

# Incidence and characterization of mastitic bovine milk antimicrobial multi-drug resistant bacteria in middle west region of São Paulo, Brazil

Incidência e caracterização de bactérias com resistência múltipla antimicrobiana em leite mastítico bovino da região centro-oeste do Estado de São Paulo, Brasil

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## SUMMARY

A study of the incidence and characterization of bacteria multi-drug resistance isolated from 750 milk samples of cows affected by subclinical mastitis in middle west region of São Paulo, Brazil, was carried out. Staphylococci (75.8%) (mainly represented by *Staphylococcus aureus* and *Staphylococcus epidermidis*) and *Escherichia coli* (7.1%) were most frequently isolated bacteria. Most milk samples yielded pure cultures, but associations of pathogens were found in some samples. *In vitro* multi-drug resistance to six antibiotics (penicillin, ampicillin, dicloxacillin, streptomycin, tetracycline and oxacillin), was observed only among Gram-negative isolates and in some *Staphylococcus aureus* (39.9%) strains. Remaining Gram positive bacteria, were all sensitive to drugs tested. Multi-drug resistance in mastitogenic bacteria remain a problem difficult to be solved or controlled, mainly by short term programmes.

UNITERMS: Bacterial pathogens; Bovine mastitis; Drug resistance

## INTRODUCTION

Bovine mastitis is an infectious disease caused by various pathogens which differ virulence, frequency and permanence in the mammary gland<sup>1</sup>. These pathogens staphylococci include staphylococci, streptococci, *Corynebacterium pyogenes* and *Enterobacteriaceae*, as well some other eventual etiological agents such as *Fusobacterium necrophorum*, *Clostridium* ssp, *Pseudomonas aeruginosa* and *Bacillus cereus*. In addition to it, the occurrence of bovine mastitis as a consequence of secondary infection by the dissemination of pathogens such as *Mycobacterium* and *Brucella* species, is a matter of great public health significance<sup>1</sup>.

Because of the lack of information<sup>14,15,16,17</sup> available concerning the characteristics of the bovine mastitis in middle west region of São Paulo and the fact that an increasing number of antibiotics has shown to be inefficient *in vivo*, the purpose of this paper was to investigate the incidence and characterization of mastitis etiological agents multi-drug resistance in breeds used mainly for milk production.

## MATERIAL AND METHODS

### Samples and Bacteriological Examination

Milk samples (750) from cows at five dairy farms located in the city of Botucatu and Avaré, middle west São Paulo, Brazil,

from February 1990 to September 1992 were collected into sterile glass flasks following teat swabbing 70% alcohol and submitted to California Mastitis Test (CMT) according to the method proposed by Schalm; Noorlander<sup>24</sup> (1957) reactions were graded from 1 to 5 as suggested by Milne<sup>21</sup> (1977). CMT-positive samples were kept refrigerated at about 4°C and dispatched to the laboratory (Dept. of Microbiology and Immunology - Instituto de Biociências - Botucatu - UNESP) without delay to be cultured within 24h. Bacterial strains were isolated and identified by standard bacteriological methods<sup>12</sup>.

### Drug Sensitivity Testing

*In vitro* antibiotic susceptibility of all bacteria was assessed using disk diffusion technique<sup>23</sup>. After subculturing each strain on 5% sheep blood agar plates, cell paste from the upper region of four or five isolated colonies was inoculated into 5.0 ml Tryptic Soy broth (Difco) and incubated for 2 to 4 h at 35°C. Turbidity of this log-phase suspension was adjusted to that of McFarland 0.5 standard before inoculation on Mueller Hinton Agar plates (Difco). The following disks (Difco) were tested: penicillin (10 IU), ampicillin (30 ug), dicloxacillin (5 ug), streptomycin (19 ug), tetracycline (30 ug) and oxacillin (5 ug). After 16 to 18 h of incubation at 35°C, zones of inhibition around all disks except oxacillin, were measured using reflected light and interpreted according to NCCLS<sup>23</sup> (1984) recommendations. Any zone around an oxacillin disk was examined using transmitted light and evaluated as follows: a) isolates with < 10 mm zones at 16 to 18 h were

defined as oxacillin-resistant; b) isolates with > 10 mm zones a 16 to 18 h were incubated for more 24 h at 30°C and reexamined. Any colonies or haze within the 10 mm zone after two days of incubation was considered significant. Strains that showed resistance to the six antibiotics tested were recorded as multi-resistant.

## RESULTS AND DISCUSSION

The incidence of known pathogens found in this research (Tab. 1) was quite similar to that reported in the literature<sup>6,7,8,10,11,13,14</sup>. Although some *Enterobacteriaceae* strains other *E. coli* and *K. pneumoniae* have not been reported as commonly involved in the etiology of bovine mastitis, prevalence of these organisms in high numbers in pure cultures suggest that they may also have a pathogenic role in development of the disease.

Staphylococci represented mainly by *S. aureus* and *S. epidermidis* showed the highest incidence (75.8%) followed by *Enterobacteriaceae* (17.8%) and streptococci (6.4%). The incidence of *S. aureus* (54.1%), correlates well with reports of other workers in various Brazilian regions<sup>6,7,9,13</sup>. In the UK, Booth<sup>1</sup> (1988) in the Third Mastitis Field Experiment and the 500-herd national mastitis survey held in 1966/67 and 1977, respectively, found *S. aureus* to be the most frequent mastitis causative agent. On the other hand, clinical significance of the coagulase-negative staphylococci is not a consensus<sup>20</sup>. Maisi *et al.*<sup>19</sup> (1987) studying subclinical mastitis in ewes, found that coagulase-negative staphylococci were able to bring inflammatory indicators to milk as effectively as coagulase-positive staphylococci.

In this study the low incidence of streptococci (6.4%), agreed to what was observed by other authors in Brazil<sup>6,14</sup>, however, it was not consistent with data by Francis; Carrol<sup>8</sup> (1986), who found that in England and Wales these organisms were the most frequent pathogens in cases of mastitis recorded as mild in dry and lactating cows.

Following *S. aureus* and *S. epidermidis*, *E. coli* was the next most frequently isolated organism (7.1%). In general, it has been regarded as an opportunistic pathogen, and attention has been drawn to its increasing incidence in some countries<sup>8,10,11</sup>. According to Hinton<sup>10</sup> (1986) *E. coli* is now one of the principal causes of mastitis in the UK, which was detected in 24% of 10,977 mastitic milk samples submitted to veterinary investigation-centres in 1982. In these countries, the reduction of coecal mastitis brought about by a combination of drug therapy and teat dipping plus increased *E. coli* challenge related to unhygienic loose housing conditions, has been the commonly offered explanation for this increase in incidence<sup>2</sup>.

Therefore, it has been recognized that bacterial resistance to antibiotics vary widely and depends on geography (country, region, or institution), use of drugs and infection control practices. In this context, some investigators in Brazil<sup>3,4</sup> have stressed the importance of widespread indiscriminate use of drugs for therapy and control of bovine mastitis. However, despite many significant data from other Brazilian regions<sup>3,4,5,6,7,13,22</sup>, no information is available on the emergence of multi-drug resistance among mastitogenic bacteria.

In this study, antibiotic susceptibilities of bacterial populations (Fig. 1), presented remarkable differences between the sta-

phylococci-streptococci group and *Enterobacteriaceae*. The majority of Gram-positive cocci, excluding some multi-drug resistant *S. aureus* strains (39.9%), were sensitive to all six antibiotics tested, whereas Gram-negative bacteria were mostly multi-drug resistant. In literature, some authors<sup>15,16,17,18,19</sup> have found little change in antibiotic sensitivity patterns of bovine mastitis *S. aureus*. However, our characterization of multi-drug resistance among some microorganism strains, is a new and troublesome animal health problem, for its being the most frequent pathogen responsible for the disease. Otherwise, antibiotic sensitivity patterns of our *E. coli* strains were somewhat different from those observed by other workers<sup>10,17</sup>, who found a low incidence of resistance among mastitogenic *E. coli*.

The main conclusions from this research are: (i) there were no substantial differences in the incidences of the most significant pathogens found here or in other Brazilian regions, as well as, only slight differences when compared to the results found in overseas countries; (ii) multi-drug resistance was observed only among Gram negative bacteria and in some *Staphylococcus aureus* strains; (iii) great regional care must be taken in the use of beta-lactamic antibiotics mainly for the control of bovine mastitis caused by Gram negative bacteria.

In summary, monitoring of resistance patterns of clinical isolates may have significance in the selection of antibiotics for therapeutic use, although *in vitro* antibiotic susceptibility of a pathogen cannot by itself lead to a successful mastitis therapy, for interactions with host tissues must be considered. However, multi-drug resistance among mastitogenic bacteria in an emerging problem in some regions, which remains difficult to be solved mainly in short term control programmes.

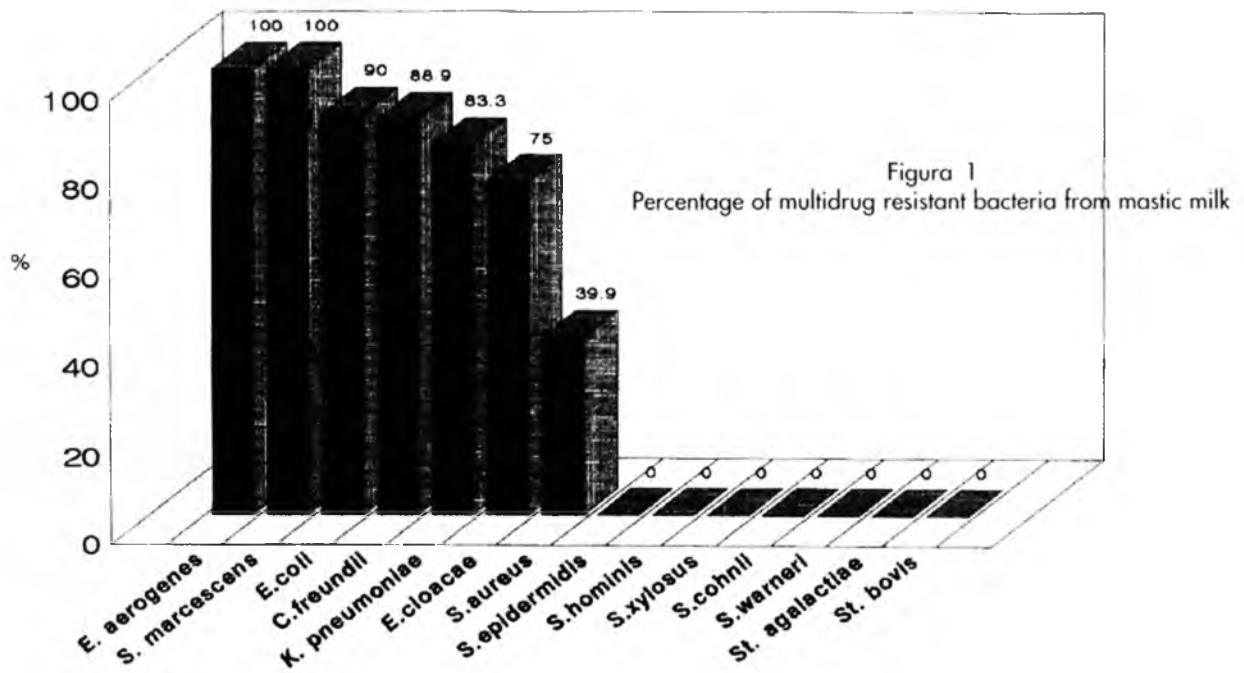
TABLE 1

Distribution of bacteria isolated from mastitic milk samples. Botucatu, SP, 1995

Organisms	Nº	%
<i>Staphylococcus aureus</i>	228	(54.1)
<i>Staphylococcus epidermidis</i>	68	(16.1)
<i>Staphylococcus hominis</i>	12	(2.8)
<i>Staphylococcus cohnii</i>	6	(1.4)
<i>Staphylococcus warneri</i>	4	(0.9)
<i>Staphylococcus xylosus</i>	2	(0.5)
<i>Streptococcus agalactiae</i>	14	(3.3)
<i>Streptococcus bovis</i>	13	(3.1)
<i>Escherichia coli</i>	30	(7.1)
<i>Klebsiella pneumoniae</i>	18	(4.3)
<i>Citrobacter freundii</i>	9	(2.2)
<i>Enterobacter cloacae</i>	8	(1.9)
<i>Enterobacter aerogenes</i>	6	(1.4)
<i>Serratia marcescens</i>	4	(0.9)
<b>TOTAL</b>	<b>422</b>	<b>(100)</b>

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## RESUMO

Foram analisadas a incidência e a multi-resistência a drogas antimicrobianas em bactérias patogênicas isoladas de 750 amostras de leite de vacas com mastite sub-clínica, na região-centro oeste do Estado de São Paulo. Os microorganismos do gênero *Staphylococcus* (75,8%), principalmente representados pelo *S. aureus* e *S. epidermidis*, e a *Escherichia coli* (7,1%) foram as bactérias mais freqüentemente isoladas. A maioria dos agentes etiológicos apresentou-se em cultura pura, embora associações de microorganismos tenham sido encontradas em algumas amostras. A resistência múltipla a drogas antimicrobianas (penicilina, ampicilina, dicloxacilina, estreptomicina, tetraciclina e oxacilina) foi observada somente entre bactérias Gram negativas e em algumas linhagens de *S. aureus* (39,9%). Os demais agentes bacterianos Gram positivos demonstraram sensibilidade às drogas testadas. Os problemas conseqüentes da resistência múltipla a drogas constituem um obstáculo à terapêutica e de difícil solução, principalmente através de programas de controle a curto prazo.

UNITERMOS: Bactérias patogênicas; Mastite bovina; Resistência a drogas

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