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REGULAR ARTICLES

Molecular and bacteriological investigation of subclinical mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* in domestic bovids from Ismailia, Egypt

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Abstract A study was carried out to establish the prevalence of subclinical mastitis (SCM) in smallholder dairy farms in Ismailia, Egypt. A total of 340 milking cows and buffaloes were sampled from 60 farms, and 50 nasal swabs were collected from consenting farm workers. Milk samples were subjected to California mastitis test (CMT) and the positive samples were examined by bacterial culture and PCR to identify etiological agents. Based on CMT, the prevalence of SCM was 71.6 % in cattle and 43.5 % in buffaloes while the prevalence was 25.2 % at cow-quarter level and 21.7 % at buffaloes-quarter level. Bacteriological analysis showed that the most frequently identified bacteria were *Staphylococcus* (*S.*) *aureus* (38.3 %) and *Streptococcus* (*Str.*) *agalactiae* (20 %). The diagnostic sensitivity of PCR compared to bacterial culture was superior with *S. aureus* and *Str. agalactiae* detection being 41 and 22.6 %, respectively. Furthermore, methicillin-resistant *S. aureus* (MRSA) strains occurred in 52.2 and 45 % of isolates of animals and workers, respectively. Subclinical mastitis due to *S. aureus* and *Str. agalactiae* is endemic in smallholder dairy herds in Ismailia. The occurrence of MRSA in animals and workers highlights a need for wide epidemiological studies of MRSA and adopting control strategies.

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Introduction

Mastitis is a multifactorial disease caused by several species of gram-negative and gram-positive bacteria, mycoplasmas, fungi, and algae (Zadoks et al. 2011).

The most common pathogens causing mastitis are *Staphylococcus* (*S.*) *aureus* and *Streptococcus* (*Str.*) *agalactiae*, which are spread among cows during milking, causing significant economic losses (Fox and Gay 1993; Benić et al. 2012).

Besides causing mastitis, the genus *Staphylococcus* contains etiologic agents responsible for approximately 45 % of toxin infections worldwide. These toxins can cause toxic shock syndrome and food poisoning (Balaban and Rasooly 2000). Methicillin-resistant *S. aureus* (MRSA) infection has emerged recently among animals and can be spread between cows and humans, posing a potential risk both for human and animal health (Juhász-Kaszanyitzky et al. 2007). MRSA strains are frequently resistant to a variety of antibiotics, such as ampicillin, oxacillin, and penicillin (Lee 2003). Furthermore, the presence of MRSA does not cause a delay in treatment but may cause failure of treatment, not only mastitis but also other bovine diseases (Soo Ko et al. 2005).

Str. agalactiae causes disease in both humans and cattle. In cattle, it causes bovine clinical mastitis (CM) and subclinical mastitis (SCM), and in humans, it is associated with infections among neonates and adults (Pinto et al. 2013). Man may be a source of infection for cattle (Zadoks et al. 2011).

In Egypt, more than 70 % of the total livestock population is owned by subsistence and small-scale farmers, who keep a few animals as a source of milk and dairy products for the

home consumer or to sell, often unpasteurized, in local markets (Aidaros 2005).

There have been many studies on the diagnosis of bovine CM which is easily detected on the basis of clinical signs (Mdegela et al. 2009). Diagnosis of SCM requires detection of the measurement of inflammatory components and isolation of the pathogens in the milk (Nielen et al. 1993). In spite of strict control standards to reduce the spread of mastitis, SCM remains the most common disease for dairy farms worldwide.

A number of diagnostic systems for diagnosis of mastitis and for determining the causative agents, including CMT, bacteriological, and molecular examination, are available. Bacteriological examination could identify the microbial pathogens, but is not highly specific (Riffon et al. 2001). Of these methods, molecular detection of pathogenic microorganisms based on DNA amplification of the target pathogen based on unique DNA sequences within the 16S subunit of the ribosomal RNA (rRNA) gene has been proven to be an accurate, reliable, and fast diagnostic method (Meiri-Bendek et al. 2002; Elias et al. 2012). The aim of this study was to investigate the prevalence of SCM caused by *S. aureus* and *Str. agalactiae* in randomly selected cattle and buffaloes, which were sampled from households and small-scale dairy farms in the Ismailia province in Egypt and compare the techniques of bacterial culture and PCR assay, as well as to gain information about MRSA prevalence in both animals and animal workers.

Materials and methods

Study area and sample collection

This study was conducted among the herds of cattle and buffalo located in Ismailia, a province located in northeastern Egypt, which has a total land area of approximately 210 km^2 and a hot desert climate. Ismailia is situated between longitude 32° 16′ 0″ E and latitude 30° 35′ 0″ N, ~120 km east of Cairo, the Egyptian capital. All herds participating in the study were in smallholder farms (n=60), having between 1 and 45 lactating animals and were randomly selected for this study. The survey was conducted on 340 of milk-producing animals (240 cows and 100 buffaloes) sampled from various small dairy farms in the Ismailia province, between March 2012 and June 2013. The cattle, which had a median age of 5.5 years, and buffalo, which had a median age of 7.5 years, were maintained under suboptimal conditions, with milking being done by hand. All animals were subjected to clinical examination and California mastitis test, and the positive samples were examined by bacteriological and PCR examination. To detect SCM, 1240 samples of milk-quarters obtained from each of the animals were examined. Before sampling for bacterial examination, the first milk stream was discarded after which routine teat cleaning and disinfection, using 70 % alcohol, as prescribed by the National Mastitis Council (NMC) (1999), was performed. This was done prior to collecting the milk samples. Samples were immediately placed on ice and transported to the laboratory of Infectious Diseases, Faculty of Veterinary Medicine, University of Suez Canal in Ismailia, Egypt. In addition, nasal swabs were collected from 50 consenting animal owners (median age 30.5 years), who were in contact with the animals and agreed to take part in this survey. These samples were transferred to the laboratory on ice with minimal delay.

California mastitis test (CMT), bacterial culture, and PCR amplification

Milk samples collected from all quarters of the animals (non-clinical) were examined by the CMT as described by the NMC (1999). An equal amount of milk and CMT solution were gently mixed for 10 s, after which the result was recorded by a single test reader. If one quarter was positive on CMT and the animal had no clinical signs of infection then it was considered to have subclinical mastitis.

Milk samples positive for SCM (score of 1+ or more) and all nasal swabs received from the owners were subjected to bacteriological examination and PCR. Samples were cultured and identified according to the Laboratory Handbook on Bovine Mastitis (NMC 1999). Briefly, 10 μl of each milk sample and nasal swabs of the animal workers moistened with sterile saline (0.9 % NaCl) were cultured on the surface of 5 % sheep blood agar and MacConkey agar plates followed by incubation at 37 °C for 18–24 h. Bacterial identification was confirmed by other laboratory tests and by PCR. *S. aureus* was identified by Gram stain, a positive catalase test, α- and β-hemolysis, and a positive tube coagulase test, while *Str. agalactiae* was identified by Gram stain, a negative catalase test, and biochemical tests. Plates containing colonies for other types instead of *S. aureus* and *Str. agalactiae* have been ignored.

For PCR amplification, DNA was extracted from bacterial cells and clinical samples using the Nucleospin® kit (MACHEREY-NAGEL, Germany), following the manufacturer's instructions. Briefly, 1.5 ml of the bacterial culture or 1 ml of liquid milk sample was transferred into an Eppendorf tube and centrifuged at 5000 rpm for 10 min. DNA was extracted from the pellets by resuspending the pelleted cells in 20 mM Tris/HCl, 2 mM EDTA, 1 % Triton X-100, and pH 8 supplemented with 20 mg/ml lysozyme and incubating for 30 min at 37 °C. Thereafter, 25 μl of Proteinase K was added and samples were incubated at 56 °C until complete lysis was obtained. The DNA was eluted off of the NucleoSpin® Tissue Column by adding 100 μl prewarmed Buffer BE (70 °C). Aliquots of DNA were stored at −20 °C until further use. The PCR was performed in a 25 μl volume with 5 μl of 5× Master Mix (Jena Bioscience, GMBH, Germany) containing

Thermostable DNA Taq polymerase buffer, dNTPs, (NH4) 2SO4, MgCl2, and 20 pmol concentrations of each of the primers. Double-distilled water was added to bring the final volume to 25 μl. Amplification of a 228 bp fragment of the *S. aureus* was achieved using 16S rRNA gene primers (F: 5-GTAGGTGGCAAGCGTTATCC-3 and R: 5-CGC ACATCA GCGTCAG-3) (Monday and Bohach 1999). Thermal cycling consisted of an initial denaturation step of 2 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final 10-min incubation at 72 °C. For PCR amplification of a 120 bp fragment of the 16S rRNA gene of *Str. agalactiae* was obtained using primers V1: 5′-TTTGGTGTTTACACTAGACTG-3′ and V2: 5′-TGTGTT AATTACTCTTATGCG-3′ (Meiri-Bendek et al. 2002; Ahmadi et al. 2009). Thermal cycling conditions were 94 °C for 4 min, 30 cycles of 94 °C, 55 °C and 72 °C for 45 s each step, and a final elongation step at 72 °C for 5 min. The amplification of 533 bp fragment of *mecA* gene in MRSA was performed using the primers *mecA1*: 5′-AAAATCGATG GTAAAGGTTGGC-3′ and *mecA2*: 5′AGTTCTGCAGTACC GGATTTGC-3′ (Murakami et al. 1991). Thermal cycling conditions were 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 5 min at 72 °C. Positive control (reference *S. aureus* 1003080MC0015 and *Str. agalactiae* 1003080MC0017, State Institute for Consumer Protection of Saxony-Anhalt, Department of Veterinary Medicine, Stendal, Germany) and negative control (no template) were utilized. The PCR products were analyzed by electrophoresis through 1.5 % agarose gel containing ethidium bromide (0.5 μg/ml), and the image of the amplified DNA was captured using a gel documentation system (Biospectrum UVP, UK).

Antimicrobial susceptibility testing

Susceptibility to methicillin was determined by modifying Kirby-Bauer disk diffusion methods in Mueller-Hinton agar according to the Clinical Laboratory Standards Institute, formerly NCCLS guidelines. Methicillin disks (5 μg) were used for the determination of methicillin-resistant strains. Results were recorded according to the zone size and interpreted in accordance with the criteria of the Clinical and Laboratory Standards Institute, 2005 (methicillin resistant=≤9 mm and methicillin susceptible=≥14 mm). *S. aureus*1003080MC0015 was used as quality control.

Statistical analysis

The kappa values were calculated to evaluate the agreement between the bacterial culture and PCR (http://vassarstats.net/kappa.html) according to Viera and Garrett (2005).

Results

Descriptive results

The overall prevalence of CM in the study was 8.8 % (30/340 heads of the animals), and the rest of the animals appeared healthy.

As tabulated in Table 1, 63.2 % (196/310 heads of the animals) and 24.2 % (300/1240 quarters) were CMT positive. Among cattle and buffaloes included in this study, cattle had the highest SCM prevalence of 71.6 % (156/218) compared to 43.5 % (40/92) in buffaloes. In addition, the individual quarter-level prevalence of SCM was 25.2 and 21.7 % for cattle and buffaloes, respectively.

Comparing *Staphylococcus aureus* and *Streptococcus agalactiae* positivity by bacteriological methods and PCR assay

A total of 300 milk samples (positive on CMT) were examined bacteriologically. Bacteria isolated were identified as *S. aureus* and *Str. agalactiae*. As tabulated in Table 2, the proportion of positive milk samples was 38.3 and 20 % by bacteriological examination and 41 and 22.6 % by PCR assay for *S. aureus* and *Str. agalactiae*, respectively. In total, bacterial culture provided 175 bacterial identifications and PCR provided 191 identifications in subclinical milk samples. PCR detected all bacterial species detected by bacterial culture, except for 16 samples were negative by bacterial culture and PCR positive. Kappa testing showed an excellent agreement between bacterial culture and PCR (Kappa=0.9, 95 % confidence interval=0.84 to 0.94) (Table 3).

Furthermore, *S. aureus* was most frequently isolated in SCM in this study by both bacteriological and PCR assays. In addition, 40 % of the nasal swabs of the workers were positive for *S. aureus* by bacterial culture and 50 % by PCR assay, but none were positive for *Str. agalactiae* by either assay.

Table 1 Prevalence of subclinical mastitis per animal and quarter using CMT

Examined animals	Examined quarters	CMT			
		Animals		Quarters	
		Positive	%	Positive	%
Cattle (*n*=218)	872	156	71.6	220	25.2
Buffaloes (*n*=92)	368	40	43.5	80	21.7
Total (*n*=310)	1240	196	63.2	300	24.2

Table 2 Results of the bacteriological culture and PCR of CMT-positive quarter milk samples and from nasal swabs of workers

Animals	Bacteriological examination			PCR		
	S. aureus Positive (%)	*Str. agalactiae* Positive (%)	Total Positive (%)	*S. aureus* Positive (%)	*Str. agalactiae* Positive (%)	Total Positive (%)
Cattle (n=220)	86 (39 %)	48 (21.8 %)	134 (60.9)	92 (41.8 %)	49 (22.3 %)	141 (59.5)
Buffaloes (n=80)	29 (36.25 %)	12 (15 %)	41 (51.2)	31 (38.75 %)	19 (23.75 %)	50 (62.5)
Total (n=300)	115 (38.3)	60 (20)	175 (58.3)	123 (41)	68 (22.6)	191 (63.6)
Nasal swabs (n=50)	20 (40)	0 (0)	20 (40)	25 (50)	0 (0)	25 (50)

Antimicrobial susceptibility testing of ***S. aureus*** isolates to methicillin

Results revealed that 52.2 % (60/115) and 45 % (9/20) of the *S. aureus* isolated from animals and humans, respectively, were phenotypically resistant against methicillin and were *mecA*-PCR positive.

Discussion

Of contagious pathogens of the udder, *S. aureus* and *Str. agalactiae* predominate in all regions of the world, causing subclinical mastitis (Benić et al. 2012), despite intensive research efforts aimed at reducing the rate of the spread. The current study aimed to estimate the prevalence of CM and SCM and using of molecular methods as an efficient and supplementary tool for diagnosis of SCM in dairy herds.

This study revealed that CM was observed in 8.8 % (30/340 heads of the animals), this finding is within the range reported by Joshi and Gokhale (2006) and higher than those reported by (Getahun et al. 2008) 1.8 %.

The prevalence of SCM as shown in the current study in cows was 71.6 %, higher than those reported in Ethiopia (22.8 %) (Getahun et al. 2008) and Tanzania (51.6 %) (Mdegela et al. 2009). The prevalence of SCM was lower on animal and quarter level in buffaloes than in cattle. This conclusion is supported by previous studies (Tsonev et al. 1975; Moroni et al. 2006), which reported that the buffaloes showed higher absolute and relative resistance to SCM.

Table 3 Comparison of bacterial culture and the PCR assay targeting bacterial identifications causing SCM

Test		Bacterial culture		Total
		Positive	Negative	
PCR	Positive	175	16	191
	Negative	0	109	109
	Total	175	125	300

Overall, about 24.2 % of samples were positive on the CMT and 58 % of the CMT positive samples were positive on bacterial culture. The high proportion of SCM of *S. aureus* (38 %) and *Str. agalactiae* (20 %) among the investigated animals concurs with that of previous studies (Gianneechini et al. 2002; Mdegela et al. 2009; Amin et al. 2011).

S. aureus was detected in 40 % of the animal workers by bacterial culture, while none of the workers were positive for *Str. agalactiae*. MRSA is an important pathogen found in milk and dairy products worldwide with variable prevalence ranging from low prevalence in Europe, USA, and Canada to high prevalence in Asia and Africa (Pexara et al. 2013). Furthermore, it was reported not only in bovine milk, but also in animal workers in the UK (Paterson et al. 2013).

The prevalence of MRSA among *S. aureus* isolates was lower than 50 % in most of the African countries, but as high as 52 % between 2003 and 2005 in Egypt (Falagas et al. 2013). To our knowledge, the data regarding MRSA in the northeastern portion of Egypt, particularly in dairy animals are missing. This study confirmed MRSA in isolates of *S. aureus* of dairy animals (52.2 %) and animal workers (45 %) and is supported by results from Ethiopia (Daka et al. 2012). The occurrence of MRSA in animals and workers in the present study has to be considered as a public health burden where resistant bacteria may spread to the community. It could be emerged because of misuse and manipulation of the antimicrobial drugs by farmers (Juhász-Kaszanyitzky et al. 2007). Thus, further studies are needed to understand the epidemiology of MRSA in endemic situations, which require careful study, and they would provide insights which would help control the disease.

This study shows a very high agreement between results of bacterial culture and PCR, although, PCR found all samples positive on bacterial culture, but the culture did not confirm all PCR-positive samples, with 16 of culture-negative milk samples (eight samples for *S. aureus* and eight for *Str. agalactiae*) being positive in PCR. This finding is supported by Keane et al. (2013), who indicated that PCR appears to be more accurate in bacterial detection. The low proportion of positive samples by bacterial culture may be related to no bacterial growth due to the low number of bacteria or because of residual antibiotic therapy (Phuektes et al. 2001; Lee et al.

2008; Taponen et al. 2009). Furthermore, it was reported that 22.6 to 49.7 % of all milk samples yield no bacterial growth (Makovec and Ruegg 2003). However, the sensitivity of PCR assays in the identification of bacterial pathogens depends on the primers used and the detection of nucleic acids instead of live cells (Koskinen et al. 2010). In the current study, the observed high proportion of positive milk samples by PCR (63.6 %, 191/300) compared to the bacteria culture (58.3 %, 175/300) is in agreement with Koskinen et al. 2010, Elias et al. 2012, and Mahmmod et al. 2013. In addition, the PCR advantages over bacterial culture derive from its ability to detect the low number of pathogens, even in a dead state, easy handle samples simultaneously, as well as, its rapidity, sensitivity, ease of analysis, and interpretation. The higher positivity of PCR could be attributed to the use of a larger amount of milk (350 μl) for PCR compared to bacterial culture (10 μl) which generally increases the chance of bacteria detection (Koskinen et al. 2010; Mahmmod et al. 2013).

Furthermore, PCR can be used routinely for the early detection of animal reservoirs and provides the veterinarian and the owners the results quickly with increased sensitivity, thus lower the cost of treatment and improve mastitis management (Barkema et al. 2006; Koskinen et al. 2010).

PCR does not distinguish whether the detection of bacterial cells is dead or alive, and this means that dead bacteria are identified as positive (Mahmmod et al. 2013). Moreover, it is unlikely that the PCR test will yield a false positive if the DNA of the pathogen is present in the sample. Thus, when taken together the results of PCR and bacterial culture, concludes that culture has provided false negative results when the sample was PCR positive and negative culture. It is significant to remark that the culture is able to detect viable cells only, and the PCR identifies both live and dead organisms (Koskinen et al. 2010). Moreover, Keane et al. (2013), who reported that the evaluation of PCR testing in the case of culture-negative samples is problematic, as the current gold standard is bacterial culture. For this, there is a need for further studies to examine the results of a PCR-positive/negative culture.

In conclusion, this study shows that SCM because of *S. aureus* and *Str. agalactiae* was prevalent among cows and buffaloes in Ismailia, Egypt, either by bacteriology or PCR assay and, therefore, reliable diagnosis should be utilized for the treatment or prevention of SCM. MRSA was observed in several cases of SCM and workers. Further investigation of these isolates by molecular analysis is needed to determine the human-livestock linkage.

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Conflict of interest None.

References

Ahmadi, M., Razavi Rohani, S. M. and Ayremlou, N., 2009. Evaluation of *Streptococcus agalactiae* detection by PCR in Milk and its comparison to other microbiological methods. Iranian journal of microbiology, 1(4), 28–31.

Aidaros, H., 2005. Global perspectives - the Middle East: Egypt. Revue scientifique et technique (International Office of Epizootics), 24 (2), 589–596.

Amin, A. S., Hamouda, R. H. and Abdel-All, A. A., 2011. PCR Assays for Detecting Major Pathogens of Mastitis in Milk Samples. World Journal of Dairy & Food Sciences, 6 (2), 199–206.

Balaban, N. and Rasooly, A., 2000. Staphylococcal enterotoxins. International Journal of Food Microbiology, 61 (1), 1–10.

Barkema, H.W., Schukken, Y.H. and Zadoks, R.N., 2006. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. Journal of Dairy Science, 89, 1877–1895.

Benić, M., Habrun, B. and Kompes, G., 2012. Clinical and Epidemiological Aspects of Cow Mastitis Caused by *Staphylococcus aureus* and its Methicillin-Resistant Strains. Rad Hrvatske akademije znanosti i umjetnosti. Medicinske znanosti, 37, 113–121.

Daka, D., Solomon, G/ silassie and Yihdego, D., 2012. Antibiotic-resistance *Staphylococcus aureus* isolated from cow's milk in the Hawassa area, South Ethiopia. Annals of Clinical Microbiology and Antimicrobials, 11, 26–37.

Elias, A. O., Cortez, A., Brandão, P. E., da Silva, R. C. and Langoni, H., 2012. Molecular detection of *Streptococcus agalactiae* in bovine raw milk samples obtained directly from bulk tanks. Research in Veterinary Science, 93(1), 34–38.

Falagas, M. E., Karageorgopoulos, D. E., Leptidis, J. and Korbila, I. P., 2013. MRSA in Africa: Filling the Global Map of Antimicrobial Resistance. PloS one, 8 (7), e68024.

Fox, L. K. and Gay, J. M., 1993. Contagious mastitis. Veterinary Clinics of North America: Food Animal Practice, 9, 475–487.

Getahun, K., Kelay, B., Bekana, M. and Lobago, F., 2008. Bovine mastitis and antibiotic resistance patterns in Selalle smallholder dairy farms, central Ethiopia. Tropical Animal Health and Production, 40, 261–8.

Gianneechini, R., Concha, C., Rivero, R., Delucci, I. and Moreno López, J., 2002. Occurrence of clinical and sub-clinical mastitis in dairy herds in the West Littoral Region in Uruguay. Acta Veterinaria Scandinavica, 43, 221–230.

Joshi, S. and Gokhale, S., 2006. Status of mastitis as an emerging disease in improved and periurban dairy farms in India. Annals of the New York Academy of Sciences, 1081 (1), 74–83.

Juhász-Kaszanyitzky, É., Jánosi, S., Somogyi, P., Dán, Á., vanderGraaf van Bloois, L., van Duijkeren, E. and Wagenaar, J. A., 2007. MRSA transmission between cows and humans. Emerging Infectious Diseases, 13 (4), 630–632.

Keane, O. M., Budd, K. E., Flynn, J. and McCoy, F., 2013. Increased detection of mastitis pathogens by real-time PCR compared to bacterial culture. Veterinary Record, 173(11), 268–268.

Koskinen, M.T., Wellenberg, G.J., Sampimon, O.C., Holopainen, J., Rothkamp, A., Salmikivi, L., Van Haeringen, W.A., Lam, T.J.G.M. and Pyörälä, S., 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. Journal of Dairy Science, 93, 5707–5715.

Lee, J. H. 2003. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Applied and Environmental Microbiology, 69: 6489–6494.

Lee, K. H., Lee, J. W., Wang, S. W., Liu, L. Y., Lee, M. F., Chuang, S. T., Shy, Y.M., Chang, C.L., Wu, M.C. and Chi, C. H., 2008.

Development of a novel biochip for rapid multiplex detection of seven mastitis-causing pathogens in bovine milk samples. Journal of veterinary diagnostic investigation, 20(4), 463–471.

Mahmmod, Y. S., Toft, N., Katholm, J., Grønbæk, C. and Klaas, I. C., 2013. Bayesian estimation of test characteristics of real-time PCR, bacteriological culture and California mastitis test for diagnosis of intramammary infections with Staphylococcus aureus in dairy cattle at routine milk recordings. Preventive Veterinary Medicine, 112 (3), 309–317.

Makovec, J.A. and Ruegg, P.L., 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. Journal of Dairy Science, 86:3466–3472.

Mdegela, R. H., Ryoba, R., Karimuribo, E. D., Phiri, E. J., Løken, T., Reksen, O., Mtengeti, E. and Urio, N. A., 2009. Prevalence of clinical and subclinical mastitis and quality of milk in smallholder dairy farms in Tanzania. Journal of the South African Veterinary Association, 80 (3), 163–168.

Meiri-Bendek, I., Lipkin, E., Friedmann, A., Leitner, G., Saran, A., Friedman, S. and Kashi, Y., 2002. A PCR-Based Method for the Detection of *Streptococcus agalactiae* in Milk. Journal of Dairy Science, 85 (7), 1717–1723.

Monday, S.R. and Bohach, G.A., 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. Journal of Clinical Microbiology, 37, 3411–3414.

Moroni, P., Sgoifo Rossi, C., Pisoni, G., Bronzo, V., Castiglioni, B. and Boettcher, P. J., 2006. Relationships between somatic cell count and intramammary infection in buffaloes. Journal of Dairy Science, 89 (3), 998–1003.

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H. and Watanabe, S., 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. Journal of Clinical Microbiology, 29:2240–2244.

National Mastitis Council, 1999. Laboratory and Field Handbook on Bovine Mastitis. 2nd Edition. National Mastitis Council, Madison, Wisconsin, USA.

Nielen, M., Schukken, Y. H., Van De Broek, J., Brand, A., Deluyker, H. A. and Maatje, K., 1993. Relations between on-line electrical conductivity and daily milk production on a low somatic cell count farm. Journal of Dairy Science, 76, 2589–2596.

Paterson, G. K., Harrison, E. M., Craven, E. F., Petersen, A., Larsen, A. R., Ellington, M. J., Török, M. E., Peacock, S. J., Parkhill, J., Zadoks, R. N. and Holmes, M. A., 2013. Incidence and Characterisation of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from Nasal Colonisation in Participants Attending a Cattle Veterinary Conference in the UK. PloS one. 8 (7), e68463.

Pexara, A., Solomakos, N. and Govaris, A., 2013. Prevalence of methicillin-resistant Staphylococcus aureus in milk and dairy products. Journal of the Hellenic Veterinary Medical Society, 64 (1), 17–34.

Phuektes, P., Mansell, P.D. and Browning, G.F., 2001. Multiple polymerase chain reaction assay for simultaneous detection of Staphylococcus aureus and streptococcal causes of bovine mastitis. Journal of Dairy Science, 84,1140–1148.

Pinto, T.C.A., Costa, N.S., Souza, A.R.V., Silva, L.G.D., Corrêa, A.B..D.A., Fernandes, F.G., Oliveira I.C., Mattos M.C., Rosado A.S. and Benchetrit, L.C., 2013. Distribution of serotypes and evaluation of antimicrobial susceptibility among human and bovine Streptococcus agalactiae strains isolated in Brazil between 1980 and 2006. Brazilian Journal of Infectious Diseases, 17(2), 131–136.

Riffon, R., Sayasith, K., Lhalil, H., Dubreuil, P., Drolet, M. and Lagace, J., 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. Journal of Clinical Microbiology, 39, 2584–2589.

Soo Ko, K., Peck, K. R., Sup Oh, W., Lee, N. Y., Hiramatsu, K. and Song. J. H., 2005. Genetic differentiation of methicillin-resistant *Staphylococcus aureus* strains from Korea and Japan. Microbial Drug Resistance,11 (3), 279–286.

Taponen, S., Salmikivi, L., Simojoki, H., Koskinen, M. T. and Pyörälä, S., 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. Journal of dairy science, 92(6), 2610–2617.

Tsonev, P., Kamburov, G. and G'l'binov, G., 1975. Interbreed and interspecies variation in the occurrence of subclinical mastitis in cows and buffaloes. Veterinarno-meditsinski nauki, 12 (9), 37–40.

Viera, A. J. and Garrett, J. M., 2005. Understanding interobserver agreement: the kappa statistic. Family Medicine, 37(5), 360–363.

Zadoks, R. N., Middleton, J. R., McDougall, S., Katholm, J. and Schukken, Y. H., 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Journal of Mammary Gland Biology and Neoplasia, 16(4), 357–372.