Revista Brasileira de Agrotecnologia
V. 11, Nº 2, p. 646-651, ANO 2021
Garanhuns, PE, Grupo Verde de Agroecologia e Abelhas
https://www.gvaa.com.br/revista/index.php/REBAGRO/index
DOI: 10.18378/REBAGRO.V12I2.8945

I CONGRESSO BRASILEIRO ONLINE DE CIÊNCIAS DOS ALIMENTOS

MILKING ANTISEPTICS AGAINST BACTERIA MULTIDRUG-RESISTANT BOVINE MASTITIS

Anti-sépticos de ordenha contra bacterias multirresistente de mastite bovina

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ABSTRACT: This work aims to verify the in vitro action of antiseptics used in the pre- and post-dipping against multidrug-resistant bacteria from bovine mastitis. Antiseptic solutions in the concentration of use of iodine, chlorhexidine and sodium hypochlorite were tested against *Staphylococcus aureus* (n=12), *Staphylococcus sciuri* (n=1), *Staphylococcus lentus* (n=1), *Streptococcus* sp. (n=1), *Enterococcus faecalis* (n=1), *Enterococcus casseliflavus* (n=1), *Kocuria kristinae* (n=2), *Kocuria varians* (n=1). Was verified the Minimal Inhibitory and Bactericidal Concentration. The cytotoxicity test complemented the work. It was found that the Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of the iodine, sodium hypochlorite and chlorhexidine was effective in the values recommended for use, however the presence of organic material reduced the activity of the tested products. Chlorhexidine showed higher efficiency of the other disinfectants.

Key words: Iodine. Sodium hypochlorite. Chlorhexidine. Broth microdilution. Milk.

RESUMO: Este trabalho tem como objetivo verificar a ação in vitro de antissépticos utilizados no pré e pós-dipping contra bactérias multirresistentes da mastite bovina. Soluções antissépticas na concentração de uso de iodo, clorexidina e hipoclorito de sódio foram testadas contra *Staphylococcus aureus* (n = 12), *Staphylococcus sciuri* (n = 1), *Staphylococcus lentus* (n = 1), *Streptococcus* sp. (n = 1), *Enterococcus faecalis* (n = 1), *Enterococcus casseliflavus* (n = 1), *Kocuria kristinae* (n = 2), *Kocuria varians* (n = 1). Foi verificada a Concentração Inibitória Mínima e Bactericida. O teste de citotoxicidade complementou o trabalho. Verificou-se que a Concentração Inibitória Mínima e a Concentração Bactericida Mínima do iodo, hipoclorito de sódio e clorexidina foram eficazes nos valores recomendados para uso, porém a presença de material orgânico reduziu a atividade dos produtos testados. A clorexidina apresentou maior eficiência dos outros desinfetantes.

Palavras-chave: Iodo. Hipoclorito de sódio. Clorexidina. Microdiluição em caldo. Leite.

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Received for publication in 20/04/2021, approved in 05/06/2021.
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INTRODUCTION

Mastitis is the most frequent and one of the main diseases of dairy cows, it commits to viability of the milk chain, affecting milk producer, industry, and final consumer (RUEGG, 2012). It is characterized by an inflammation of the mammary gland related to physical, chemical, thermal, or microbial aggressions, with 90% of mastitis caused by bacteria (LIMA et al., 2018).

Today, the rapid emergence of multi-resistant bacteria is occurring worldwide (CASTELANI et al., 2019; SOUZA et al., 2019; MONISTERO et al., 2020). This affects the problem with the treatment of the animal and the dissemination of the bacteria to human. To avoid these problems, an alternative is the decontamination of the teats and consequently of milk.

Milk production with quality and quantity is indispensable for health. Washing teats dipping teat (before and after milking) remains a widespread management practice in the dairy farm to prevent and control mastitis pathogens at the time of milking (RUEGG, 2012; MORRIL et al., 2019; SKOWRON et al., 2019). Soaking the teats before milking is essential to disinfect the teat skin before milking to reduce the risk that the open end of the teat will be exposed to environmental pathogens during milking. Soaking the teats after milking is useful to disinfect the teats from contagious pathogens to which the cow was exposed during milking and to protect the teat end from environmental pathogens before the teat end closes after the cow leaves (PANKEY et al., 1984; TITZE and KROMBER 2020). Disinfecting the skin of the teat after milking helps to reduce the spread of mastitis pathogens, killing them before they can settle in the mammary gland (KUMAR, BHARATHI and PORTEEN, 2012; ROWE et al., 2018), additionally, they must leave teat skin in good condition (HEMLING, 2002). Miseikiene et al (2020) indicate that application with pre- and post-milking teat disinfectants provided an impact also on the reduction of SCC in milk.

The most studied and used active disinfectant substances for teats disinfection are iodine, chlorhexidine, chlorine, sulfonic acid, peroxide, chloride acid, lauric dine (MEDEIROS et al., 2009). The big question is whether disinfectants or antiseptics of routine use in dairy production can eliminate these resistant microorganisms.

Considering the importance of the correct choice and applicability of the antiseptic product for use in the disinfection of teats, the present work was carried to evaluate three disinfectants utilized often in Brazil in pre- and post-dipping: sodium hypochlorite, iodine, and chlorhexidine.

MATERIAL AND METHODS

For this, 20 bacterial isolates of subclinical bovine mastitis, previously identified by the assimilation of carbohydrates by the Vitek² System and stored in the Laboratory of Inspection of Animal Products of the Veterinary School of UFPEL were tested. They were selected for their resistance to seven or more antibiotics in the antibiogram test.

Bacteria was tested with antiseptic solutions (concentration of use) of iodine, chlorhexidine, and sodium hypochlorite through Minimal Inhibitory and Bactericidal Concentration (CLSI-M7-A6 methodology adapted). The concentrations were 1 to 0.0019% for iodine and chlorhexidine and 1.25 to 0.0024% for sodium hypochlorite. Whit CIM50 and CIM90 calculation, isolates were classified as Sensitive (S), Intermediate (I) and Resistant (R) and analysed by the chi square frequency test.

Isolates were reactivated in BHI (Brain-Heart Infusion), seeded in BHA (Brain-Heart Agar), and after 20 h the inoculum was prepared by diluting it in saline (NaCl 0.9%) in a concentration between 10^{7-8} CFU / mL. Each disinfectant was disposed in dilutions 1:2 in the wells of the same line with ten concentrations in the presence of the inoculum. The reading was performed after 18-20 h of incubation at 35°C. Assays were performed in duplicate with three repetitions.

To Kinetics or Efficiency Disinfectant Test, five bacterial were selected: *Staphylococcus aureus* (3), *Enterococcus faecalis* (1) and *Kocuria kristinae* (1). The reactivated isolates were diluted in saline (NaCl 0.9%) to 10^{-8} CFU/mL. 100 µL aliquot was taken and diluted in a tube containing 0.9 mL of Muller Hilton (MH). The disinfectant was prepared in two test tubes, containing 2 mL of disinfectant test, for each bacterium: one with 1 mL of milk (simulating organic material) and no other 1 mL saline solution.

50 µL of the inoculum were used, stirred and 100 Ll were removed every 30 and 60 s, 5 and 15 min and added in 1.5 mL of chemical disinfectant neutralizers. 50 µL of these tubes were seeded in three Petri dishes containing BHI. The plates were incubated for 24 h (36°C) and the colonies were counted. Negative control was performed with culture medium and disinfectants, and positive control with bacterial culture of all test solutions. The statistical analysis was descriptive, calculating the absolute and relative frequencies.

The cellular toxicity of antiseptics in MDBK Cells (Madin-Darby bovine kidney) was verified in microplates of 96 wells in an oven at 37ºC with 5% CO_2. After cell confluence, 100 µl of serial dilutions of disinfectants were added to each plate. The dye was solubilized by the addition of 100 µL of a solution containing 50% ethanol, acetic acid 1% and distilled water qsp. The optical densities were read in a spectrophotometer at a wavelength of 540 nm.

RESULTS AND DISCUSSION

The Table 1 present the 20 bacterial isolates of subclinical bovine mastitis, previously identified by the assimilation of carbohydrates, and selected for their resistance to seven or more antibiotics in the antibiogram test.

MIC, MBC, and susceptibility of each isolate to each antiseptic are shown in Table 2. Chlorhexidine was the antiseptic with the highest number of multidrug-resistant bacteria sensitive (65%), followed by sodium hypochlorite (50%) and iodine (45%). The three antiseptics have no significant difference with intermediate resistance: chlorhexidine 20%, sodium hypochlorite in 30% and iodine 35% of the isolates. The

antiseptic with the most resistant isolates was iodine and hypochlorite (15% each) and chlorhexidine (10%).

Table 1 - Bacterial isolates from bovine mastitis subclinical stored in the Laboratory of Inspection of Animal Products of the Veterinary School of UFPEL and their susceptibility to antibiotics

Bacteria	N^0 R	AMP	AMO	BAC	CFE	CTF	ENO	GEN	NEO	NOR	PEN	TET	TRI
1	7	R	S	S	R	R	S	R	R	R	S	R	R
1	7	R	S	S	R	R	S	R	R	R	S	R	R
1	8	R	S	S	R	R	R	R	R	R	S	R	R
1	9	R	R	R	R	R	R	S	R	S	R	R	R
1	10	R	S	R	R	R	R	R	R	R	R	R	R
1	11	R	R	R	R	R	R	R	R	R	R	R	R
1	9	R	R	R	R	S	S	R	R	R	R	R	R
1	9	R	S	R	R	R	S	R	R	R	R	R	R
1	10	R	R	R	R	R	S	R	R	R	R	R	R
1	11	S	S	R	R	R	R	R	R	R	S	S	R
1	7	R	R	S	R	R	S	R	R	S	R	R	R
1	9	R	R	S	R	S	R	R	R	R	R	R	R
2	7	R	R	S	S	S	S	R	R	S	R	R	R
3	12	R	R	R	R	R	R	R	R	R	R	R	R
4	11	R	R	R	R	R	NG	R	R	R	R	R	R
5	10	R	S	R	R	R	R	R	R	R	R	R	R
6	11	R	R	R	R	R	NG	R	R	R	R	R	R
7	7	R	S	S	R	R	S	R	R	R	S	R	R
7	10	R	R	R	R	R	R	S	R	S	R	R	R
8	12	R	R	R	R	R	R	R	R	R	R	R	R

1- *Staphylococcus aureus* (n=12); 2- *Staphylococcus* sciuri (n=1); 3- *Staphylococcus lentus* (n=1); 4- *Streptococcus* sp. (n=1); 5- *Enterococcus faecalis* (n=1); 6- *Enterococcus casseliflavus* (n=1); 7- *Kocuria* kristinae (n=2); 8- *Kocuria varians* (n=1);

AMP – Ampicillin; AMO – Amoxicillin; BAC – Bacitracin; CFE – Cephalexin; CTF – Ceftiofur; ENO – Enrofloxacin; GEN – Gentamicin; NEO – Neomicin; NOR – Norfloxacin; PEN – Penicillin ; TET – Tetracycline ; TRI- Trimethoprim;

R- Resistant; S- Sensibility; NG – No Growing.

Table 2 - Minimal Inhibitory Concentration (MIC) and susceptibility profile from three antiseptics used in pre- and post-dipping against 20 bacterial isolates from subclinical bovine mastitis

Bacteria	**Hypochlorite**		**Iodine**		**Chlorhexidine**	
	MIC/MBC	**SP**	**MIC/MBC**	**SP**	**MIC/MBC**	**SP**
Staphylococcus aureus	0,156	S	0,125	S	0,125	I
S. aureus	0,156	I	0,031	S	0,031	S
S. aureus	0,156	I	0,5	R	0,5	R
S. aureus	0,078	S	0,25	I	0,031	S
S. aureus	0,039	S	0,125	S	0,625	S
S. aureus	0,078	S	0,625	S	0,625	S
S. aureus	0,078	S	0,25	I	0,625	S
S. aureus	0,019	S	0,25	I	0,031	S
S. aureus	0,156	I	0,5	R	0,5	R
S. aureus	0,078	S	0,25	I	0,625	S
S. aureus	0,078	S	0,625	S	0,125	I
S. sciuri	0,313	R	0,625	S	0,625	S
S. lentus	0,156	I	0,25	I	0,625	S
Enterococcus casseliflavus	0,156	I	0,125	S	0,125	I
Enterecoccus faecalis	0,078	S	0,5	R	0,125	I
Streptococcus sp.	0,078	R	0,125	S	0,031	S
Kocuria kristinae	0,313	R	0,125	S	0,031	S
Kocuria kristinae	0,039	S	0,25	I	0,031	S
Kocuria varians	0,156	I	0,25	I	0,031	S

SP – Susceptibility profile

In the kinetic test (Table 3), iodine (2%) reduced the five bacteria by 100% after 60 s of exposure to the antiseptic in saline. Only *Staphylococcus aureus* and *Enterococcus faecalis* did not decrease after 30 s of contact. In the presence of organic material (milk), the bacteria were eliminated from 50% after exposure in 5 min. Two *Staphylococcus aureus* reduced 100% after 15 min of exposure.

Sodium hypochlorite (0.625%) inhibited the five bacteria on exposure for 5 min in saline and only *Staphylococcus aureus* was inhibited on contact in 30 s. Exposure to hypochlorite in the presence of organic material (milk), only *Staphylococcus aureus*, showed a reduction in 15 min of exposure. The others at no time showed a reduction.

Chlorhexidine (2%) in saline reduced all isolates by 30 s. In the presence of organic material (milk), all isolates reduced

100% by 30 s, except one isolate of *Kokuria kristinae* and *Staphylococcus aureus* which were inhibited with 5 min of exposure.

In cytotoxicity test, sodium hypochlorite-maintained cell viability at 100% in the concentrations of use (1.25 to 0.0024%). However, chlorhexidine in the lowest concentration studied (0.0019%) maintained cell viability at 80%. Only the 15,000-fold dilution of Chlorhexidine maintained 100% cell viability.

Table 3 - kinetic test: CFU reduction (%), of five bacteria from bovine mastitis subclinical, after contact with each antiseptic (iodine, sodium hypochlorite, chlorhexidine) at different times of exposure (30”, 60”, 5’, 15’)

Antiseptic	Time	*S. aureus*		*S. aureus*		*E. faecalis*		*S. aureus*		*K. kristinae*	
		M	S	M	S	M	S	M	S	M	S
Iodine	30”	89	100	92	99	72	98	92	100	86	99
	60”	97	100	93	100	72	99	94	100	90	99
	5’	98	100	99	100	95	100	98	100	93	100
	15’	100	100	100	100	98	100	99	100	97	100
Sodium	30”	98	95	38	100	56	97	81	99	81	99
Hypochlorite	60”	99	99	46	100	58	99	75	99	87	98
	5’	95	100	47	100	54	100	73	100	93	100
	15’	100	100	34	100	55	100	66	100	96	100
	30”	96	100	72	100	100	100	82	99	79	100
Chlorexidine	60”	100	100	100	100	100	100	93	100	92	100
	5’	100	100	100	100	100	100	100	100	98	100
	15’	100	100	100	100	100	100	100	100	100	100

M – Milk; S – Saline solution; ‘= min; “= s; values % reduction.

What can we learn from these results? About each antiseptic with two methodologies, it was observed that all multi-resistant bacteria were sensitive to antiseptics in concentrations below those recommended by the manufacturer. However, in the second test, the exposure time of the microorganism with the antiseptic and the presence of organic material influenced the effectiveness of the product. In both, Chlorhexidine had better results.

Chlorhexidine was the antiseptic with the highest number of multidrug resistant bacterial with sensibility and iodine with the highest number of multidrug resistant bacterial with resistance. These results were observed in only culture medium. The concentrations were according with Fonseca & Santos (2001) recommendation: iodine 0.7-1.0%, chlorhexidine 0.5-1.0% and 4% hypochlorite of sodium. Medeiros et al., (2009); Coutinho et al., (2012); Ramalho et al., (2012), also obtained better disinfectant activity for chlorhexidine.

According to Medeiros et al. (2009), the longer the exposure time, the better the action of chlorhexidine. So, this fact was observed with Kinect test when Chlorhexidine reduced all bacterial multidrug-resistant in 30 s in saline solution. Chlorhexidine is widely used for the treatment of superficial infections of teats in cows due to its cumulative and continuous effect, remaining on the skin for at least six hours, in addition, it acts in the presence of organic material, it is easy to apply and economical, when in comparison with iodine causes less tissue reaction at the recommended dilutions (MEDEIROS et al., 2009; RAMALHO et al., 2012) and this result was observed when used milk in culture medium in Kinect test. Coutinho et al (2012), also suggests that this disinfectant should be used as one of the first options in the pre- and post-dipping.

As for sodium hypochlorite in our study, it presented 30% of resistant isolates. It is an antiseptic used in dairy farms in Brazil, due to its low cost. However, its disadvantage is its lower stability, inactivated in the presence of proteins (AMARAL et al., 2004). Sodium hypochlorite is a non-toxic compound, but it has an irritating effect on tissues and its action is reduced by the presence of organic material (COUTINHO et al., 2012). Sodium hypochlorite has a high degree of affinity for organic material such as fees, blood and pus that can neutralize its microbicidal action in the routine practice of pre- and post-dipping. In this work it only reduced 100% growing bacteria post 15 min in mil. The results show that chlorine with milk, possibly became unstable, and had its disinfectant action reduced. Furthermore, the application of disinfectant to un-cleaned teats may have impacted on the effectiveness of the disinfectant products (GLEESON et al., 2018). Concentration and contact time are factors that can interfere the action of the antiseptic, and this binomial is fundamental for the success of the disinfectant effect of chlorine-based compounds (MEDEIROS et al., 2009).

Iodine showed efficiency in Staphylococcus aureus, inhibiting its growth by 30 s in saline, but with milk only reduced after 15 min. Iodine has greater activity in vitro at 2% and 1% against pathogenic microorganisms isolated from cases of clinical bovine mastitis (PEDRINI & MARGATHO, 2003). Medeiros et al. (2009) presented the same results.

In this study, there was a significant difference in the performance of the antiseptic when used in the presence of organic material. Agreeing with Medeiros et al. (2009). The next step is to verify in the herd how these antiseptics act in vivo, because in practice Skowron et al. (2019) shows that the reduction in the percentage rate in the number of bacteria after the use of selected immersion agents remained at a level above 90%. Thus, to be used in the milking routine of cattle, these antiseptics must be used on the microorganisms of mastitis, used the correct time and without presence of organic materials, such as dirt, milk or urine, hinder the effective use of the product (PANKEY et al., 1984).

CONCLUSION

In this study, the concentrations studied and when used in animals, were not toxic in the cytotoxicity test and among them, Chlorhexidine showed greater efficiency than other disinfectants in bacteria resistant to multiple drugs, but with a harmful effect in the presence of organic material.

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