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Prevalence and public health hazards of subclinical mastitis in dairy cows

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Abstract

Subclinical mastitis (SCM) is an asymptomatic udder infection distributed worldwide that causes significant losses in the dairy industry. The study aims to detect the prevalence of this pathological condition and to identify the most prevalent related pathogens. A total of 440 quarter milk samples from 110 dairy cows were subjected to California mastitis test (CMT) and Modified Whiteside test (MWST) to quantify their efficacy in detecting subclinical mastitis in dairy cows. Quarter-wise prevalence of subclinical mastitis (SCM) was detected in 30.23% and 28.64% samples by CMT and MWST, respectively, while animal-wise prevalence of SCM was recorded in 60% and 55.45% by CMT and MWST, respectively. The left and right forequarter were most susceptible to SCM than other quarters. All positive samples by field tests were subjected to microbiological examinations. *Staphylococcus aureus (S. aureus)* (48.51%) which considered the primary pathogens among the bacterial isolates followed by Coagulase negative Staphylococci (40.09%), *Escherichia coli* (*E. coli*) (38.12%) and *Streptococcus agalactiae* (*S. agalactiae*) (13.37%). The sensitivity and specificity of the CMT and MWST were 100%, respectively. The results revealed a strong association between these parameters and the diagnosis of subclinical mastitis in milk samples. In conclusion, the bacteria isolated from SCM play an important role on food poisoning especially *S. aureus* and *E. coli.*

Keywords:

California mastitis test, *E. coli*, Modified Whiteside test, *S. aureus*, Subclinical mastitis.

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Introduction

Mastitis is defined as an inflammation of the udder that affects many dairy cows (Dufour et al., 2019). Mastitis can be classified into two forms: clinical and subclinical. Clinical mastitis (CM) is an infection characterized by a sudden occurrence, a change in composition and appearance of milk, decreased milk output, and signs of local inflammation in the affected mammary quarters. Subclinical mastitis (SCM), on the other hand, is an infection that does not show apparent symptoms of local inflammation or systemic involvement. SCM has no visible indications in the milk or on the udder, yet it reduces milk production. This type of disease is more widespread and prevalent than clinical mastitis (Kader et al., 2003; Abebe et al., 2016). SCM is responsible for 70% of economic losses and is one of the major factors limiting milk production (Heleili et al., 2012).

Various screening methods are used for diagnosis of SCM during lactation, based on physical and chemical changes of milk (Sharma et al., 2010). Field tests as California mastitis test (CMT) and Modified White side test (MWST) are preferred as screening tests for subclinical mastitis due to their ease of use and ability to yield rapid and satisfactory results (Tilahun and Aylate, 2015). Furthermore, bacteriological culture of milk samples served as a gold standard method that necessary for definitive diagnosis of subclinical mastitis and evaluation of intra-mammary infection (Badiuzzaman et al., 2015; Sumon et al., 2017).

Over a hundred different microorganisms have been isolated from bovine mastitis, Staphylococci, Streptococci, and Gram-negative bacteria being the most often isolated pathogens (Oliver et al., 2004; Hussain et al., 2012 & 2013). Staphylococci are thought to be one of the most important causative agents of subclinical mastitis in dairy cows (Unal and Yildirim, 2010). The purpose of this study is to investigate the prevalence and public health hazards of pathogens causing SCM in the milk of dairy cows located in Qena Governorate, Egypt.

Materials and methods

Ethical approval:

All experimental procedures in the present study were performed and approved in accordance with the Ethics Committee of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.

Materials and methods

Samples Collection:

A total of four hundred and forty quarter milk samples were collected aseptically from all quarters of 110 dairy cows with apparently healthy udders from different dairy farms in Qena Governorate, Egypt, according to the procedure recommended by Quinn et al. (2002). The collected samples were labeled and kept in an ice box and transported to the laboratory without delay for microbiological examination.

Field tests to detect subclinical mastitis

A) California Mastitis Test (CMT) according to (Schalm et al., 1971):

A plastic vessel with four shallow wells was used for collecting approximately 2 ml of milk from each udder quarter; then equal amount of alkali reagent (Schalm reagent) was added. A gentle circular motion was applied to the mixtures in horizontal plane for 5 seconds and the different degrees of gel was recorded.

B) Modified Whiteside Test (MWST) according to (Murphy and Hanson, 1941):

Five drops of milk were added to 2 drops of NaOH 4% on clean glass plate placed on dark black ground and mixed

well and the reaction was graded according to precipitation and gel formation.

Microbiological Examination:

All the quarter milk samples that showed positive results with field tests were subjected to microbiological examination.

1. Isolation and identification of *S. aureus*:

Enrichment procedure was done according to (APHA, 1985) as the milk samples were inoculated into NaCl broth 10% and then incubated at 37 °C for 24 hrs., then loopful from the incubated broth was streaked (AOAC, 2000) on mannitol salt agar selective agar and then incubated for 24 hrs. at 37 °C. Suspected colonies were picked up onto nutrient agar slants for further identification using colonial morphology (Collins et al., 1991), Gram stain (APHA, 2004), catalase activity test (Bailey and Scott, 1994) and coagulase test (Cruickshank et al., 1975).

2. Detection of *S. agalactiae*:

By the using Hotis test according to (Hotis and Miller, 1936), 9.5 ml milk and 0.5 ml of sterile aqueous solution of bromocresol purple 0.5% were mixed thoroughly (purple color appeared, pH 6.5) and incubated at 37 °C for 24 hrs.; the positive result was indicated by appearance of yellow color and flakes of canary yellow color at the side of test tube and if negative further incubation for 24 hrs. was applied. A loopful from the positive tubes was inoculated into a slope Tryptic soya agar for further examination using Gram stain (A.P.H.A., 2004) catalase test (Cruickshank et al., 1975) Hippurate hydrolysis (Mahon and Manuselis, 1995) CAMP test (Quinn et al., 1994).

3. Total coliforms, Fecal coliforms, and *E. coli* count by using Most Probable Number technique (MPN) (FAO, 1992):

a) Presumptive test for coliforms group (FAO, 1992):

1 ml of each 1:10, 1:100 and 1:1000 of the milk sample dilutions was inoculated into 3 replicate tubes of lauryl sulphate tryptose (LST) broth (high media) supplied with inverted Durham's tubes and incubated at 35± 0.5 °C for 48 hrs. Tubes showed gas in Durham's tubes within 48±2 hrs. (positive tubes) were submitted for confirmatory test. Negative tubes re-incubated for additional 24 hrs. and reexamined for gas production.

b) Confirmatory test for coliforms group (FAO, 1992):

This was done at all positive LST broth tubes showing gas in Durham's tubes after 48±2 hrs. A loopful after gently agitation was inoculated into brilliant green lactose bile 2% (BGLB) broth (high media) tubes with inverted Durham's tube and incubated at 35±0.5 °C for 48±2 hrs. The BGLB broth tubes that showed gas in the Durham's tubes was recorded and considered positive for coliforms. The number of coliforms/ml was calculated from the most probable number (MPN) table for 3 tubes dilutions.

c) Confirmatory test for Fecal coliforms (FAO, 1992):

From all the positive LST broth tubes, a loopful was inoculated into *Escherichia coli* broth (EC broth) (high media) tubes with inverted Durham's tubes and incubated at 45.5±0.5 °C for 48±2 hrs. Tubes showed gas production in Durham's tubes were recorded and considered positive for fecal coliforms. Negative tubes were examined again after 48±2 hrs. The number of fecal coliforms /ml was calculated using MPN table for 3 tubes dilutions.

d) Confirmatory test for *E. coli* count (FAO, 1992):

A loopful from the positive EC broth tubes showed gas production was subculture by streaking on Levine's Eosin Methylene Blue (L-EMB) (high media) plates and incubated at 35 °C for 18- 24 hrs. The typical nucleated dark center colonies with metallic sheen were recorded as *E. coli* positive. The numbers of *E. coli*/ml were calculated from MPN tables for 3 tubes dilutions.

4. Yeasts and molds (ISO, 2008):

Loopful from milk sediment was streaked on Sabouraud dextrose agar (high media) in form of C shape (not in zigzag shape, to obtain pure colony). The plates were incubated at 25 °C for 3-5 days. Suspected colonies were picked up on agar slant for further purification and identification.

Result:

Based on the results showed in Table (1), it was found that 133 out of 440 quarter (30.23%) and 126 out of 440 quarter (28.64%) of examined milk samples were positive for CMT and MWST at quarter level. While out of 110 animals, 66 (60%) and 61 (55.45%) of examined cows were positive for SCM using CMT and MWST, respectively.

The numbers and percentages of cows showing subclinical infection in one, two, three and all four quarters out of 110 cows were 18, 15 (16.36 and 13.64%), 32, 30 (29.09 and 27.27%), 13, 13 (11.82 and 11.82%) and 3, 3 (2.73 and 2.73%) according to CMT and MWST, respectively (Table 2).

As shown in (Table 3), the prevalence of SCM was highest in the left quarter 75, 74 (34.09 and 33.64%) than right one 58, 52 (26.36 and 23.64%) according to CMT and MWST, respectively.

Table 1. Quarter and cows -wise prevalence of subclinical mastitis in cow's milk samples based on the result of CMT& MWST:

Source of milk samples	Number of quarters / cows	CMT positive		MWST positive	
		No.	%	No.	%
Quarters	440	133	30.23	126	28.64
Cows	110	66	60	61	55.45

Table 2. Prevalence of subclinical mastitis in the examined cows according to the affected quarters:

Field tests	No. of examined animals	One quarter		Two quarters		Three quarters		Four quarters	
		No.	%	No.	%	No.	%	No.	%
CMT	110	18	16.36	32	29.09	13	11.82	3	2.73
MWST		15	13.64	30	27.27	13	11.82	3	2.73

Table 3. Individual quarter affected with subclinical mastitis in the examined cows:

Quarter position		No. of screened quarters	CMT		MWST	
			No. of positive	Prevalence (%)	No. of positive	Prevalence (%)
Right	Fore	110	38	34.55	34	30.91
	Hind	110	20	18.18	18	16.36
	Total right	220	58	26.36	52	23.64
Left	Fore	110	44	40	43	39.09
	Hind	110	31	28.18	31	28.18
	Total left	220	75	34.09	74	33.64

Table (4) showed that the quarter-wise prevalence of S. aureus, CNS, *S. agalactiae*, E. coli and yeasts and molds in the examined samples was (22.27, 18.41, 6.14, 17.5, and 27.27%), respectively. Conversely, the cow-wise prevalence of S. aureus, CNS, *S. agalactiae*, E. coli and yeasts and molds was (53.64, 45.45, 21.82 39.09, and 50.91%), respectively.

Table (5) illustrated that the highest frequency distribution of quarters milk samples was showed for coliforms 54 (40.60%) that lied in the range of 10 - $\leq 10^2$ cfu/ml, while the rest of the positive samples were lied in between < 3, 3 - ≤10, 10^2 - $\leq 10^3$ and 10^3 - $\leq 10^4$ cfu/ml, respectively. Moreover, the highest frequency distribution for fecal coliforms was 36 (27.07%) lied in the range of 10 - $\leq 10^2$ cfu/ml. While the rest of the positive samples were distributed in between < 3, 3 - ≤10, 10^2 - $\leq 10^3$ and 10^3 - $\leq 10^4$ cfu/ml, respectively. Concerning E. coli, the highest frequency distribution was 56 (42.11%) lied in the range of < 3 cfu/ml. While the rest of the positive quarter milk samples were lied in between 3 - ≤10, 10^2 - $\leq 10^3$ and 10^3 - $\leq 10^4$ cfu/ml, respectively.

As shown in (Table 6), (90.98, 57.89 and 73.68%) of the examined SCM milk samples for Coliforms, E. coli, and S. aureus, were unacceptable according to the limits recommended by the Egyptian standards (2005).

Table 4. Prevalence of the isolated bacteria causing subclinical mastitis in the examined cow's milk samples (quarters and cow level):

Isolated species	Quarters level		Cow level	
	No. / 440	%	No. / 110	%
S. aureus	98	22.27	59	53.64
CNS	81	18.41	50	45.45
S. agalactiae	27	6.14	24	21.82
Coliforms	128	29.09	65	59.09
Fecal coliforms	119	27.05	63	57.27
E. coli	77	17.5	43	39.09
Yeasts and molds	120	27.27	56	50.91

Table 5. Frequency distribution of positive quarter milk samples in relation to Coliforms, Fecal coliforms and *E. coli* counts:

Count / ml	No. of quarters show Coliforms		No. of quarters show Fecal coliforms		No. of quarters show *E. coli*	
	No. / 133	%	No. / 133	%	No. / 133	%
< 3	5	3.76	14	10.53	56	42.11
3 - ≤ 10	7	5.26	27	20.30	33	24.81
10 - ≤ 100	54	40.60	36	27.07	39	29.32
100 - ≤ 1000	25	18.8	22	16.54	5	3.76
1000 - ≤ 10000	42	31.58	34	25.56	-	-
Total	133	100	133	100	133	100

Table 6. Summarized results of bacteriological examination of milk samples as compared with the Egyptian standards (Egyptian Organization for Standardization and Quality Control (EOSQC, 2005):

Organisms	Standards	Milk samples			
		Unacceptable		Acceptable	
		No.	%	No.	%
Coliforms	Not more than 10 /ml	121	90.98%	12	9.02%
E. coli	Free	77	57.89%	56	42.11%
S. aureus	< 100	98	73.68%	35	26.32%

Discussion

Mastitis is one of the most serious economic and health problems affecting the eventual milk production of dairy cows and consider a major cause for excessive culling of cows in dairy herds (Cobirka et al., 2020). One of the aims of this study is to evaluate the prevalence of SCM. From the obtained data in Table (1), it was clear that the quarter-wise prevalence of SCM based on the results of CMT was (30.23%). Nearly similar results of (29.1 and 30%) were recorded by (Asmare and Kassa, 2017) and (Youssef, 2017). Lower results of 22.66, and 20.18% were obtained by Lakew et al., (2019) and (Mourya et al., 2020). Higher results of 59.68 and 43.1% were reported by (Badiuzzaman et al., 2015) and (Ndahetuye et al., 2019). The differences in prevalence between studies might be due to differences in milking practice, environmental conditions, and animals' immune status (Qayyum et al., 2016). Moreover, the cow-wise prevalence of SCM based on the results of CMT was (60%). This result somewhat agreed with that mentioned previously by (Pumipuntu et al., 2019) as they recorded that 59% of tested cows were sub-clinically mastitic. Lower results of 26 and 31.55% were stated by (Ait-Kaki et al., 2019) and (Mourya et al., 2020). While higher results of 72.07 and 76.2% were detected by (Badiuzzaman et al., 2015) and (Ndahetuye et al., 2019).

In regarding the quarter and cow-wise prevalence of SCM based on the results of MWST. It was found that the quarter-wise prevalence in milk samples was (28.6%). Lower prevalence of 13.19% was revealed by (Zahid, 2004). Higher result of 49% were obtained by (Bakr et al., 2019). Moreover, the cow-wise prevalence of

SCM was (55.45%). Higher finding of 64.86% was detected by (Badiuzzaman et al., 2015). While lower finding of 27.5% was recorded by (Islam et al., 2011). These variations in the occurrence of SCM in cows could be attributed to the nature of mastitis as a complex disease including interactions of numerous factors as management, environment, and factors relating to animal and causative organisms (Constable et al., 2017).

Prevalence of SCM in the examined cows according to the affected quarters stated in Table (2). Numbers and percentages of cows showing subclinical infection in one, two, three and all four quarters were (16.36 and 13.64%), (29.09 and 27.27%), (11.82 and 11.82%) and (2.73 and 2.73%) according to CMT and MWST, respectively. These findings were agreed with (Dasohari et al., 2017) who recorded that the highest percentage of subclinical infection by using CMT and MWST was in two quarters (43.33, and 50.62%), followed by single quarter (30, and 30.86%), then, three quarters (20, and 14.82%) and the least one was in four quarters (6.67, and 3.7%). (Patil et al., 2000) also reported a higher number of infected cows in one quarter. It may be due to behavior of animal when laying out or due to unhygienic practice in the farm.

Results presented in Table (3), illustrated the prevalence of SCM in individual udder quarters depending on CMT and MWST. According to CMT, the prevalence was highest in the left forequarter 40% (44 out of 110 quarters) followed by 34.55% (38 out of 110 quarters) in the right forequarter 28.18% (31 out of 110 quarters) in the left hindquarter and the least prevalence was18.18% (20 out of 110 quarters) in the right hindquarter. Concerning the results of MWST, the prevalence of SCM was the highest in the left forequarter 39.09% (43 out of 110 quarters) followed by 30.91% (34 out of 110 quarters) in the right forequarter, 28.18% (31 out of 110 quarters) in the left hindquarter and the least prevalence was of 16.36% (18 out of 110 quarters) in the right hindquarter.

Regarding the affected quarter's results, the percentage of SCM in the examined milk samples were 44 (33.08%) for left forequarter 38 (28.57%) for right forequarter, 31 (23.31%) for left hindquarter and 20 (15.04%) for right hindquarter. The obtained data were supported by (El- Kholy et al., 2018) and (Mourya et al., 2020) who scored a higher prevalence of SCM in forequarters than hindquarters.

As shown in Table (4), the quarter-wise prevalence of *S. aureus*, CNS, *S. agalactiae*, *E. coli* and yeasts and molds in the examined samples was (22.27, 18.41, 6.14, 17.5, and 27.27%), respectively. However, the animal-wise prevalence of *S. aureus*, CNS, *S. agalactiae*, *E. coli* and yeasts and molds in the examined cows was (53.64, 45.45, 21.82 39.09, and 50.91%), respectively. Among the total isolates, *S. aureus* was the most predominant isolates with a prevalence of (48.51%). While CNS, *S. agalactiae* and *E. coli* were 40.09, 13.37, and 38.12, respectively. At quarter level, relatively lower results of 9.20 and 3.45% of *S. aureus* and *E. coli* were achieved by (Janevski et al., 2020). Moreover, higher results were obtained by (Sztachańska et al., 2016) (31.6 and 15.6%) for CNS and *S. agalactiae*. Furthermore, at cow level, lower results of 35.5, 25.5, 11.8% and 0.91 of *S. aureus*, CNS, *S. agalactiae* and *E. coli* were detected by (Mureithi and Njuguna, 2016).

S. aureus is the most important contagious pathogen causing mastitis with

high penetrating power forming deep settled foci in infected organs (Ranjan et al., 2011). *S. aureus* can cause serious problems and economic losses in dairy cows (Dego et al., 2002). The high prevalence of *S. aureus* in this study might be related to improper hygienic practice of milkers' hands before and during milking process, absence of teat dipping after milking, lack of culling of chronically infected cows and absence of dry cow therapy in the dairy herds (Abebe et al., 2016). CNS produce mild form of mastitis, and usually remains subclinical so, these pathogens were identified as a minor mastitis pathogen especially when compared with major one (*S. aureus*, *Streptococci* and Coliforms) (Taponen et al., 2006).

Mastitis caused by *S. agalactiae* can be successfully reduced with eradication program depending on antimicrobial agent-treatment strategy and adequate herd management to limit the incidence of new infection (Reyes et al., 2014). E. coli is responsible for more than 80% of coliform mastitis cases (Fahim et al., 2019). The variations of E. coli incidence may be associated with unhygienic practice in the farms such as poor cleanliness, faulty in drainage and manure disposal, ineffective udder washing, improper drying before milking, using of dirty washing towels and absence of post milking teat dipping (Ayano et al., 2013).

The results presented in Table (5), showed that the highest frequency distribution of quarters milk samples was showed for coliforms 54 (40.60%) that lied in the range of 10 - $\leq 10^2$ cfu/ml, while the rest of the positive samples were distributed as follow 3.76%, 5.26%, 18.8%, and 31.58% lied in between < 3, 3 - ≤ 10, 10^2 - $\leq 10^3$ and 10^3 - $\leq 10^4$ cfu/ml, respectively. Lower results were detected by (Bakr et al., 2019). Moreover, it is clear from the results in Table (5), that the highest frequency distribution of quarters milk samples showed fecal coliforms was 36 (27.07%) lied in the range of 10 - $\leq 10^2$ cfu/ml. Relatively lower finding was detected by (Bakr et al., 2019). While the rest of the positive samples were distributed as 10.53%, 20.30%, 16.54%, and 25.56% lied in between < 3, 3 - ≤ 10, 10^2 - $\leq 10^3$ and 10^3 - $\leq 10^4$ cfu/ml, respectively. Higher result of the distribution < 3 was obtained by (Bakr et al., 2019). Concerning E. coli, it is evident from the obtained results in Table (5), that the highest frequency distribution of quarter milk samples for E. coli was 56 (42.11%) lied in the range of < 3 cfu/ml. Nearly similar result of the distribution < 3 was recorded by (Bakr et al., 2019). While the rest of the positive quarter milk samples were distributed as 24.81%, 29.32%, and 3.76% lied in between 3 - ≤ 10, 10^2 - $\leq 10^3$ and 10^3 - $\leq 10^4$ cfu/ml, respectively. Lower results were detected by (Bakr et al., 2019). The variations in frequency may be associated with unhygienic practice in the farms such as poor cleanliness, faulty in drainage and manure disposal, ineffective udder washing, improper drying before milking, using of dirty washing towels and absence of post milking teat dipping (Ayano et al., 2013).

From the present study, it was clear that the quarter-wise prevalence of SCM in cow's milk samples based on the results of microbiological examination was (30.22%). These results were nearly like that evaluated by (EL-Bassiony et al., 2009) (28.50%). While the prevalence of SCM in cow's milk samples was 60%. A higher result of 67.5% was recorded by (Abdel-Ghani, 2005). These variations in the occurrence of SCM in quarters and cows could be attributed to the nature of mastitis as a complex disease including interactions of numerous factors as management, environment, and factors

relating to animal and causative organisms (Constable *et al.*, 2017).

The analysis of results obtained in the present study revealed that there is a close confident relationship between field tests (CMT & MWST) and isolation of bacteria from examined milk samples on a quarters and cow's level. As approximately all milk samples that were positive to these tests, were microbiologically positive and different bacteria were isolated. These findings were in harmony with that reported by (El- Kholy et al., 2018). In this concern, CMT was identified to be a good diagnostic and the most reliable test in the early detection of SCM in the dairy farms (Bitew et al., 2010).

In the light of the above, it was found that the sensitivity and specificity of CMT and MWST were 100% and the agreement between these tests and microbiological examination was also 100%. Similar result was obtained by (El- Kholy et al., 2018).

According to the limits recommended by the Egyptian standards (2005), we found that, 121 (90.98%), of the examined SCM milk samples for Coliforms, 77 (57.89%) for *E. coli* and 98 (73.68%) for *S. aureus*, failed to comply with the limits, (Table 6). Higher results of 60% and 80% for *E. coli*, and *S. aureus* were recorded by (Kandil et al., 2018). The variation between results may attributed to difference in hygienic practice and environmental condition.

Conclusion

The subclinical occurrence of the mastitis continues to be a major problem for dairy producers. The result of the present study indicated a relatively high prevalence of subclinical mastitis in dairy cows in Qena governorate. CMT and MWST findings were certified as a reliable cow side screening tests for detection of SCM in cows with no clinical indications of mastitis

Conflict of interest statement

The authors declare that they have no conflict of interest.

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