Species identification and antimicrobial susceptibility profile of bacteria causing subclinical mastitis in buffalo[1]

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ABSTRACT.- Vásquez-García A., Silva T.S., Almeida-Queiroz S.R., Godoy S.H.S., Fernandes A.M., Sousa R.L.M. & Franzolin R. 2017. **Species identification and antimicrobial profile of bacteria causing subclinical mastitis in buffalo.** *Pesquisa Veterinária Brasileira 37(5):447-452*. Departamento de Zootecnia, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Av. Duque de Caxias Norte 225, Pirassununga, SP 13635-900, Brazil. E-mail: rfranzol@usp.br

Microorganisms causing subclinical mastitis in water buffalo were isolated from 20 buffalo milk samples at four dairy farms located in central region of São Paulo State, Brazil, through testing of somatic cell count (SCC), standard plate count (SPC), biochemical, PCR assays and antimicrobial profile. The SCC showed average of 721,000 cells/mL in the milk, indicating the presence of subclinical mastitis. The overall average for SPC was 1.8 x 10^4 CFU/mL. The microorganism most frequently isolation according to biochemical tests were: *Staphylococcus epidermidis* (17%), *Staphylococcus aureus* (15%), *Bacillus* spp. (14%), *Acinetobacter* spp. (12.5%); with intermediate frequency: *Pseudomonas aeruginosa* (9.5%); *Shigella flexneri* (7.0%), *Streptococcus* spp. (5.5%), *Corynebacterium spp*. (5.0%), *Escherichia coli* (4.5%), *Serratia marcescens* (4.0%), *Stenotrophomonas maltophilia* (4.0%), and low incidence: *Klebsiella rhinoscleromatis* (0.5%), *Klebsiella ozaenae* (0.5%), *Tatumella ptyseos* (0.5%), *Enterobacter cloacae* (0.5%). The molecular analysis indicated that samples positive by culture method of the genera *Staphylococcus*, *Streptococcus* and *E. coli* were positive by PCR. Para *S. aureus* and *S. epidermidis* the highest percentages of observed sensitivity were gentamicin (100%) and vancomycin (100%); for the genus *Streptococcus* to gentamicin and oxacillin and *E. coli* to Ampicilin. These findings may help in the control and treatment of subclinical mastitis in buffaloes and contribute to improving the efficiency and quality of the milk produced.

INDEX TERMS: Antimicrobial profile, mastitis, buffalo, antibiotics, bacteria, somatic cell count, milk.

RESUMO.- [**Identificação de espécies e perfil de susceptibilidade antimicrobiano de bactérias causadoras de mastite subclínica em búfalos.**] Microrganismos causadores de mastites subclínicas em búfalas foram isolados desde 20 amostras de leite de búfalos de quatro granjas leiteiras localizadas na região central do Estado de São Paulo, Brasil, através dos testes contagem de células somáticas (CCS), contagem padrão em placas (CPP), provas bioquímicas, reações de PCR e perfil antimicrobiano. A CCS apresentou uma mediana de 721.000 cel/mL no leite, indicando presença de mastite subclínica. A média geral de CPP foi de 1,8x10^4 UFC/mL. Os microrganismos com maior frequência de isolamento segundo os testes bioquímicos foram: *Staphylococcus epidermidis* (17%), *Staphylococcus aureus* (15%), *Bacillus* spp. (14%), *Acinetobacter* spp. (12,5%); frequência intermediaria: *Pseudomonas aeruginosa* (9,5%); *Shigella flexneri* (7,0%), *Streptococcus* spp. (5,5%), *Corynebacterium* spp. (5,0%), *Escherichia coli* (4,5%), *Serratia*

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marcescens (4,0%), *Stenotrophomonas maltophilia* (4,0%), e baixa incidência: *Klebsiella rhinoscleromatis* (0,5%), *Klebsiella ozaenae* (0,5%), *Tatumella ptyseos* (0,5%), *Enterobacter cloacae* (0,5%). A análise molecular indicou que as amostras positivas pelo método de cultura dos gêneros *Staphylococcus, Streptococcus* e *Escherichia coli* foram positivas por PCR. Para *S. aureus* e *S. epidermidis* os maiores percentuais de sensibilidade observados foram gentamicina (100%) e vancomicina (100%); para o gênero *Streptococcus* à gentamicina e oxacilina e para *E. coli* à ampicilina. Este resultados podem ajudar no controle e tratamento da mastite subclínica em búfalos e contribuir para melhorar a eficiência e qualidade do leite produzido.

TERMOS PARA INDEXAÇÃO: Susceptibilidade antimicrobiano, bactérias, mastite subclínica, búfalos, antibióticos, contagem de células somáticas, leite.

INTRODUCTION

The buffalo milk production is a highly important activity in many countries, among which are highlighted Asian countries, Italy and Brazil (Bernardes 2007). In Brazil, the buffalo has been exploited for milk and meat, but the main economic activity is the dairy industry, especially the mozzarella cheese produced exclusively with buffalo milk (Andrighetto et al. 2005, Bernardes 2007, Araujo et al. 2012). Buffalo milk has a great potential for commercial production due mainly contain particular physicochemical characteristics with high total solids, fat and protein (Amaral et al. 2005).

The importance of identifying the aspects related to the health of the mammary gland and milk products of buffaloes has been highlighted in the world literature as in Brazil (Medeiros et al. 2011), India (Tiwari et al. 2011), Pakistan (Hussain et al. 2013), Nepal (Dhakal et al. 2007), Italy (Fagiolo & Lai 2007) and Germany (Braun & Preuss 2007). Mastitis is an inflammation of the mammary gland parenchyma due to an infectious process predominantly caused by many microorganisms, particularly bacteria, and may also be involved fungi and yeast (Baloch et al. 2011). The effective and accurate diagnosis is extremely important to control this severe disease in buffalo (Viana et al. 2010). However, the absence of macroscopic changes in the tissues or secretions in cases of subclinical mastitis, does not allow the identification of infected mammary quarters before milking, once routine diagnostic methods include only physical examination, and fluid secretion (Bonini Pardo et al. 2007). Several factors have been identified as predisposing to subclinical mastitis in buffalo, as level of milk production, body weight, calving period, udder type and hygiene conditions for milking (Hussain et al. 2013).

Along the lack of information on the buffalo species, the same animal management techniques for cattle milk production are used for the control of mastitis in buffalo milk production, resulting in lack of success since they have peculiar habits of each ruminant species (Carvalho et al. 2007, Medeiros et al. 2011). Indeed, most of mastitis prevalence was found in cow's milk (32%) than in buffalo milk (22%) (Khan et al. 2013). Thus, subclinical mastitis unfortunately has not been diagnosed with frequency and consequently its etiology has not been widely investigated (Fagiolo & Lai 2007). In addition, Brazilian literature has presented a considerable number of publications about the buffalo mastitis, but when compared to the number of papers on bovine mastitis is small. A fact that needs more research efforts because buffalo has attracted growing interest of breeders and research institutions as an alternative for dairy farming (Langoni et al. 2001, Jorge et al. 2005, Medeiros et al. 2011).

The objective of this work was the isolation and phenotypic characterization of the main microorganisms that cause mastitis subclinical in buffaloes (*Bubalus bubalis*) raised in four dairy farms located in central region of Sao Paulo state, Brazil, as well as the molecular characterization and evaluation of bacterial sensitivity profile for the isolated species.

MATERIALS AND METHODS

This study was carried out at four commercial buffalo farms in the central region of São Paulo State, Brazil, using animals reared in a loose housing system with supplementation of concentrated ration according to the stage of lactation. Water and mineral supplementation were available *ad libitum*. The procedures involved in this experiment were approved by Comissão de Ética no Uso de Animais (CEUA/FZEA) (Protocol #13.1.2338.74.7). In order to select buffaloes more prone to subclinical mastitis, sanitary control data carried out by the property were evaluated and Somatic Cell Count test (SCC) of all of the female buffaloes in the herd at previous month were taken in account. Thus, milk samples were selected from twenty animals that showed SCC values above to 200,000 cells/mL (Dhakal 2006). Selected animals were dairy buffaloes of Murrah breed, in the second or third stage of lactation, with average production of 7 kg of milk/animal/day, in two daily milking. After the physical examination of the mammary gland, twenty milk samples were collected by combining the four quarters of the mammary in duplicate with all necessary hygiene, properly identified and packed in insulated boxes with ice packs.

One of the samples, collected in a bottle containing Bromopol (Microtabs®) was designed to determine the SCC by flow cytometry using Somacount 500 equipment. The second sample was collected aseptically for microbiological analysis and antimicrobial susceptibility testing. For the Standard Plate Count (SPC), the milk samples were diluted in sterile peptone water solution (Himedia®, India) at 0.1% (w/v). Aliquot 0.1 mL of each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) was inoculated into Brain Heart Infusion agar (BHI - Difco, USA) and incubated at 37°C under aerobic conditions for 48 hours. Then, the read of the plates was carried out after the incubation. Ten colonies with different characteristics were picked up randomly per animal and seeded by exhaustion for the isolation of colonies in different culture media: blood agar (Acumedia®, USA), Eosin Methylene Blue Agar (Himedia®, India), Mannitol Agar (Himedia ®, India) and MacConkey Agar (Himedia®, India). The microorganism pure of the respective boards were identified based on Gram stain, morphology and macroscopic characteristics.

Biochemical tests were subsequently carried out in accordance with bacterial groups identified in previous tests. Strains of *Staphylococcus* spp. were submitted to the free coagulase tests (Plasma Coagulase EDTA, Coagu LB - Laborclin, Brazil), DNase (DNase Agar -Difco®, USA), latex agglutination particles (Laborclin, Brazil) and catalase test. The tests of glucose fermentation

(aerobiosis) and mannitol (aerobiosis and anaerobiosis) were performed according (Macfaddin 1980) and isolates classified in accordance with (Baird-Parker 1990).

Gram negative bacteria isolated were identified using Bactray Kit I, II and III® of biochemical identification (Laborclin, Brazil). The genus Bacillus was featured on nutrient agar by the formation of colonies rounded, smooth and irregular border with creamy consistency, gram stain and rod shape with catalase and oxidase test. The bacteria whose colonies had become small, round, whitish or creamy, with rough surface, measuring 1 to 2 mm in diameter, gram-positive rods, absence of hemolysis on blood agar, catalase production, were classified as *Corynebacterium* spp. Tests for *Streptococcus* genus identification included absence of catalase production, growth in Bile Agar Esculin (Himedia®, India), type of hemolysis on blood agar (α, β and γ hemolytic) with 5% defibrinated blood sheep and tolerance to tellurite.

The molecular characterization of isolated *Staphylococcus* spp., *Streptococcus* spp. and *E. coli* were performed according to Shome et al. (2011) with some modifications. Isolated microorganisms of cultures incubated for 18 hours at 37°C in Brain Heart Infusion agar (BHI - Difco, USA) were replicated in one mL of broth in BHI and incubated at 37°C under stirring for 24 hours. After centrifugation at 10,000 x g for 10 min, the supernatant was discarded and the pellet was solubilized in 100 uL of MilliQ water, homogenized and incubated at 95°C for 10 min. Then the wells were centrifuged at 12,000 x g for 2 min at room temperature, and the supernatant was used as a substrate for PCR reactions (Fang & Hedin 2003). The concentration of genomic DNA was determined using genequant pro RNA/DNA calculator, GE Healthcare, EUA and stored at 20°C until use. Six pairs of *primers* were selected for amplification of genomic fragments of bacterial strains belonging to the genera *Staphylococcus* (*23S rRNA, sodA, rdr* and *gap* genes), three pairs of *primers* to the genera *Streptococcus* (*16S rRNA* and *cpn60* genes), and one pair of *primers* to the *E. coli* (*phoA* gene) (Shome et al. 2011) (Table 1).

For the PCR reactions were used GoTaq® Green Master Mix kit (Promega Corporation, USA) according to the manufacturer's recommendations. Briefly, the PCR reaction has consisted of a solution containing around 200ng of DNA; 12.5μL of GoTaq® Colorless Master Mix 2X; 0.4μM of each specific sense primer; 0.4μM of each specific antisense primer and 9.5μL of nuclease-free water (GE Healthcare, USA) totaling 25.0μL. The thermocycling protocol (Swift® MaxPro Thermal Cycler, Esco Technologies Inc., USA) was: initial denaturation at 94°C for 5 min, and 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec and final extension at 72°C for 10 min (Shome et al. 2011). The resulting amplicons were subjected to electrophoresis in agarose gel 2% in Tris-acetate/EDTA buffer (TAE 1X) in 8μL volume per sample, adding a 2μL of a solution containing 10mM Tris- HCl (pH 7.5), 50mM EDTA (pH 8.0), 0.03% (w/v) bromophenol blue, 0.03% xylene cyanol FF and 15% Ficoll® 400 (Blue / Orange Loading Dye, 6X, Promega, USA).

Subsequently, the gel was subjected to staining solution of SYBR® Gold nucleic acid gel stain (Life Technologies, USA) and observed under UV light, using a photo documentation system L-Pix ST and L-PixImage software (Loccus Biotechnology, Brazil). The size of the fragments was determined by comparison of the pattern of electrophoretic migration of a molecular weight marker 100pb (GE Healthcare, USA). The standard strains *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 43895 were used as controls of the reactions.

The antimicrobial profile was determined using eight isolates of *S. aureus, S. epidermidis*, *S. agalactiae* and *E. coli* previously identified in biochemical and molecular tests. After growing in BHI incubated at 37 °C for 24 hours, the bacterial cultures were plated on Mueller Hinton Agar (Himedia®, India) for carrying out the antibiograms, through the simple method disk, according to the technique described by Bauer (1966). The following antibiotics and dosages for *Staphylococcus* and *Streptococcus* genera were used: cefepime (30μg) clindamycin (2μg), erythromycin (15μg), gentamicin (10μg), oxacillin (1μg), penicillin G (10μg), rifampicin (34μg), sulphazotrim (25 μg), tetracycline (30μg) and vancomycin (30μg) and for *E. coli*: ampicillin (10μg), amoxicillin + clavulanate (30μg), ceftazidime (30μg), cefepime (30μg), cefoxitin (30μg), cefuroxime (30μg), gentamicin (10μg), meropenem (10μg), cephalothin (30μg) and Trimethoprim + sulphazotrim (25μg). The plates were incubated for 24 hours at 37°C. After reading the halos formed around the discs, we determined the sensitivity profile and resistance of isolated according to the manual for antibiogram diffusion in Kirby-Bauer disk (Laborclin, Brazil).

Table 1. Sequence of primers used in the confirmation of the most frequent microorganisms in the isolation of subclinical mastitis in buffaloes

Microorganism	Primer	Gene	Orientation	Sequence of primer 5´-3´	Product (pb)
Staphylococcus aureus	SAS2F	*23S*	sense	AGCGAGTCTGAATAGGGCGTTT	894
	SAS2R	*rRNA*	antisense	CCCATCACAGCTCAGCCTTAAC	
Staphylococcus chromogenes	SCHS1F	*sodA*	sense	GCGTACCAGAAGATAAACAAACTC	222
	SCHS1R		antisense	CATTATTTACAACGAGCCATGC	
Staphylococcus haemolyticus	SHS1F	*sodA*	sense	CAAATTAAATTCTGCAGTTGAGG	214
	SHS1R		antisense	AGAGCCCATTGTTCTTTGA	
Staphylococcus epidermidis	SERF	*rdr*	sense	AAGAGCGTGGAGAAAAGTATCAAG	130
	SERR		antisense	TCGATACCATCAAAAAGTTGG	
Staphylococcus sciuri	SSCGF	*gap*	sense	GATTCCGCGTAAACGGTAGAG	306
	SSCGR		antisense	CATCATTTAATACTTTAGCCATTG	
Staphylococcus simulans	SSMF	*gap*	sense	AGCTTCGTTTACTTCTTCGATTGT	472
	SMR		antisense	AAAAGCACACAAGCTCACATTGAC	
Streptococcus agalactiae	STAGF	*16S*	sense	GCTAATACCGCATAAGAGTAATTAAC	317
	STAGR	*rRNA*	antisense	GGTAGATTTTCCACTCCTACCAA	
Streptococcus dysgalactiae	STDGF	*16S*	sense	GGGAGTGGAAAATCCACCAT	572
	STAGR	*rRNA*	antisense	AAGGGAAAGCCTATCTCTAGACC	
Streptococcus uberis	STUBF	*cpn60*	sense	TCGCGGTATTGAAAAAGCAACAT	400
	STUBR		antisense	TGCAATAATGAGAAGGGGACGAC	
Escherichia coli	ECPF	*phoA*	sense	GGTAACGTTTCTACCGCAGAGTTG	468
	ECPR		antisense	CAGGGTTGGTACACTGTCATTACG	

RESULTS

The average Somatic cell count was 721,000 cells/mL of milk (minimum: 205,000, maximum: 2.264 million), indicating the presence of subclinical mastitis. All positive -samples by culture method were also positive by PCR that confirmed the identity of the *Staphylococcus aureus, S. epidermidis* and *E. coli* species with amplimers electrophoretic pattern compatible with the described species. Two isolates have showed an amplification product (500 bp) specific to *Streptococcus dysgalactiae* and eight isolates have showed an amplification product of 300pb in PCR for *Streptococcus. agalactiae*.

All twenty samples showed bacterial growth in the BHI agar. The overall average of standard plate count obtained in this study was $1.8x10^4$ CFU/mL. Two hundred isolates recovered from milk samples culture were submitted to phenotypic and biochemical characterization (Table 2). *S. epidermidis* (17%) was the most frequently isolated organism, followed by *S. aureus* (15%). As further relates to Gram-positive pathogens, there was high isolation *Bacillus* spp. in buffalo milk (14%). However, bacteria of the genus *Streptococcus* spp. had a lower frequency of isolation (5.5%) as well as the gram negative bacteria *E. coli* (4.5%).

Table 2. Bacteria number and frequency of isolates (%) in buffalo milk samples with subclinical mastitis

Microrganism	Number Frequency (%)
Staphylococcus epidermidis	34 (17.0%)
Staphylococcus aureus	30 (15.0%)
Bacillus spp.	28 (14.0%)
Acinetobacter spp.	25 (12.5%)
Pseudomonas aeruginosa	19 (9.5%)
Shigella flexneri	14 (7.0%)
Streptococcus spp.	11 (5.5%)
Corynebacterium spp.	10 (5.0%)
Escherichia coli	9 (4.5%)
Serratia marcescens	8 (4.0%)
Stenotrophomonas maltophilia	8 (4.0%)
Klebsiella rhinoscleromatis	1 (0.5%)
Klebsiella ozaenae	1 (0.5%)
Tatumella ptyseos	1 (0.5%)
Enterobacter cloacae	1(0.5%)
Isolation total	200

Table 3. *In vitro* susceptibility profile of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus* spp. isolated from buffalo milk samples with subclinical mastitis

Antimicrobian	Sensibility profile					
	Staphylococcus aureus		*Staphylococcus epidermidis*		*Streptococcus* spp.	
	N	%	N	%	N	%
Gentamicin (10µg)	8/8	100.0	8/8	100.0	8/8	100.0
Vancomycin (30µg)	8/8	100.0	8/8	100.0	2/8	25.0
Clindamycin (2µg)	4/8	50.0	4/8	50.0	2/8	25.0
Erythromycin (15µg)	4/8	50.0	4/8	50.0	2/8	25.0
Penicillin G (10µg)	2/8	25.0	3/8	37.5	1/8	12.5
Sulphazotrim (25 µg)	4/8	50.0	4/8	50.0	6/8	75.0
Oxacillin (1µg)	4/8	50.0	4/8	50.0	8/8	100.0
Cefepime (30µg)	4/8	50.0	4/8	50.0	2/8	25.0
Tetracycline (30µg)	2/8	25.0	3/8	37.5	-	-
Rifampicin (34µg)	4/8	50.0	4/8	50.0	2/8	25.0

N = evaluated number of microorganisms.

Table 4. In vitro susceptibility profile of *Escherichia coli* isolated from buffalo milk samples with subclinical mastitis

Antimicrobian	Sensibility profile of *Escherichia coli*	
	N	%
Cephalothin (30µg)	6/8	75.0
Ampicillin (10µg)	8/8	100.0
Meropenem (10µg)	6/8	75.0
Trimethoprim-sulfamethoxazole (25 µg)	6/8	75.0
Cefuroxime (30µg)	6/8	75.0
Amoxicillin + clavulanate (30µg)	5/8	62.5
Cefoxitin (30µg)	6/8	75.0
Cefepime (30µg)	6/8	75.0
Ceftazidime (30µg)	6/8	75.0
Gentamicin (10µg)	4/8	50.0

N = evaluated number of microorganisms.

The study of bacterial sensitivity (Table 3) has showed that *S. aureus* and *S. epidermidis* bacteria were more sensitive to antibiotics gentamicin (100%) and vancomycin (100%). For the genus *Streptococcus*, gentamicin and oxacillin were the best action antibiotics, followed by sulphazotrim. In contrast, gentamicin was a less effective antimicrobial (50%) for *Escherichia coli* isolates (Table 4).

DISCUSSION

The higher average obtained from the SCC (721,000 cells/mL) was expected, since the sampling was restricted to buffaloes that had high SCC in order to obtain samples with microorganisms that may cause subclinical mastitis. It was high compared to average values of 63,000 cells/mL observed in 2693 buffalo milk samples belonging to a single herd from state of Sao Paulo (Cerón-Muñoz et al. 2002) and 63,380 cells/mL mean value identified in lactating buffaloes also in Brazil (Jorge et al. 2005). Subclinical mastitis in buffalo milk samples was previously described with SCC greater than 200,000 cells/mL and positive bacterial growth in culture in 21.7% (52/200) of the evaluated animals (Dhakal 2006). High SCC has been obtained in crossbred Murrah buffaloes, with values of 171,000 cells/mL for animals without mastitis, 799,000 cells/mL for animals with subclinical mastitis and 6,039,000 cells/mL with clinical mastitis (Dhakal et al. 2008).

The most often agents isolated in the samples *Staphylococcus epidermidis* (17%) and *S. aureus* (15%) (Table 2) were similar to those obtained in 49 adult Murrah buffalo at different stages of lactation in herds from state of Pernambuco, Brazil (Oliveira et al. 2004). In that study, the *Staphylococcus* genus was considered the main etiological agent, being of great epidemiological importance in buffalo mastitis. Indeed, the genus *Staphylococcus* has been predominantly isolated in samples from buffalo Murrah crossbred with subclinical mastitis (Dhakal et al. 2008) and clinical mastitis (Pizauro et al. 2014). Khan and Muhammad (2005) obtained similar prevalence for microbiological analysis in milk samples of buffalos from Pakistan: *Staphylococcus* spp. (45%), *Streptococcus* spp. (23%) and *Bacillus* spp. (14%). However, Khan et al. (2013) observed a greater prevalence of *S. aureus* in cow milk samples (90%) than in buffalo milk (84%). Langoni et al. (2001) observed

prevalence of *S. epidermidis* (30.1%) and *S. aureus* (4.8%) in buffaloes with mastitis in São Paulo, Brazil. Other studies have also identified a high prevalence of *S. epidermidis* (Tenhagen et al. 2009, Sampimon et al. 2009, Pankaj et al. 2013), indicating the importance of this group of microorganisms which are commonly described as lower prevalence of pathogens in the case of mastitis. In addition, *S. epidermidis* is a component of the normal flora of the skin of the udder and its prevalence, as observed in this study may be a result of poor hygienic practices for milking.

Acinetobacter was isolated with a frequency also considered high of 12.5% in this study. Similar results were found in cows with clinical mastitis and milk samples with mastitis in Korea (Nam et al. 2009, 2010, Gurung et al. 2013). The association of this bacterium with subclinical mastitis in buffaloes presents a new challenge for the treatment and control of disease. There is need for further studies to evaluate its role as a pathogen potential and identify possible sources of contamination.

The percentage of *Pseudomonas aeruginosa* isolated, can be related to the occurrence of the contamination in milk during and/or after milking, such as contaminated water used for washing the teat, cleaning and during intramammary therapy (Fernandes et al. 2009, Langoni et al. 2009).

The incidence of *Bacillus* spp. observed (14%) is found within a wide occurrence in buffalo milk samples ranging from 2.4 to 32.4% (Langoni et al. 2001, Oliveira et al. 2004, Dhakal et al. 2008). This level of detection may differ according to the type of sample of milk, herd and region. This kind of bacteria is widely distributed in soil, water, air, feces and vegetation.

Although *Streptococcus* are considered less frequent agents of mastitis, as also obtained in this study, these opportunistic pathogens are widespread in the environment and can be found on the ground, water and manure. However, some studies have identified the *Streptococcus* spp. as the major etiologic agent isolated from buffalo subclinical mastitis (Fagiolo & Lai 2007).

Low frequency of *E. coli* was observed in this study (4.5%) being in agreement with the data obtained by Saini et al. (1994) that observed lower frequency of microorganisms *E. coli* (12.9%) in buffalo milk samples from Punjab, India. Moreover, Kumar (2009) observed buffalo milk samples with high incidence of mastitis *E. coli* (30%).

Other species of bacteria as *Lactococcus garvieae* and *Enterococcus gallinarum* have been isolated from milk samples of buffalo subclinical mastitis (Vianni & Lazaro 2003). *Arcobacter* species also has considered an important source of bacteria in cow and buffalo milk with risk to public health (Yesilmen et al. 2014).

Conventional bacterial culture is relatively slow performance, since incubation of primary cultures often requires 48 hours (or 72 hours) to be completed, and additional confirmatory tests are relatively time-consuming. The PCR assay for the identification of microorganisms in milk samples with mastitis require an analysis time of 3 to 4 hours. In the case of subclinical mastitis such quick results may allow the identification of animals with this disease and indicate the treatment, while optimizing the results with appropriate use of antibiotics and reduce the indiscriminate usage. Moreover, PCR has been shown to be more sensitive and specific for the diagnosis of microorganisms in subclinical mastitis than conventional culturing technique (Shahzad et al. 2013).

Gentamicin was the antibiotic of choice for *Staphylococcus* and *Streptococcus* bacteria (Table 3) partially agreeing with the findings of Cunha et al. (2006) who observed a wide gentamicin action with high antimicrobial efficacy (97.98%) on various bacteria and association including *Staphylococcus* spp., *Streptococcus* spp. and *E. coli*. However, gentamicin was one of the less effective antimicrobial (50%) for *E. coli* isolates (Table 4). But, this finding agrees with the results of Costa (2008) who observed that aminoglycosides and sulfonamides were the antimicrobials less effective for *E. coli*, highlighting the sensitivity percentage of 46.2% and 41.8% for gentamicin and neomycin, respectively.

For the genus *Streptococcus*, gentamicin and oxacillin were the best action antibiotics, followed by sulphazotrim (Table 3), confirming the data of Langoni et al. (2001) who have demonstrated a better efficiency of gentamicin (96%) and oxacillin (95%) on the isolated agents with more frequency: *Corynebacterium bovis, Staphylococcus epidermidis* and *Streptococcus agalactiae*.

The somatic cell count and standard plate count can perform an assessment of the health status of the buffalo mammary gland with subclinical mastitis and emphasize the importance of a normative specific for buffalo that allows the control and inspection of milk, since the parameters used for cattle may not be suitable for monitoring mastitis in buffalo herds.

CONCLUSION

This study highlights the importance of some pathogens involved subclinical mastitis in buffaloes and the possibility of specific antimicrobial use in the control and treatment of this serious problem, promoting increased quality and milk production with effective reduction of production costs and improvement in health foods.

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