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ANTIMICROBIAL DRUG RESISTANCE OF *Staphylococcus sp* ISOLATED FROM HEALTHY LACTATING COWS OF AN ORGANIC FARM

(RESISTÊNCIA ANTIMICROBIANA DE CEPAS DE Staphylococcus sp *ISOLADAS DE GADO LACTANTE SAUDÁVEL DE UMA FAZENDA ORGÂNICA)*

(RESISTENCIA ANTIMICROBIANA DE CEPAS DE Staphylococcus sp *AISLADAS DE GANADO LACTANTE SALUDABLE DE UNA HACIENDA ORGÁNICA)*

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SUMMARY

A total of 95 *Staphylococcus sp* strains, isolated from 129 apparently healthy lactating cows from an organic farm situated in São Paulo State, Brazil, were characterized for resistance to 7 antimicrobial drugs. Resistance to penicillin was most frequently (96.8%) found, followed by resistance to streptomycin (67.3%), norfloxacin (31.5%) and vancomycin (5.2%). β-Lactamase production was found in 21.0% of the isolates. Multidrug resistant isolates were common and two patterns of phenotypes were found in 78.9% of the isolates. These results showed that cows not exposed to antimicrobial drugs for five years still presented a high level of antimicrobial resistance among the colonizing bacteria of these animals.

KEY-WORDS: *Staphylococcus sp.* Antimicrobial drug resistance. Homeopathic treatment. Multidrug resistance.

RESUMO

Um total de 95 cepas de *Staphylococcus sp,* isoladas de 129 vacas leiteiras aparentemente saudáveis em uma fazenda orgânica situada no Estado de São Paulo, foram caracterizadas em relação à resistência a 7 drogas antimicrobianas. Entre as 95 cepas obtidas, resistência à penicilina foi a mais freqüentemente encontrada (96,8%), seguida pela resistência a estreptomicina (67,3%), norfloxacina (31,5%) e vancomicina (5,2%). A produção de β-lactamase foi encontrada em 21,0% das cepas isoladas. Resistência a múltiplas drogas foi comumente encontrada e dois modelos de fenótipos foram encontrados em 78,9% das cepas. Estes resultados mostraram que mesmo em vacas que não foram expostas a antimicrobianos há 5 anos, foi possível observar uma alta incidência de resistência a antimicrobianos entre as cepas bacterianas colonizadoras destes animais.

PALAVRAS-CHAVE: *Staphylococcus sp*. Resistência antimicrobiana. Tratamento homeopático. Resistência múltipla.

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RESUMEN

Un total de 95 cepas de *Staphylococcus sp* aisladas de 129 vacas lecheras, aparentemente saludables, en una hacienda orgánica situada en el Estado de São Paulo, Brasil, fueron caracterizadas en relación a su resistencia a 7 drogas antimicrobianas. Entre las 95 cepas obtenidas, la resistencia a penicilina fue la más frecuentemente encontrada (96,8%), seguida por la resistencia a estreptomicina (67,3%), norfloxacina (31,5%) y vancomicina (5,2%). La producción de β-lactamasa fue encontrada en 21,0% de las cepas aisladas. Resistencia a múltiples drogas fue comúnmente encontrada y dos modelos de fenotipos fueron encontrados en 78,9% de las cepas. Estos resultados mostraron que aunque las vacas no fueron expuestas a antimicrobianos hace 5 años, fue posible observar alta incidencia de resistencia a antimicrobianos entre las cepas bacterianas colonizadoras de estos animales.

PALABRAS-CLAVE: *Staphylococcus sp*. Resistencia antimicrobiana. Tratamiento homeopático. Resistencia múltiple.

INTRODUCTION

Staphylococci are one of the most common causes of infections in cattle, mostly caused by coagulase positive *staphylococci*, in special *Staphylococcus aureus*; coagulase negative *staphylococci* seem also to be associated with the infections (WATTS, 1988).

Research has been extensively focused on the control of *S. aureus* mastitis in dairy cows due to the contagious nature of this organism and its poor response to antibiotic therapy (BRAMLEY e DODD, 1984, SCHOCKEN-ITURRINO et al, 1996, CARDOSO et al, 2000). Coagulase-negative staphylococci (CNS) are often the most prevalent organism isolated from herds when mastitis control procedures are used (BIRGERSSON et al, 1992; FTHENAKIS et al, 1994).

Extensive and often indiscriminate use of antimicrobial agents in medicine and animal husbandry has a selective effect on the emergence and maintenance of drug resistance to bacteria (LYON e SKURRAY, 1987). A survey among US veterinarians reported that antibiotics were the drugs most often used to treat lactating dairy cows; the most common reason for treatment of mastitis (MITCHELL et al, 1998).

There exists an increasing concern about the problem of antimicrobial resistance and variability in antibiotic susceptibility to bacteria isolated from cows (BEZEK, 1998). Complementary and alternative therapies, including homeopathy, have at present a definitive place in veterinary medicine. Supporters or “believers” in homeopathy claim that the method involves treatment of the individual or patient, as a whole using a so-called holistic concept (LOKEN, 2001). The basic principle of homeopathy is the “law of similar”, which prescribes the treatment of a disease with very small quantities of preparations which cause effects similar to those of the conditions itself. The principles and practice of this treatment of cattle were described by MacLeod (1981). A number of oral vaccines described as nosodes are available, they are based on extracts of the etiological organism or of toxins that they produce. The extracts are subjected to serial dilutions and impact shaking termed “succession”, and are usually in alcoholic solution. They are in general preventative medicines, used in a manner similar to conventional vaccines.

In organic farming, there exists the desire to avoid the use of chemicals, as a part of a general striving towards naturalness (*ie*, none or a severely limited use of antimicrobials).

The purpose of the present work was to characterize strains of *Staphylococcus sp* isolated from healthy homeopathic-treated cattle investigating their resistance to antimicrobial drugs.

MATERIAL AND METHODS

Animals: The animals selected for this study were 129 apparently healthy, lactating cows, from a farm situated in Monte Alto, São Paulo, that had not been exposed to antimicrobial drugs for at least five years. A mastitis control program had been set up for homeopathic preventive treatment since 1996. When confronted with a cow with less severe symptoms- *e.g*, showing slightly abnormal milk or a slightly swollen udder gland, the animal was separated from the herd and a milk sample collected for microbiological analyses. The five major bacteria isolated were grown in an appropriate medium and serially diluted in water 1:10 or 1:100, for between, 10 and 100 times. Five drops of the specific dilution were mixed with raw glucose and used once a week for oral treatment of the affected cow, and once a month for treatment of the herd. For a curative purpose, the affected cow was given the 1:10 dilution further diluted 10 times; for a preventive purpose 1:100 dilution further diluted 100 times was used in the herd. The concept that “like cures like”, that states that a drug which induces intoxication is able to cure the same disease, was the basis of the use of these autogenous oral vaccines.

Sample collection: Cow teats and udders were washed and thoroughly dried with single-use papers towels. Saline-moistened cotton swabs were rolled over the teat surface, put into sterile vials, placed on ice and transported to the laboratory to be cultured without delay. Cultures were obtained from swabs rolled on the surface of Ni agar (ITO et al, 1969.) and incubated for 48 h at 37°C. Primary cultures were evaluated by visual examination of the morphology of the bacterial colonies. Isolated colonies suspected to be of staphylococci were picked out for subcultivation and further identification using Gram-stained cell morphology, catalase and coagulase activities (AARESTRUP et al, 1995, KONEMAN et al, 1997). A single one colony was selected from each plate and the antimicrobial susceptibility patterns of each colony were determined.

Drug susceptibility testing: For the determination of minimum inhibitory concentrations (MICs), test plates were prepared by incorporating serial twofold dilutions of each drug in 20 ml (final volume) of Muller Hinton medium (MHM) (Acumedia Manufactures, Baltimore, Maryland , USA) or MHM with 4% Na Cl (in the case of methicillin). Drug concentration range in the plate was between 0.0312 and 256 µg ml^{-1}. Strains were grown overnight in Brain Heart Infusion broth (Difco, Detroit, MI, USA), incubated at 37 °C until their turbidity had reached a density corresponding to the McFarland nephelometer standard n° 0.5. Cultures were diluted 1:10 in 0.85% NaCl solutions and 1ìl of each bacterial suspension was applied with a Steers replicator to the antimicrobial-containing agar plates, yielding an inoculum of approximately 10^4 CFU/spot. Plates were incubated overnight at 35 °C and the MIC was taken as the lowest drug concentration that completely prevented the appearance of visible growth. *Staphylococcus aureus* ATCC 29213 was used as reference control. The antimicrobial agents tested, with their respective NCCLS breakpoints (NCCLS, 1997) were: penicillin (>0.25µg/ml); streptomycin, gentamicin, neomycin (>16µg/ml); vancomycin (>32µg/ml); norfloxacin (>16µg/ml) and methicillin (> 6µg/ml).

Penicillinase production test: Penicillin-sensitive *Micrococcus luteus* (ATCC 9341) and 0.2 ml of the culture/ 100ml of MHM and 0.03U penicillin per ml of a freshly prepared penicillin G solution were added to cooled Mueller Hinton Medium (Difco). The mixture was poured on Petri dishes (10.0ml) and allowed to stiffen. *Staphylococci* isolates to be tested were streaked by ellipse form loopfull and the plates incubated at 37 °C for 24h. Strains that allowed the growth of *M. luteus* colonies surrounding the ellipse (HAIGHT e FINLAND, 1952) were considered penicillinase-producers.

Screening for methicillin-resistance: Methicillin-resistance was verified by the NCCLS oxacillin screening test (NCCLS, 2000). *Staphylococci* isolates were inoculated using a Steers replicator onto oxacillin screening agar plates (Mueller Hinton agar; Na Cl 4%; 6µg/ml oxacillin) and after incubation at 35°C for 24h, strains that grew on this agar were counted as methicillin-resistant.

RESULTS AND DISCUSSION

Animal welfare is a major goal of organic dairy farming. A reduced need for disease treatment reflects good animal health and welfare. The rules for organic farming contain restrictions with regarding medication especially of antimicrobials, in order to encourage disease prevention, or even better, health care.

We studied *Staphylococcus sp* isolates from the teat skin of 129 apparently healthy cows from a farm where the antimicrobial agents has not been used for 5 years. According to the farmer, there was a significantly lower incidence of a need for mastitis treatments of the herd, following introduction of homeopathic therapy compared with that needed prior to this therapy. However as shown on table 1 we found a high level of resistance for penicillin (96.8%) and for streptomycin (67.3%) among the 95 isolates of *Staphylococcus sp* studied.

Resistance to penicillin in animal *Staphylococci* isolates is a problem persisting since the mid-1950's, resistance to other antibiotics being less common (SMITH, 1971, FRANCIS e CARROL, 1986). Lange et al (1999) reported a study with 66 isolates of *S. aureus* obtained in southern Brazil from milk samples of dairy cows suffering from subclinical mastitis, in which the predominant resistance to penicillin and ampicilin was observed in 43.9% of the isolates, either alone or in combination with resistance to others antimicrobials. Schocken-Iturrino e Nader Filho (1984), Nader Filho et al (1986), Nader Filho et al (1992), Schocken-Iturrino et al (1996) and Cardoso et al (2000) working with isolates from clinical or subclinical mastitis, reported 100.0%; 86.5%; 98.4%; 100.0% and 64.9% rates respectively, for penicillin resistance. However, Pereira e Siqueira-Junior (1995) reported a high level of penicillin resistance (80.4%) among isolates of *S. aureus* from healthy cattle in Brazil. Thus, a high level of penicillin resistance is common in both, mastitc and healthy cattle, probably as a consequence of the intensive use of this drug during the last decades; nearly the same result is shown for streptomycin.

A high level of effectiveness on *Staphylococci* isolates from cattle has been shown by gentamicin: in this work, 96.8%; in work by respectively, Pereira e Siqueira Junior (1995) – 100.0%; by Cardoso et al (2000) – 100.0% and by Brito et al (2001) – 100.0%; thus this aminoglycoside can be considered a good choice for treatment of *Staphylococci* infection.

Surprising results of the present work were norfloxacin resistance (31.5%) and vancomycin resistance (5.2%) (Table 1). The fluorquinolone norfloxacin is a new drug that has not been intensively used in cattle treatments

Table 1 - Antimicrobial resistance of 95 *Staphylococcus sp* isolates obtained from teat skin of healthy cattle treated with a homeopathic oral vaccine made from major mastitis pathogens in a organic farm at São Paulo State, Brazil during October 2001.

Antimicrobial drugs	Resistance Number of strains (%)
penicillin	92 (96.8)
streptomycin	64 (67.3)
norfloxacin	30 (31.5)
vancomycin	5 (5.2)
neomycin	1 (1.0)
gentamicin	1 (1.0)
methicillin	6 (6.3)

Table 2 - β-lactamase production amongst *Staphylococcus sp* strains isolated from teat skin of healthy cattle treated with a homeopathic oral vaccine made from major mastitis pathogens in a organic farm at São Paulo State, Brazil during October 2001.

Staphylococcus sp strains	
β-lactamase producers number (%)	β-lactamase non-producers number (%)
20 (21.0)	75 (79.0)

Table 3 - Patterns and phenotypes of drug resistance amongst 95 *Staphylococcus sp* isolates from teat skin of healthy cattle treated with a homeopathic oral vaccine made from major mastitis pathogens in a organic farm at São Paulo State, Brazil during October 2001.

Patterns	Phenotypes
Resistant to none (0)*	Sensitive (0)
Resistant to 1 drug (10)	pen (7)
	str (3)
Resistant to 2 drugs (75)	pen , str (53)
	pen, nor (22)
Resistant to 3 drugs (7)	pen, str, nor (5)
	pen, str, van (1)
	pen, van, nor (1)
Resistant to 4 drugs (2)	pen, str, van, neo (1)
	pen, str, van, nor (1)
Resistant to 5 drugs (1)	pen, str, van, nor, gen (1)

* Number of isolates is given between parentheses. Pen-penicillin; str-streptomycin; nor- norfloxacin; van-vancomycin; neo-neomycin; gen-gentamicin.

(BRITO et al., 2001); its high level of resistance found in this work is unexpected. Vancomycin, a drug being preserved for human use is not expected to lead to resistance.

β-lactamase production has not been usually tested against animal origin *Staphylococci* penicillin-resistant isolates; however, Brito et al. (2001) reported that all *S. aureus* penicillin-resistant strains isolated from intramammary infection, were also β-lactamase producers, in contrast to the results reported here (Table 2), showing that only 21.0% of the penicillin resistant isolates were also â-lactamase producers, this result is not easy to explain.

The results in Table 3 confirming the characteristics of a contagious contaminant of *Staphylococci* strains, by showing that two resistant-phenotypes, pen^R str^R (53 isolates) and pen^R nor^R (22 isolates) were predominant, what could reflect local environmental pressures.

Focus on animal welfare and health promotion is a fundamental goal in organic animal husbandry. This should be the primary effort for every herd, and their improvement should initiate and direct reductions in medication. However, it still remains a controversial point. Vaarst e Bennedsgaard (2001) reported that no significant differences could be found regarding incidence of mastitis treatment or somatic cell counts in 27 organic and 57 conventional herds. Tikofsky et al. (2003), working with *S. aureus* strains isolated from milk samples from 22 organic and 16 conventional dairy farms, reported that strains from the organic farms were more susceptible to antimicrobial drugs than isolates from conventional farms.

The present work accentuates this controversy because the farmer questioned claimed not to have been using antimicrobial drugs on the herd for the last five years; nevertheless, we isolated strains of *Staphylococcus sp* from his cows showing high level of resistance for at least some drugs, suggesting that resistance genes could be coming across the environment by contaminated water or food. Our results highlight the need for deeper investigations on bacterial strains isolated from cattle living in organic farms.

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