in cattle with clinical or subclinical presentation; lumps, swelling, clotted milk and increased somatic cell counts were the salient features ***(Shwimmer et al., 2007).***

This study was undertaken to investigate the incidence of subclinical mastitis in dairy cows and the importance of screening tests as well as isolation of Yersinia spp. and detection of the virulence gene (*virF*) in *Yersinia enterocolitica* using PCR.

2. Materials and Methods

Collection and preparation of samples (APHA, 1992):

Forty six random samples of raw cow's milk were collected from different localities at Sharkia Governorate, Egypt. Collected samples (about 500 ml. each) were transferred directly to the laboratory with a minimum of delay in an ice box (at 4ºC) to be examined chemically and microbiologically. Each sample was mixed thoroughly before being divided into two parts. The first was examined for incidence of mastitis and the second was used for microbiological examination.

Incidence of mastitis:

I-California Mastitis Test (CMT): (APHA, 2004):

Equal volume of milk samples and CMT reagent were mixed thoroughly in a cup of plastic paddle. The mixture was gently swirled by circular motion of paddle and results were recorded after 10 seconds and judged according to ***Radostitis et al., 2000.***

Ii-Somatic cell count (SCC):

Milk samples were examined automatically using ***somatic cell counter MT05*** apparatus. The sample was warmed at 40˚C for 5 minutes, and then mixed before reading ***(Radostitis et al., 2000).***

Iii-White side Test (WST) Schalm et al. (1971): Five drops of milk were added to two drops of NaoH 4% on clean glass plate placed on dark black ground and mixed well and the reaction was graded according to the Scandinavian recommendations ***(Klastrup and Schmidt Madsen, 1974).***

Iv-Determination of chloride %:(Sanders, 1939):

Titration of chloride in milk (silver nitrate, nitric acid, and ferric alum indicator, is added to the sample and the percentage of chloride is determined by titrating with standardized ammonium thiocyanate).

Microbiological examination:

I-Preparation of decimal dilution (APHA, 2004):

One ml. of milk sample was aseptically transferred to 9 ml. of sterile 1/4 strength Ringer solution and well mixed to obtain 1/10 dilution. One ml from first dilution was added to 9 ml. of sterilized diluents to obtain ten-fold serial dilution.

Ii-Isolation of Yersinia spp. (Schiemann, 1987)

ii.a. Pre-enrichment:

One ml of each milk sample was enriched in 9 ml of phosphate buffered saline then incubated at 25˚C for 1– 2 days.

ii.b. Selective enrichment broth:

One ml of pre-enrichment culture was added to 9 ml of Bile Oxalate Sorbose (BOS) broth followed by incubation at 22˚C for 2–5 days.

ii.c. Plating procedure:

A loopful of each enrichment liquid culture was streaked on the surfaces of the selective medium, Cefsulodin-Irgasan-Novobiocin (CIN) then incubated at 22˚C for 48hrs. Five colonies, appearing as typical Yersinia (deep red center with a rather sharp border and translucent outer zone) were picked and streaked onto slopes of tryptone soy agar medium supplemented by yeast extract (TSAYE). The slopes were incubated at 30˚C for 24hrs.

Identification of suspected isolates:

Isolated colonies were purified and subjected to identification tests according to ***Krieg and Holt (1984).***

Iii-Detection of virF gene in Yersinia enterocolitica using PCR (Cecilia et al., 2011):

iii.a. DNA extraction: The *Y. enterocolitica* strains were inoculated onto trypticase soy agar and incubated for 48 hrs at 25ºC. Three colonies were suspended in one milliliter of trypticase soy broth and the DNA extraction was carried out. This nucleic acid isolation method was performed according to the manufacturer's instructions. After the extraction, the DNA concentration was determined and 10 ng/ml of DNA were used in PCR and PCR ribotyping procedures.

iii.b. PCR for virulence marker:

Virulence marker was assayed by specific PCR for detection of virF genes.

Primer used to detect the (virF) gene in *Yersinia enterocolitica*:

Gene	Sequence(5'→3')	Amplicon size(bp)	References
VirF	TCA TGG CAG AAC AGC AGT CAG ACT CAT CTT ACC ATT AAG AAG	590	Versalovic *et al.* (1991) Falcao *et al.*(2006)

A step-by-step empirical approach was used to determine the optimal annealing temperature, raising the temperature between 55 – 60ºC with 1 ºC increments, and the concentration of primers, $MgCl_2$ and deoxynucleoside triphosphates in the reaction mixture. The amplification conditions were: an initial denaturation of 94 ºC for 1 min, followed by 30 cycles of 94 ºC for 45 s, 57 ºC for 45 s and 72 ºC for 45 s, with a final extension at 72 ºC for 4 min.

iii.c .Gel electrophoresis of amplified products:

PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide (0.5 mg/ml) and visualized by UV light. In order to check the reproducibility of the technique, the PCR assays were repeated three times.

Statistical analysis (Foster, 2001):

Data were analyzed by statistical package for social science (SPSS) version 10 software package. Quantitative variable were expressed as mean and standard deviation, comparison of two means were estimated by 2-tailed test. Correlation is significant at the 0.01 level.

3. Results

Table (1): Statistical analytical results of CMT in examined cow milk samples.

Examined samples	No.	Negative CMT samples		Positive CMT samples		Scores of positive CMT samples							
						Score ±		Score +		Score ++		Score +++	
		No	%	No	%	No	%	No	%	No	%	No	%
Cow milk samples	46	13	28.3	33	71.7	0	0.0	12	26.1	11	23.9	10	21.7

Table (2): Statistical analytical results of WST in examined cow milk samples

Examined samples	No	Negative WST samples		Positive WST samples		Scores of positive CMT samples					
						Score +		Score ++		Score +++	
		No.	%	No.	%	No.	%	No.	%	No.	%
Cow milk samples	46	12	26.1	34	73.9	17	37.0	11	23.9	6	13.0

Table (3): Statistical analytical results of somatic cell count (MSCC)/ml in examined cow milk samples.

Examined samples		No.	Min.	Max.	Mean ±S.E.M.
Cow milk samples	Normal	13	3×10^4	3.55×10^5	$1.7 \times 10^5 \pm 1.3 \times 10^5$
	Mastitis	33	1.5×10^5	6.8×10^6	$3.2 \times 10^6 \pm 3.8 \times 10^5$

Table (4): Frequency distribution of somatic cell count (MSCC)/ml in examined cow milk samples.

Intervals	Normal (13)		Mastitis (33)	
	No.	%	No.	%
$<200\times10^3$	7	53.8	1	3.0
200×10^3--$<500\times10^3$	6	46.2	3	9.1
500×10^3--$<1500\times10^3$	0	0.0	8	24.3
1500×10^3--$<5000\times10^3$	0	0.0	11	33.3
$>5000\times10^3$	0	0.0	10	30.3
total	13	100.0	33	100.0

Table (5): Statistical analytical results of chloride % in examined cow milk samples.

Examined samples		No.	Min.	Max.	Mean ±S.E.M.
Cow milk samples	Normal	13	0.07	0.14	0.105±0.027
	Mastitis	33	0.15	0.18	0.168±0.002

Table (6): Frequency distribution of chloride % in examined cow milk samples.

Intervals	Normal (13)		Mastitis (33)	
	No.	%	No.	%
≤0.14	13	100.0	0	0.0
>0.14	0	0.0	33	100.0
total	13	100.0	33	100.0

Table (7): Paired analysis of somatic cell count and chlorine % of normal and mastitis cow milk samples.

		Pearson Correlation	2-tailed	Significance
SCC X Chlorine	Normal	0.968**	0.000	S.**
	Mastitis	0.910**	0.000	S.**

**. Correlation is significant at the 0.01 level (2-tailed).

Table (8) Incidence of *Yersinia* Species in examined cow milk samples.

Samples	Positive samples n=46		
	No.	%	No. of isolates
Cow milk samples	24	52.2	38

Table (9) Incidence of isolated *Yersinia* species in examined cow milk samples (n=46)

Yersinia Species	No.	%
Y. enterocolitica	18	39.1
Y. pseudo tuberculosis	5	10.9
Y. intermedia	6	13.1
Y. Kristensenii	8	17.4
Y. frederiksenii	1	2.2

Table (10): Frequency distribution of virulence-associated genes among strains of *Yersinia enterocolitica.*

Type of examined Samples	No. of examined strains	No. of strains bearing virulence-associated genes	%
Cow milk samples	18	10	55.6

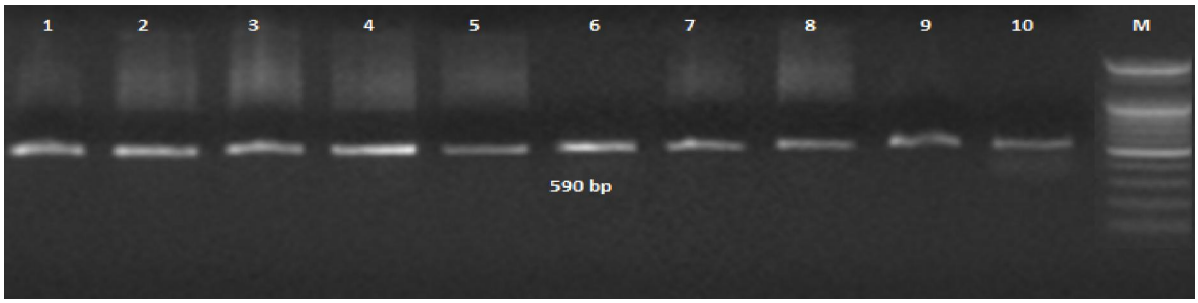

Figure (1): Agarose gel electrophoresis analysis of DNA products with specific primers for the virF gene of *Y. enterocolitica* produced a PCR product of 590-bp:
Lane M: DNA marker (100-bp ladder)
Lane 1to 10: Positive strains

Lane 1to 8: Negative strains

4. Discussion

Mastitis is the most costly disease of dairy cattle due to economic losses from reduced milk production, treatment costs, increased labour, milk withheld following treatment, premature culling and, in severe cases, death of producing animals ***(Miller et al., 1993)***. Early detection of mastitic cows is important for most dairy farmers to reduce these losses and to enhance prospects of recovery where changes in the udder tissue take place much earlier than they become apparent ***(Sharma et al., 2010)***. While diagnosis of clinical mastitis is straight forward as it is based on the local and systemic reactions and the obvious changes in milk (e.g. off color, watery, bloody appearance and presence of flakes, clots and pus), diagnosis of subclinical mastitis is more problematic since milk appears normal and no clinical signs are observed.

Various methods, based on physical and chemical changes of milk and cultural isolation of organisms, are used for diagnosis of subclinical mastitis ***(Emanuelson et al., 1987)***. The diagnosis of mastitis according to the International Dairy Federation (IDF) recommendations is based on SCC and microbiological status of the quarter. Indirect methods such as CMT and WST are available for the diagnosis of mastitis under field conditions, as cow side test ***(Sharma et al.., 2011)***.

Detection of subclinical mastitis:

1- **California Mastitis Test (CMT):**

Results listed in table (1) showed that 46 examined cow milk samples were classified into 13 (28.3%) CMT negative and 33 (71.7%) CMT positive samples and among the positive samples, the highest incidence was recorded in CMT (+) as (26.1%) and the lowest in CMT (+++) as (21.7%) while CMT (++) was (23.9%).Nearly similar findings were reported by ***Getahun et al. (2008)*** and ***Bhutto et al. (2012)***. Lower results were reported by ***Morsi et al. (2000); Ahlner and Axelsson (2002); Al-Hawary et al. (2003); Elango et al. (2010)*** and ***Jin-bo et al. (2012)***while higher results were recorded by ***Karimuribo et al. (2006)*** and ***Varatanović et al. (2010).***

CMT could have a useful role in dairy herd monitoring programs as a screening test to detect cows with intramammary infection caused by major pathogens ***(Sargeant et al., 2001)***. Also, it is a better and rapid indirect diagnostic tool for the detection of subclinical mastitis in cows***(Tiwari and Sisodia, 2001)*** as well as it gives a sharp discrimination between normal and subclinical mastitis milk samples of different scores **(*Marwa, 2007)***. While, ***Leach et al. (2008)*** reported that CMT is used on farms to identify subclinical mastitis by an indirect estimation of SCC in milk.

Nazem and Azab (1998) reported that the sensitivity of CMT was 88.02%. ***Maiti et al. (2003)*** reported 70.37% incidence of sub clinical mastitis in cows, while ***Joshi and Gokhale (2004)*** reported that the incidence of subclinical mastitis varied from 10 to 50% in cows in improved and peri urban dairy farms in India. While, ***Sharma et al. (2010)*** reported that the incidence of subclinical mastitis in crossbred dairy cows was 67.76%.

The difference in prevalence observed between the reports from different localities and the present study may be due to differences in management and husbandry conditions in the area and lack of awareness of farmers to the loss caused by mastitis ***(Al-Hawary et al., 2003)***.

2- **White side test (WST):**

Results tabulated in table (2) showed that 46 examined cow milk samples were classified into WST negative (26.1%) and WST positive (73.9%) samples. Among the positive samples, the highest incidence was recorded in WST (+) as (37.0%) and incidence in WST (++) and WST (+++) were recorded by (23.9%) and (13.0%), respectively.

Ali et al. (2011) recorded lower results, as they found that overall prevalence of subclinical mastitis using Whiteside test was 44%. Also, ***Lafi and Hailat (1998); Getahun et al. (2008); Muhammed et al. (2010); Pitkala et al. (2004)*** and ***Iqbal et al. (2004)*** recorded an incidence of 92, 54.7, 54.37, 32.85 and 23.18%, respectively. The variation in prevalence of mastitis might be due to the different regions, therapeutic practices, management conditions and presence of microorganisms in environment ***(Ali et al., 2011)***. ***Iqbal et al. (2004)*** reported that White Side Test was not very sensitive in detecting mastitis positive cases.

3- **Milk somatic cell count (MSCC/ml):**

Somatic cells are always present in milk and they increase due to mammary gland infections. When udders are healthy, somatic cell count (SCC) in milk lies between 50,000 and 100,000 cells/ml up to 200,000 cells/ml. ***(Skrzypek et al., 2004).*** If the SCC is greater than 200,000 cells/ml, it is assumed a threshold distinguishing a healthy udder from a diseased udder ***(Harmon, 2001*** and ***Skrzypek et al., 2004).*** High SCC in milk reduces the quality of both milk and dairy products, and also affects milk shelf life and flavor, as well as cheese and butterfat yield.

Table (3) revealed that the minimum MSCC of normal cow milk samples (CMT negative samples) was 3×10^4, the maximum was 3.55×10^5 with a mean value of $1.7 \times 10^5 \pm 1.3 \times 10^5$, while the CMT positive samples showed a minimum $1.5 \times 10^5$and a maximum $6.8 \times 10^6$with a mean value of $3.2 \times 10^6 \pm 3.8 \times 10^5$.The highest frequency distribution (53.8) of MSCC for normal cow milk samples were $<200 \times 10^3$ while, in case of mastitis cow milk samples we found that the highest frequency distributions of MSCC was 33.3% lies in the range of 1500×10^3--$<5000 \times 10^3$ (Table 4).

Nearly similar findings were reported by ***Varatanović et al. (2010)***. While, lower results for CMT negative samples reported by ***Elango et al. (2010)*** and higher figures reported by ***Sharif et al. (2007).*** Higher values of MSCC/ml. for mastitis milk samples were recorded by ***Sharif et al. (2007)*** and ***Elango et al. (2010)*** while lower figures were recorded by ***Spakauskas et al. (2006)*** and ***Bhutto et al. (2012). Sharma et al. (2010)*** found that out of 335 quarter milk samples, 180 (53.73%) milk samples were positive by MSCC.

Reneau (1986) opined that the mammary gland infection is the most important factor affecting somatic cell count in milk in sub-clinical mastitis. While, ***McDougall et al. (2001)*** reported that SCC was a better predictor of bacteriological status than either CMT score or impedance. Also, ***Gronlund et al., 2005*** *and* ***Friggens et al., 2007*** stated that beside the microbiological testing, Somatic Cell Counts in milk is often used to define subclinical mastitis.

4- **Chloride test (CT):**

Table (5) declared that the minimum chloride% of normal cow milk samples (CMT negative samples) was 0.07, the maximum was 0.14 with a mean value of 0.105±0.027, while the CMT positive samples showed a minimum 0.15 and a maximum 0.18 with a mean value of 0.168±0.002.The highest frequency distribution (100%) of chloride % for normal cow samples were ≤0.14 while in case of mastitis cow samples, the highest frequency distribution (100%) of chloride % was lies in the range of >0.14 (Table 6).

Higher values of chloride content (0.12) for normal milk samples were recorded by ***Elango et al. (2010)*** while ***Sharma et al. (2011)*** found that the chloride content of normal milk sample was 0.91.

Elango et al. (2010) reported that the normal range of chloride content of healthy animal's milk is 0.08 to 0.14%. While, ***Sharma et al. (2011)*** found that the chloride content of normal milk sample was 0.91%. ***Batavani et al. (2007)*** found that milk from quarters with subclinical mastitis showed elevated chloride (>0.14 vs<0.14 g/dl) which is significantly higher in the milk of inflamed quarter than those in normal ones (P<0.01). ***Schalm et al. (1971)*** reported that bacterial infection of udder leads to opening up of the alveolar junction and an increased permeability of capillaries. Sodium and chloride which were higher in extracellular fluid poured into lumen of alveolus. ***Morsi et al. (2000)*** showed that bacteria causing mastitis had an effect on chloride content causing its increase.

The comparisons by 2-tailed test represented in table (7) compare the mean values for MSCC/ml and

chlorides% in normal and mastitis milk samples which found that there is a significant difference of mean at 0.01 levels.

The interpretation is due to the higher mean values of MSCC as a result of damage occurred in the mammary epithelial synthetic cells of the infected udder by microbial toxin due to mastitis and high mean value of chlorides to compensate lower osmotic pressure resulting from decreased lactose% so higher amount of minerals penetrate cellular walls. Also, indicates that these pathogens can be considered from the main causative agents of subclinical mastitis in our study.

Bacteriological examination:

1-Isolation and identification of *Yersinia* species

Yersinia organisms are Gram negative, psychrotrophic milk borne enteric pathogens. These organisms are widespread in the environment and are indigenous to the gastrointestinal tracts of warm blooded animals including dairy cattle ***Bahout and Moustafa (2004).***

Data presented in table (8) reveal that *Yersinia* species could be detected in (52.2%) of examined cow milk samples. Lower incidence was reported by ***Jayarao et al. (2006); Barakat (2007)*** and ***Awad Allah (2010).***While higher percentages obtained by ***Hamama et al. (1992)*** and ***Yucel and Ulusoy (2006).***

Results summarized in table (9) showed that *Yersinia enterocolitica*, *Y. pseudo tuberculosis, Y. intermedia, Y. kristensenii and Y. frederiksenii* were isolated from cow milk samples in a percentage of (39.1%, 10.9%, 13.1%, 17.4% and 2.2%), respectively.

The obtained results are inagreement with those reported by ***Hamama et al (1992)*** and ***Awad (2002)*** while it is partially different with that obtained by ***Yucel and Ulusoy (2006); Barakat (2007)*** and ***Awad Allah (2010)***.

Yersinia enterocolitica and *Y. pseudo tuberculosis*, both members of family enterobacteriaceae, are comprised of strains with different degrees of pathogenecity. Both pathogenic and nonpathogenic strains are frequently isolated from various animals as well as from the environment ***(Thoerner et al., 2003).***

Consumption of raw or inadequately pasteurized milk has been associated with outbreaks of enteric infections. *Y. enterocolitica* can grow to large numbers at refrigeration temperatures, so, milk contaminated with that organism could become a significant health risk for consumers ***(Dallal et al., 2004).***In humans, *Y. enterocolitica* and *Y. pseudo tuberculosis* are well known food borne pathogens and are mainly transmitted through ingestion of contaminated milk or water, yersiniosis frequently occurs in young children as enterocolitis with fever, diarrhea and abdominal cramps ***(Thoerner et al., 2003).***

Molecular genetic studies have emphasized the importance of a virulence plasmid (pYV) that encodes various virulence genes, among them *virF*, which is an important transcriptional regulator of other plasmid genes, as well as the role of chromosomal virulence genes that mediate cell invasion (*inv* and *ail*) and produce a thermostable enterotoxin (*ystA*), among others ***(Cornelis et al., 1998).*** A problem with the detection of strains of *Y. enterocolitica* is that they are only pathogenic to man in the presence of specific virulence factors. The best characterized virulence factor is encoded by the *virF* gene mapped to a 70 kbp plasmid ***(Kapperud, 1991).***

Results presented in table (10) showed that 10 (55.6%) out of 18 examined *Y. enterocolitica* strains isolated from 46 cow milk samples were positive for the presence of the Virulence related gene (*vir*F). Nearly similar results were reported by ***Bhaduri and Cottrell (1997); Sakai et al. (2005)*** and ***Awad Allah (2010)*** who could isolate *Y. enterocolitica* from raw milk and the strains found to be virulent by having the *virF* gene.

The application of PCR was shown to be an efficient tool for the identification of pathogenic *Y. enterocolitica*, separating the pathogenic serotypes from non-virulent ones. Our work highlights the role of milk as transmission vehicles of potentially pathogenic *Y. enterocolitica* strains, with consequent risks for consumer's health.

Conclusion

The problem of mastitis is encountered in its subclinical form. So, earlier identification of subclinically infected gland is urgently required for reduction of production losses, enhancing the prospects of recovery and successful control of mastitis in dairy animals.

SCC, CMT, WST and intramammary infection are associated significantly; therefore these parameters provide the necessary information to evaluate udder health status in cows. Also, chloride composition is linked to the subclinical mastitis due to changing of the ionic environment because of inflammation of mammary gland.

Therefore, to improve the hygienic quality of milk and to safeguard the consumer from being infected, the existing situation must be improved, and this can be achieved by applying highly recommended hygienic practices and regulations, such as on-site pasteurization and implementation of HACCP following established standards.

References

1. ***Ahlner, S. and Axelsson, A. (2002):*** Analysis of bacterial growth and the prevalence of sub-clinical mastitis from bulk tank samples and individual samples from dairy herds in a region in Uruguay. Minor-Field-Studies-International-Office, Swedish-University-of-Agricultural Sci., 214: 16
2. ***Al-Hawary, I.I.; Sobeih, A.M.K. and Aman, I. (2003):*** Further studies on the prevalence of subclinical mastitis in dairy cows in El-Gharbia and Kafr El-Sheikh Governorates with special observation to antibiotic sensitivity. Kafr El-Sheikh Vet. Med. J., 1(1): 331-343.
3. ***Ali, M. A.; Ahmad, M. D.; Muhammad, K. and Anjum, A. A. (2011):*** Prevalence of sub clinical mastitis in dairy buffaloes of Punjab, Pakistan. J. Anim. Plant Sci., 21(3): 477-480.
4. ***American Public Health association "APHA" (1992):*** Standard method for the examination of dairy products. 16th Ed., New York.
5. ***American Public Health association "APHA" (2004):*** Compendium of Methods for the Microbiological examination of food. 17th ed., APHA, Washington D. C. USA.
6. ***Awad Allah, M. A. I. (2010):*** Microbiological status of raw and pasteurized milk in Sharkia Governorate. Ph.D. Thesis, Faculty of Veterinary Medicine, Zagazig University, Egypt.
7. ***Awad, E. E. (2002):*** Studies on food-poisoning bacteria with special reference to Campylobacter jejuni in milk. Ph.D. Thesis, Faculty of Veterinary Medicine, Zagazig University, Egypt.
8. ***Bahout, A.A. and Moustafa, A.H. (2004):*** Occurrence of *Yersinia* species in raw and pasteurized milk in Dakahlia Governorate. Assuit Vet. Med. J., 50(101): 57-63.
9. ***Barakat, W.M.A. (2007):*** Prevalence of Gram negative bacteria in bulk tank milk in Sharkia Governorate. M.V.Sc Thesis, Faculty of Veterinary Medicine, Zagazig University, Egypt.
10. ***Barbano, D. M.; Ma, Y. and Santos, M. V. (2006):*** Influence of raw milk quality on fluid milk shelf life. J. of Dairy Sci., 89 (1): 15-19.
11. ***Batavani, R. A.; Asri, S and Naebzadeh, H. (2007):***The effect of subclinical mastitis on milk composition in dairy cows. Iranian Journal of Veterinary Research, University of Shiraz, 8 (3): Ser. No. 20, 205-211.
12. ***Bhaduri, S. and Cottrel, B. (1997):***Direct detection of plasmid bearing virulent serotypes of *Y. enterocolitica* from various foods. Appl. and Envir. Microbiol., 63(2): 4952-4955.
13. ***Bhutto, A.L.; Murray, R.D. and Woldehiwet, Z. (2012):*** California mastitis test scores as indicators of subclinical intra-mammary infections at the end of lactation in dairy cows. Research in Veterinary Sci., 92: 13–17.
14. ***Blowey, R. and Edmondson, P. (1995):*** Mastitis control in dairy herds, on illustrated and practical guide, Farming Press Book, UK.
15. ***Busato, A.; Trachsel, P.; Schallibaum, M. and Blum. J. (2000):*** Udder health and risk factors for subclinical mastitis in organic dairy farms in Switzerland. Prev. Vet. Med., 44(3-4):205-20.
16. ***Cecilia, S.M.; Velázquez, L.D.C.; Escudero, M.E.; Favier, G.I.; Lazarte, V and Guzmán, A.M.S. (2011):*** Pulsed field, PCR ribotyping and multiplex PCR analysis of *Yersinia enterocolitica* strains isolated from meat food in San Luis Argentina. Food Microbiol., 28: 21-28.
17. ***Contreras, A.; Sierra, D.; Corrales, J. C.; Sanchez, A. and Marco, J. (1996):*** Physiological threshold of somatic cell count and California mastitis test for diagnosis of caprine sub clinical mastitis. Small Rumin. Res., 21: 259-264.
18. ***Cornelis, G. R.; Boland, A.; Boyd, A. P.; Geuijen, C.; Iriarte, M.; Neyt, C.; Sory, M. P. and Stainier, I. (1998):*** The virulence plasmid of *Yersinia*, an anti host genome. Microbiol. Mol. Biol. Rev., 62: 1315–1352.
19. ***Dallal, M.M.S.; Tabarraie, A. and MoezArdalan, K. (2004):*** Comparison of four methods for isolation of *Yersinia enterocolitica* from raw and pasteurized milk from northern Iran. Intern. J. of Food Microbiol., 94: 87– 91.
20. ***Emanuelson, U.; Olsson, T.; Holmberg, O.; Hageltorn, M.; Mattila, T.; Nelson, L. and Astrom, G. (1987):*** Comparison of some screening tests for detecting mastitis. J. of Dairy Sci., 70: 880–886.
21. ***Elango, A.; Doraisamy, K. A.; Rajarajan, G. and Kumaresan, G. (2010):*** Bacteriology of sub clinical mastitis and anti bio gram of isolates recovered from cross bred cows. Indian J. Anim. Res., 44 (4): 280 – 284.
22. ***Falcao, J .P.; Falcao, D. P.; Pitondo-Silva, A.; Malaspina, A. C. and Brocci, M. (2006***): Molecular typing and virulence markers of Yersinia enterocolitica strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. J. Med. Microbiol., 55: 1539-1548.
23. ***Foster, J. J. (2001):***Data Analysis Using Spss For Windows: A Beginner's Guide (Second Edition) London Sage.
24. ***Friggens, N.C. and Rasmussen, M.D. (2007):*** Milk quality assessment in automatic milking systems: accounting for the effects of variable intervals between milkings on milk composition. Livestock Prod. Sci., 73: 45–54.
25. ***Getahun, K.; Kelay, B.; Bekana, M. and Lobago, F. (2008):*** Bovine mastitis and antibiotic resistance patterns in Selalle smallholder dairy farms, central Ethiopia. Trop Anim. Health Prod., 40: 261–268.
26. ***Gronlund, U.; Hallensandgren, C. and Person Waller, K. (2005):*** Hepatogloblin and serum

amyloid A in milk from dairy cows with chronic subclinical mastitis. J. of Vet. Res., 36:191-198.

27. ***Gröhn, Y. T.; Wilson, D. J.; Gonzalez, R. N.; Hertl, J. A.; Schulte, H.; Bennett, G. and Schukken, Y. H. (2004):*** Effect of pathogen–specific clinical mastitis on milk yield in dairy cows. J. of Dairy Sci., 87: 3358-3374.
28. ***Hamama, A.; Marrakchi, A. and Othmani, F. (1992)*:** Occurrence of *Yersinia enterocolitica* in milk and dairy products in Morocco. Int. J. Food Microbiol, 16 (1): 69 – 77.
29. ***Harmon, R.J. (1994):*** Physiology of mastitis and factors affecting somatic cell counts. J. of Dairy Sci., 77: 2103-2112.
30. ***I D F (International Dairy Federation) (1987):*** Bovine mastitis: Definition and Guidelines for Mastitis Diagnosis Bulletin–International Dairy Federation no 211, Brussels, Belgium.
31. ***Iqbal, M.; Khan, M. A.; Daraz, B. and Siddique, U. (2004):*** Bacteriology of mastitic milk and in vitro anti bio gram of the isolates. Pakistan Vet. J., 24(4): 161-164.
32. ***Jayarao, B.M.; Donaldson, S.C.; Straley, B.A.; Sawant, A.A.; Hegde, N.V. and Brown, J.L. (2006):*** A survey of food borne pathogens in bulk tank milk and raw milk consumption among families in Pennsylvania. J. Dairy Sci., 89(7): 2451-2458.
33. ***Jin-bo, Y.; Neng, W. and Liu-fa, W. (2012):***Month-wise prevalence of subclinical mastitis in dairy cows in Guangdong Province, China. J. of Integrative Agri., 11(1): 166-169.
34. ***Joshi, H. D.; Joshi, B. R. and Shrestha, H. K. (1998):*** Epidemiological investigation on subclinical mastitis in cattle and buffaloes in the western hills f Nepal. Vet. Rev. (Kathmandu), 136: 12-15.
35. ***Kapperud, G. (1991):****Y. enterocolitica* in food hygiene. Intern. J. Food Microbiol., 12:53–66.
36. ***Karimuribo, E. D.; Fitzpatrick, J .L.; Bell, C. E.; Swai, E. S.; Kambarage, D. M.; Ogden, N. H.; Bryant, M. J. and French, N. P. (2006):***Clinical and subclinical mastitis in smallholder dairy farms in Tanzania: Risk, intervention and knowledge transfer. Preventive Veterinary Medicine, 74: 84–98.
37. ***Khalil, M .R. I. (2007):*** Screening tests for detection of subclinical mastitis milk M.V.Sc. Thesis (Milk hygiene), Fac. Vet. Med., Cairo University.
38. ***Klastrup, O. and Schmidt, M. P. (1974):*** Nordic recommendations concerning mastitis control of quarter samples. Nord. Vet. Med.,26: 197–204.
39. ***Krieg, N.R. and Holt, J.N. (1984):*** Bergey's manual of systemic bacteriology. Vol.1 Wilkins Company, Baltin, M.D., 21202, USA.
40. ***Lafi, S. Q. and N. Q. Hailat (1998):*** Bovine and ovine mastitis in Dhuleil valley of Jordan. VeterinarskiArhiv., 68: 51–57.
41. ***Leach, K. A.; Green, M. J.; Breen, J. E.; Huxley, J. N.; Macaulay, R.; Newton, H. T. and Bradley, A. J. (2008):***Use of domestic detergents in the California mastitis tests for high somatic cell counts in milk . Vet. Rec.8; 163(19): 566-70.
42. ***Magda, F. E. (2007):*** Some Studies on bacterial causes of mastitis in buffaloe. J. Egypt. Vet. Med. Assoc., 67(3): 129-139.
43. ***Maiti, S. K.; Sharma, N. and Awasthy, B. K. (2003):*** Studies on sub clinical mastitis in cattle and buffaloes of Durg area of Chhattisgarh. Vet. Practitioner, 4(2): 90.
44. ***Marwa, R. I. K. (2007):*** Screening tests for detection of subclinical mastitis milk. M. V. Sc. Thesis, Fac. of Vet. Med., Cairo University, Egypt.
45. ***McDougall, S.; Murdough, P.; Pànkey, W.; Delaney, C., Barlow, J. and Scruton, D. (2001):*** Relationships among somatic cell count, California mastitis test, Impedance and bacteriological status of milk in goats &sheep in early lactation. Small Ruminant, 40(3):245-254.
46. ***Miller, G. Y.; Bartlett, P. C.; Lance, S.E. Anderson, J. and Heider, L.E. (1993):*** Costs of clinical mastitis and mastitis prevention in dairy herds. J. of the Amer. Vet. Med. Assoc., 202: 1230–1236.
47. ***Morsi, N. M.; Saleh, Y.; El Ghazzar, H. and Hanafi, A. (2000):*** Effect of mastitis on milk fat content. Pakistan J. of Biological Sci., 3(2): 196-200.
48. ***Muhammed, G.; Naureen, A.; Asi, M.N.; Saqib, M. and Rehman, F.U. (2010):*** Evaluation of a 3% surf solution (surf field mastitis test) for the diagnosis of subclinical bovine and bubaline mastitis. Trop Anim Health Prod., 42:457–464.
49. ***Nazem, A. M. and Azab, M. H.(1998):*** Detection of apparently normal milk by screening and confirmatory methods. 8th Sci. Con. 15-17 Nov. Alex. Fac. Vet. Med.
50. ***Pitkala, A., M. Haveri, S. Pyorala, V. Myllys and Buzalski, T. H. (2004):*** Bovine Mastitis in Finland, Prevalence, Distribution of Bacteria, and Antimicrobial Resistance. J. Dairy Sci., 87: 2433-2441.
51. ***Radostitis, O. M., Gay, C. C., Blood, D. C., Hinchcliff, K. W. (2000):*** Veterinary Medicine. A Textbook of Diseases of Cattle, Sheep, Pigs, Goats and Horses. Ninth ed. W. B. Saunders, London, pp. 603–660.
52. ***Radostitis, O. M.; Gay, C. C.; Hinchcliff, K. W.; Constable, P. D. (2007):*** A text book of the diseases of cattle, sheep, pigs, goats and horses. Vet. Med. 10th Ed.
53. ***Reneau, J. K. (1986):*** Evaluation of some in directs tests for detecting of subclinical mastitis. J. Dairy Sci., 69: 1708-1720.
54. ***Sakai, T.; Nakayama, A.; Hashida, M.; Yamamoto, Y.; Takebe, H. and Imai, S. (2005):*** Outbreak of food poisoning by *Y. enterocolitica*

serotype O8in Nara prefecture: The first case report in Japan. Division of bacteriology, Nara prefectural Institute for hygiene and environment, Nara, 630-8131, Japan.

55. ***Salem, A. A.; Saad, Marcel, El-Ebeedy, A. and Zaki, Mervat, A. (1993):*** Some studies on subclinical mastitis in sheep &goats .J. Egypt. Vet. Med. Associ., 53(1&2): 261-265.
56. ***Sanders, G.P. (1939):*** The determination of chloride in milk. J. of Dairy Sci., 22(10): 841-852.
57. ***Sargeant, J. M.; Leslie, K. E.; Shirley, J. E.; Pulkrabek, B. J. and Lim, G.H. (2001):*** Sensitivity and specificity of somatic cell count and California mastitis test for identifying intramammary infection in early lactation. J. of Dairy Sci., 84: 2018–2024.
58. ***Schalm, O.W.; Carroll, E. J. and Jain, N .C. (1971):*** Bovine Mastitis. 1st Ed. Lea and Febiger, Philadelphia, USA, 132-360.
59. ***Schiemann, D.A. (1987*)**: *Yersinia enterocolitica* in milk and dairy products. J. Dairy Sci., 70(2):383-391.
60. ***Sharif, A.; Ahmad, T.; Bilal, M.Q.; Yousaf, A.; Muhammed, G.; Urrehman, S. and Pansota, F.M. (2007):*** Estimation of milk lactose and Somatic Cells for the diagnosis of sub-clinical mastitis in dairy Buffaloes. Intern. J. Agri. Biol., 9 (2): 267-270.
61. ***Sharma, N.; Pandey, V. and Sudhan, N.A. (2010):*** Comparison of some indirect screening tests for detection of subclinical mastitis in dairy cows. Bulgarian J. of Vet. Med., 13(2): 98−103.
62. ***Sharma, N.; Singh, N. K. and Bhadwal1, M. S. (2011):*** Relationship of Somatic Cell Count and Mastitis: An Overview. Asian-Aust. J. Anim. Sci., 24(3):429-438.
63. ***Shwimmer, A.; Freed, M.; Blum, S.; Khatib, N.; Weissblit, L.; Friedman, S. and Elad, D. (2007):*** Mastitis caused by *Yersinia pseudo tuberculosis* in Israeli dairy cattle and public health implications. Zoon. Publ. Health, 54: 353–357.
64. ***Skrzypek, R.; Wtowski, J. and Fahr, R.D. (2004):*** Factors affecting somatic cell count in cow bulk tank milk - a case study from Poland. J. Vet. Med. A, Physiol. Pathol. Clin. Med., 20.
65. ***Sloth, K.; Friggens, N.; Lovendahl, P.; Andersen, P.; Jensen, J. and Ingvartsen, K. (2003):*** Potential for improving description of bovine udder health status by combuned analysis of milk parameters. J. Dairy Sci., 86: 1221-1232.
66. ***Smith, K.L.; Todhunter, D.A. and Schoenberger, P.S. (1985):*** Environmental mastitis: cause, prevalence, prevention. J. Dairy Sci., 68: 1531-1553.
67. ***Špakauskas, V.; Klimienė, I. and Matusevičius, A. (2006):*** A comparison of indirect methods for diagnosis of subclinical mastitis in lactating dairy cows. VeterinarskiArhiv., 76 (2): 101-109.
68. ***Thoerner, P.; Binkingombe, C.I.; Bogli-Stuber, K.; Bissig-Choisat, B.; Wassenaar, T.M.; Frey, J. and Jemmi, T. (2003):***PCR detection of virulence Genes in *Y. enterocolitica* and *Y. pseudo tuberculosis* and investigation of virulence gene distribution. Appl. and Envir. Microbiol., 69 (3): 1810-1816.
69. ***Tijare, D. B.; Singh, A. K.; Chaturvedi, V. K., Srivastava, A. B.; Dhanesar, N. S. and Mehara, K. N. (2000):*** Staphylococcal subclinical mastitis in bovines. Indian Vet. J.; 77(11): 969-971.
70. ***Tiwari, A. and Sisodia, R. S. (2001):*** California mastitis test Vis. A. Vis new gradation under Indian conditions. Indian Vet. J., 78(5): 413-41.
71. ***Varatanovic, N.; Podzo, M.; Mutevelic, T.; Podazo, K.; Cengic, B.; Hodzic, A. and Hodzic, A. (2010):***Use of California mastitis test, somatic cells count and bacteriological findings in diagnostics of subclinical mastitis. Biotechnol. in Anim. Husb., 26 (1-2): 65-74.
72. ***Versalovic, J.; Koeuth, T. and Lupski, J. R. (1991):*** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res., 19: 6823–6831.
73. ***Wenz, J. R.; Barrington, G. M.; Garry, F. B.; Ellis, R. P. and Magnuson, R. J. (2006):*** Escherichia Coli isolates, serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. J. Dairy. Sci., 89 (9): 3408-3412.
74. ***Yucel, N. and Ulusoy, H. (2006):***A Turkey survey of hygiene indicator bacteria and *Yersinia enterocolitica* in raw milk and cheese samples. Food Control, 17: 383–388.

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