ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

RETROSPECTIVE STUDY ON MASTITIS PATHOGENS AND THEIR SUSCEPTIBILITY TO ANTIBIOTICS IN DAIRY FARMS OF SÃO MIGUEL (AZORES) – ASSOCIATION WITH HERDS' HOLDINGS AND MANAGEMENT PRACTICES

Andreia Filipa Gomes Vieira

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LIST OF ABBREVIATIONS

AJAM - Associação de Jovens Agricultores Micaelenses

Antibiotic – AB

IMM - Intramammary

CNS – Coagulase-negative *Staphylococci*

PFGE- Pulsed-field gel electrophoresis

Retrospective study on mastitis pathogens and their susceptibility to antibiotics in dairy farms of São Miguel (Azores) – association with herds' holding and management practices

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ABSTRACT

Bovine mastitis is the most prevalent disease in the production of dairy cattle worldwide.

The present study aimed to characterize the main etiological agents of mastitis and the corresponding susceptibility patterns in 700 milk samples submitted to the Azorean regional laboratory in 2013. A questionnaire was also performed to determine the relationship of the isolated agents with different types of management practices and features of the dairy farms that produced the analyzed milk samples.

Environmental agents were the ones more frequently isolated namely *Escherichia coli* and *Streptococcus uberis*. Contagious agents were less frequently isolated, of which *Staphylococcus aureus* was the most frequently isolated. Amoxicilin plus clavulanic acid was the antibiotic combination to which a larger number of agents was susceptible to.

Some management practices were significantly associated with the isolation of certain bacteria in mastitic milk samples, namely the extension of the dry periods and the use of internal teat sealant.

KEY WORDS

Antibiotic susceptibility; Bovine; Management; Mastitis; Pathogens; Azores' herds.

1. INTRODUCTION

Bovine mastitis is the most prevalent disease found in the production of dairy cattle worldwide and accounts for several negative effects (Seegers *et al.,* 2003).

This disease is characterized by an inflammation of the mammary gland, caused mostly by bacteria but might also have fungi and algae as causative agents (Spanamberg *et al.*, 2009; Peixoto *et al.*, 2010). Udder pathogens have been divided in environmental and contagious organisms based in the transfer of organisms from the reservoirs to the teats between milkings or in the transmission from the carrier cow or quarter to the teats of non-infected cows/quarters during the milking process, respectively (Blowey & Edmondson, 2010)..

Milk yield and composition may be altered only in the short time in less severe mastitis cases. However, in more severe cases the changes can remain in the long-term. These effects may even occur in the following lactation (Seegers *et al.*, 2003).

In Portugal, the only available estimation of the costs of mastitis in dairies, refer to the Northern mainland region of Entre-Douro e Minho and are about 249€/mastitis/year. The reasons that mostly contribute to these losses are mortality and premature culling (Aires, 2010).

Due to occurrence of mastitis, there is an increase of the somatic cell count in the milk. A healthy udder and consequently a low level of somatic cells, avoids pecuniary penalization of the producers and, simultaneously, benefits the consumer and the dairy industry since both will have a product of greater quality (Blowey & Edmondson, 2010).

Currently, antibiotic (AB) therapy remains an important component of herd health programs to control intramammary (IMM) infections in dairy cows (Guérin-Faublée *et al.*, 2002). The knowledge of the type of agent, as well as the susceptibility profile to several AB agents, can improve the success rates of the IMM infection treatment as well as contribute to the reduction of bacterial resistance (Silva *et al*., 2004).

It is well established that the indiscriminate use of AB agents to treat mastitis or other type of infections may produce an increased level of resistance of many bacteria (Silva *et al.*, 2004). AB resistances have become an increasing problem in human and veterinary medicine, which can embody serious implications in terms of public health, since it may cause failures in treatments, death and prolonged illness, as well as increased costs associated with the treatment (Rangel & Marin, 2009).

The agricultural sector in the Azorean archipelago accounts for 10% of the region's economy, of which 54% refers to milk production. About 80% of the milk produced in Azores is exported and represents 27% of the total Portuguese dairy production (Ponte, 2009). In the year of 2013, in the Azorean archipelago, 536,074,200 liters of milk were produced, of which 65.1% were produced in the island of São Miguel (SREA, 2004).

The present study was drawn in view of the significance of the dairy farms to the local and national economy, and the absence of similar studies applied to this important milk-producing region. The main objectives were to characterize the main etiological agents identified in the laboratory submitted milk samples, as well as to determine their relationship with different types of management practices and the features of the dairy farms of the Azorean island of São Miguel. Furthermore, AB susceptibility patterns of major mastitis pathogens isolated were assessed, based on the laboratory records of one of the local dairy farmers association, throughout the previous year of 2013.

2. MATERIALS AND METHODS

2.1. Characterization of the region and farms

The farms from which the animals originated were located in the island of São Miguel, also known as the "green island". This is the largest island of the Portuguese archipelago of Azores (composed by nine islands), situated in the Atlantic Ocean with their respective coordinates 37°47'N and 25°30'O. S. Miguel is located in the Oriental group of the archipelago and has an area of about 746,82km^2. The climate in this region typically features a mild temperature with a high humidity, usually overcast with a regularly distributed rainfall throughout the year (Sousa, 2007).

Pastures occupy about 90% of the agricultural area in São Miguel. Terrain has steep slopes that sometimes hamper the access to farms and difficult agricultural work. The soils are very rich in potassium while very deficient in phosphorus, and, in a smaller scale, in calcium and magnesium. Regarding the micronutrients, studies reveal scarcity in selenium, zinc, copper, cobalt and iodine (Sousa, 2007).

The feeding regime of dairy cows is based primarily in pasture grazing. Currently, in São Miguel, most animals are not housed and are rather in pasture the whole year, which means that they are exposed to adverse weather conditions. Nevertheless, a small number of farms have started to build covered collective or individual areas with sand or straw. Mobile outdoor milking equipment is the most widely used. It is during milking that most farmers supply concentrate feeds.

The predominant breed on all herds is Holstein-Friesian although some producers are incorporating the Jersey breed in their farms, as they are animals that are better adapted to high and sloping pastures due to their low weight and height (Sousa, 2007).

2.2. Sample and data collection

Data was collected from one of the two existing farmers' associations on the island of São Miguel, the *Associação de Jovens Agricultores Micaelenses* (AJAM). The AJAM gathers almost half of the dairy farms of the island, providing technical support and veterinary assistance. However, the laboratory of the AJAM serves other farms, beyond the ones registered in the association.

Laboratory data were collected retrospectively, in January 2014, and accounted for results of all samples submitted to the AJAM laboratory throughout the year 2013, by 133 different farms. Data records on the laboratory's database for each of the total 700 individual samples included individual animal identification, farm identification and farm location, as well as microorganism identification based on culture and their corresponding susceptibility for the following AB agents: marbofloxacin (5μg), danofloxacin (5μg), cefoperazone (75μg), cefquinome (30μg), kanamycin (30μg), amoxicillin plus clavulanic acid (25μg), cefazolin (30μg), spiramycin (100μg), lincomycin (75μg), gentamicin (120μg), ampicillin (10μg), trimethoprim sulfa (1,25μg), pirlimycin (2μg) and tylosin (30μg). An disk additionally employed comprised three AB agents, penethamate hydroiodide, dihydrostreptomycin sulphate and framycetin sulphate. Records were not obtained for resistance patterns.

In addition to the laboratory records, a questionnaire was held concerning the characteristics and management practices of *n*=105 holdings. Only AJAM-associated farms were considered given that the questionnaire was performed during the scheduled veterinary support visits to the farms between September 5th and December 11th of 2013. Previously informed producers consented for both collection and publication of data. The total number of cows of all enrolled herds was approximately 6,000 and the average farm size was about 170 animals, being the minimum herd size 23 animals and the maximum 320. The questionnaire was aimed at various aspects, in particular: feeding regime, existence of cowshed and cow maternity, number of milking cows, duration of dry periods and use of internal teat sealants and IMM AB at drying off. Particular aspects of milking hygiene were also abridged, such as the use of gloves during milking procedures, pre- and post-dipping with teat disinfectants, type of milking machine and criteria for milking cows with mastitis.

2.3. Statistical analysis

Available data were submitted to several exploratory analyzes through the ExcelTM and SASTM (Statistical package) programs. Frequencies were determined for factors studied through the program Proc Freq SAS (SAS Institute, 2004).

The probability of occurrence of the various environmental and contagious agents was analyzed by logistic regression, with the Proc Logistic (SAS). The following factors were also evaluated: region, type of exploration, dry periods, teat sealant, teat dipping, criteria for milking cows with mastitis, type of milking machine, period of the year and number of animals.

Statistical significance was defined previously for test results with a p-value <0.01 and <0.05, for the different tests.

The calculation of other percentages was performed with Microsoft Excel 2010 software.

3. RESULTS AND DISCUSSION

3.1. Mastitis pathogens

Overall, a single agent was isolated in 65% (455/700) of the analyzed samples, whereas in 25% (176/700) two agents were isolated and in 1% (7/700) three agents. No growth was observed in about 9% (62/700) of the milk samples (Figure 1).

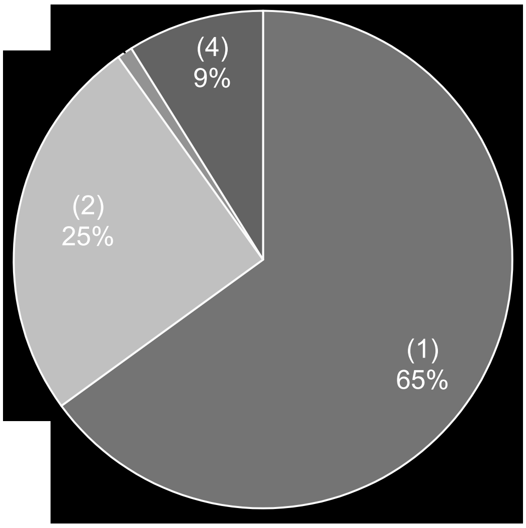

Figure 1. Isolated agents in analyzed samples, (1) – Samples with single agent isolates; (2) – Samples with 2 agents isolated; (3) – Samples with 3 agents isolated; (4) – Samples with no growth.

Of all agents isolated (*n*=826) through culture, the majority corresponded to environmental agents (83%; *n*=689) and, nearly 17% (*n*=137) to contagious agents. The main reservoir of mastitis environmental agents is the environment of the herds and the exposure of healthy quarters to these agents can occur at any time during the life of a cow. On the other hand, for contagious mastitis, the first reservoir is the cow itself, and exposure of uninfected mammary quarters to contagious agents is restricted to the milking process (Zadocks *et al*., 2001). As mentioned before, most cows in the herds of São Miguel are at pasture throughout the year, where they are exposed to environmental agents. An overcrowded pasture also contributes to a greater contamination by environmental agents (Blowey & Edmondson, 2010).

Considering the environmental agents only (*n*=689; Figure 2), *Escherichia coli* (*E. coli*) corresponded to 30% (207/689) of isolates, whereas it corresponded to circa 25% (207/826) of the total positive samples. It should be noted, however, that the occurrence of this agent can be underestimated due to the false negatives resulting from the short duration of the infection, the fast immune response and its ensuing rapid elimination (Morn, 2009; Blowey & Edmondson, 2010). The bacteria *E. coli* is ubiquitous, being the most prevalent agent in farms, since it is present in feces and can contaminate different materials, such as bedding and water. This agent causes mastitis when teats are exposed to fecal contaminated material. Thus prevalence of *E. coli* in farms increases with poor hygiene or high animal density. Rainfall and low frequency of pasture rotation can also influence the extent of exposure to *E. coli* (Morn, 2009; Rangel & Marin, 2009). These features are present in the herds of São Miguel, since most of the cows are at pasture and the climate presents regular rainfall throughout the year.

Coagulase-negative staphylococci (CNS) were isolated in nearly 14% (95/689) of the total isolated environmental agents, corresponding to 11.5% (95/826) of the total positive samples. CNS comprises 9 species and 16 subspecies. These agents are usually found in teat skin, teat end and teat canal of animals (Thorberg *et al.*, 2009; Blowey & Edmondson, 2010).

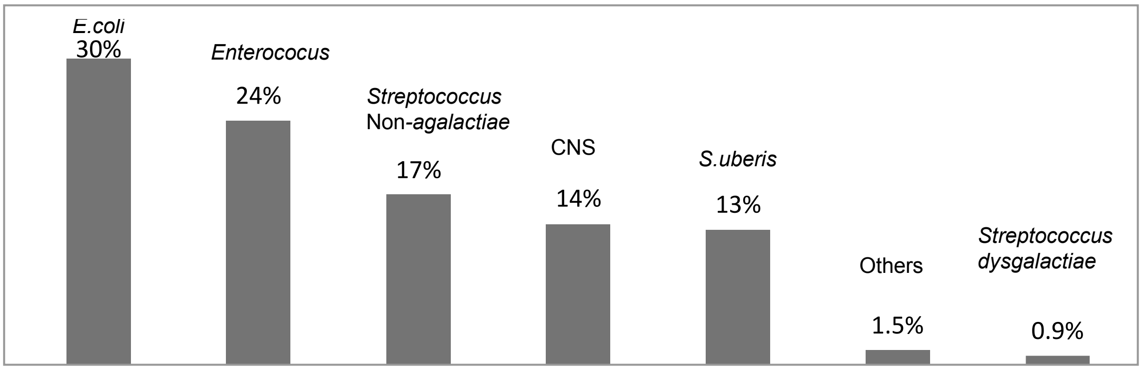

Figure 2. Percentage distribution of isolated environmental agents (*n*=689) in the analyzed milk samples (CNS, Coagulase-negative *Staphylococci*).

Streptococcus uberis (*S. uberis*) was isolated in 13.2% (91/689) of the total environmental agents' isolates, corresponding to 11% (91/826) of the total positive samples. *S. uberis* is the most common causative agent of mastitis in modern dairy herds according to Zadocks *et al*. (2007), although only the 5th most frequently isolated agent in the farms of São Miguel. The capacity of this agent to survive in the environment may be enhanced by the presence of hyaluronic acid capsule (Lopez-Benavides *et al*., 2007). Bedding with sand and straw is associated with high levels of *S. uberis*, as once contaminated with milk, urine or feces, will allow bacterial proliferation. However, it can also be found in the mouth, vulva, groin and axilla of cows. Furthermore, this agent can also affect cows in pasture, since it is present in feces. Nevertheless, its frequency of isolation is not comparable with the one of *E.coli* (Blowey & Edmondson, 2010). Zadocks (2007) refer that *S. uberis* can behave either as an environmental agent or as a contagious agent. Indeed, it has been observed in several farms that many animals within a herd had the same or closely related strain of *S. uberis.* This could be the result of a predominance of a determined strain in the environment or otherwise a contagious transmission. Rato *et al*. (2013) reported heterogeneity in pulsed-field gel electrophoresis (PFGE) patterns between *S. uberis* isolates of different farms; as, a patterns were specific in the same farm, a direct transmission of *S. uberis* among animals within the same farm can thus be suggested.

The findings of the present study, referring to the herds of the São Miguel island, reinforce the need to prevent mammary infections namely by environmental agents like *E. coli.* Measures to prevent environmental mastitis include rotating pasture, avoiding overstocking, paying attention to hygiene around feeding areas, and creating maternities in order to avoid contamination at the time of parturition (Blowey & Edmondson, 2010).

Regarding classical contagious agents (*n*=137), and as displayed in Figure 3, *Staphylococcus aureus* (*S. aureus*) corresponded to almost 60% (82/137) of the total isolated contagious agents and to nearly 10% (82/826) of the total positive samples. IMM infections by this agent are chronic and subclinical, with periods of clinical mastitis. When established, infection is extremely hard to eliminate. As the excretion of this agent is intermittent, a negative culture does not necessarily imply that the cow is free of *S. aureus*. Therefore, it is possible that the real occurrence of this agent is higher, taking into account the possibility of intermittent negative results during culture. These infections usually are spread from cow to cow at milking (Morn, 2009; Blowey & Edmondson, 2010).

Streptococcus agalactiae (*S. agalactiae*) was isolated in 22.6% (31/137) of the total isolated contagious agents, corresponding to 3.8% (31/826) of the total positive samples *and* is a major contagious pathogen. Despite an observed trend of decreasing occurrence, in the past few years, *S. agalactiae* remains a significant cause of mastitis in herds with poor management (Merl *et al*., 2003). Blowey & Edmondson (2010) reported that the isolation of this agent is a good indicator of hygiene breaching during milking.

Corynobacterium bovis was isolated in 15% (21/137) of the total isolated contagious agents, corresponding to 2.5% (21/826) of the total positive samples. This agent can be isolated from the teat canal (Blowey & Edmondson, 2010). Comparing two techniques of milk collection, Bexiga *et al*. (2010) reported that milk samples collected with a cannula surpassing the teat canal decreased the isolation of *Corynobacterium bovis* when compared with milk samples collected with the standard technique, probably because the first technique avoids contamination of the milk samples with teat canal flora.

Mycoplasma was the less frequently isolated agent. Considering the total isolated contagious agents, *Mycoplasma* was found in 2.2% (3/137), which corresponded to 0.4% (3/826) of the total positive samples. According to Pinho (2013), the prevalence of *Mycoplasma* in dairy herds of Northwestern Portugal was determined as not higher than 3%.

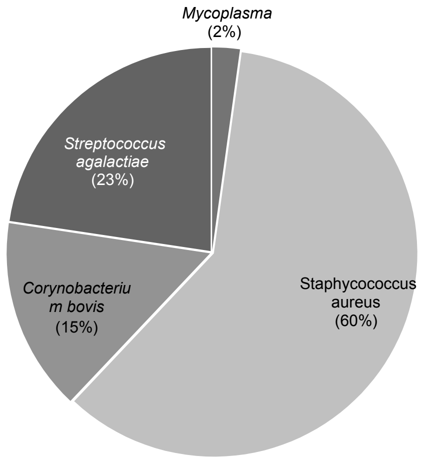

Figure 3. Percentage distribution of isolated contagious agents (*n*=137).

3.2. Antibiotic susceptibility patterns

AB susceptibility was determined in 842 isolates as summed up in Table 1. Amoxicillin plus clavulanic acid (aminopenicillins) was the AB to which a larger number of agents was sensible to, specifically 15.9% (134/842). Considering *E. coli* isolates only, 28.6% (73/255) were susceptible to marbofloxacin, 14% (36/255) to danofloxacin (which represents a sensitivity of 42.6% of strains to the tested fluoroquinolones), and almost 12% (30/255) were susceptible to kanamycin (of the aminoglycoside group). Previously, Saini *et al*. (2012) reported the resistance of this agent to kanamycin, ampicillin, streptomycin and tetracycline. Although in the present study nearly 12% of the *E. coli* isolates were susceptible to kanamycin, it is not possible to assume that remaining isolates were resistant to this drug, as resistance patterns were not available. Rocha (2013) reported resistance of this bacteria to amoxicillin plus clavulanic acid in 30% of herds evaluated in mainland Portugal. In the present study,susceptibility of the agents to this combination of AB was only 8.6%, but again patterns of resistance were not available to allow comparison with mainland.

In relation to *S. uberis* isolates, 19.6% (22/112) were susceptible to kanamycin, 18.8% (21/112) to amoxicillin plus clavulanic acid and approximately 11.6% (13/112) to spiramycin, a macrolide. Rocha (2013) reported resistance of *S. uberis* to gentamicin (71%) and cloxacillin (22.1%) in herds from the mainland of Portugal. In the routine antibiograms performed in the laboratory of AJAM, cloxacillin is not included. Moreover, sensibility of *S. uberis* to gentamicin in the São Miguel Island was only 1%. According to Blowey & Edmonson (2010), the low efficacy treatment of *S. uberis* and long refractory period in the udder, may be due to factors such as resistance to phagocytosis, maintenance inside the cells where many ABs cannot act, permanency in the mammary lymph node, kept as a reservoir of infection.

The results obtained for *S. aureus* showed that 24.6% (16/65) of isolates were susceptible to amoxicillin plus clavulanic acid, 20% (13/65) to cefoperazona (a third-generation cephalosporin) and 16.9% (11/65) to tylosin (macrolide). Rocha (2013) refer an increased resistance to the AB combination of amoxicillin plus clavulanic acid from 2004 (0.7%) to 2012 (26.6%), in dairy cattle from the mainland of Portugal. Once more, as only the susceptibility patterns were available for the herds of São Miguel, comparison of resistance patterns is not possible. Due to the production of enzymes and endotoxins by *S. aureus* strains, damage is caused in mammary cells which results in fibrosis and abscess formation. Some strains can also form biofilms in the udder. Consequently, IMM infections by *S. aureus* rarely resolve spontaneously and are difficult to treat with ABs (Morn, 2009; Melchior, 2009). Nevertheless, in a first-time infection the cure rates may be improved (Blowey & Edmondson, 2010). Through Polymerase Chain Reaction (PCR) it is possible to detect the presence of AB resistance genes or presence of genes that have been correlated with biofilm formation in *S. aureus*. The presence of these genes decreases the treatment success (Melchior *et al*., 2009).

S. agalactiae has a good response to antibiotic therapy and almost all antibiotics are effective according to Blowey & Edmondson (2010). In this study, 21.6% (11/51) of *S. agalactiae* isolates were sensible to amoxicilin plus clavulanic acid and spiramycin. Previously, Rocha (2013) reported the lowest AB resistance proportions (0 to 11.4%) of *S. agalactiae* in comparison with the remaining tested bacterial isolates.

Table 1. Number of bacterial isolates susceptible to each tested AB.

	E. coli (*n*=255)	*S.* Non-*agalactiae* (*n*=121)	*S. uberis* (*n*=112)	*Enterococcus* (*n*=168)	*S. aureus* (*n*=65)	CNS (*n*=43)	*C. bovis* (*n*=1)	*S. agalactiae* (*n*=51)	*S. dysgalactiae* (*n*=10)	Others (*n*=15)	Total (*n*=842)
Marbofloxacin	73	5	0	6	0	2	0	0	0	2	**88**
Danofloxacin	36	4	0	1	0	3	0	0	0	0	**44**
Cefoperazone	28	12	5	29	13	11	1	3	0	4	**106**
Cefquinome	19	2	1	1	4	3	0	0	0	0	**30**
Kanamycin	30	11	22	0	3	1	0	5	1	1	**75**
Amoxicillin + clavulanic acid	22	20	21	26	16	9	0	11	3	9	**134**
Cefazolin	1	3	12	0	2	0	0	2	3	3	**23**
Spiramycin	7	11	13	21	7	3	0	11	1	2	**76**
Lincomycin	18	24	9	50	4	3	0	10	1	3	**121**
Gentamicin	0	0	1	0	1	0	0	0	0	0	**2**
Ampicillin	4	10	10	7	1	4	0	2	0	0	**38**
Trimethoprim	6	5	3	8	1	2	0	2	1	1	**28**
Disk with 3 AB *	11	12	13	19	2	2	0	4	0	0	**63**
Pirlimycin	0	0	1	0	0	0	0	0	0	0	**1**
Tylosin	0	0	1	0	11	0	0	1	0	0	**13**

(AB, Antibiotic; CNS, Coagulase-negative *Staphylococci; S.* Non-*agalactiae*, *Streptococcus* non-*agalactiae*; *C. bovis*, *Corynobacterium bovis; S. dysgalactiae, Streptococcus dysgalactiae;* *, comprising penethamate hydroiodide, dihydrostreptomycin sulphate and framycetin sulphate;)

The purpose of the treatment of mastitis is to guarantee that effective AB concentrations are reached at the site of infection. The most frequently used route of administration for treatment of mastitis is IMM, as a large concentration of ABs in the mammary gland is reached because the AB is administered directly into the site of infection. With parenteral administration, it is difficult to achieve and maintain a concentration in milk or tissue. Systemic treatment is recommended in some situations, like in clinical mastitis due to *S. aureus* (in combination with IMM treatment) and severe coliform mastitis (Pyorola, 2006). Some classes of ABs are not available in preparation for IMM administration in the Portuguese market, as is the case of quinolones and macrolides. In the present study, the overall AB susceptibility of isolated bacteria to quinolones was nearly 16%.

3.3. Relationship between udder pathogens, herd's holding and management practices

According to results of the questionnaire carried out in 105 holdings, some management procedures were significantly associated with the isolation of certain bacteria in mastitic milk samples.

The use of internal teat sealants had a positive association with the milk samples tested positive for *E.coli* ($p<0.05$). According to such result, holdings that always use internal teat sealants have a higher probability to have *E. coli* mastitis, whereas, holdings that rarely use internal teat sealants have less probability to have this type of mastitis. This finding is in contrast with previous studies that reported significantly fewer IMM infection using teat sealants (Berry & Hillerton, 2002). This result can also be explained by the fact that teat sealant is being mostly used by farms that have greater mastitis problems. In addition, if aseptic techniques are not warranted during sealant installation, pathogens present around the teat, in the environment or in the disinfectants and utensils can reach the teat ends and the teat canal, promoting infection (Huscley *et al*., 2002). As such, in future studies it is necessary to ascertain in more detail this association.

Additionally, the herd size had a statistically significant association with isolation of *E. coli* and *Enterococcus* ($p<0.01$). Regarding *E. coli*, the frequency of isolation increases as the number of animals in the herd increases, whereas for *Enterococcus*, the positivity of isolation decreases when the number of animals in the herd increases. Likewise, already previously, Rangel and

Marin (2009) reported that *E. coli* increases in farms featuring a greater animal density and larger herds might be in greater risk for pasture overcrowding.

Milking order of mastitic cows also showed a statistically significant association with *Enterococcus* ($p<0.05$) isolation. Statistical analysis showed that farms where cows with mastitis were left to be milked last were less prone to test positive for *Enterococcus*. This is in accordance with the recommended measures for prevention and control of mastitis in dairy cows (as recently reviewed by Blowey and Edmondson, 2010). But given that *Enterococcus* is not a contagious agent it is surprising that this association has not occurred with contagious agents, as infections with contagious agents usually are spread from cow to cow at milking.

Possible effects of dry period length were also investigated, since it is necessary, for normal cell turnover and regeneration between lactations. Thus average length variation can affect future production level and, eventually, udder health (Collier *et al.*, 2012). Watters *et al*. (2008) refer that cows submitted to a dry period between 60 and 69 days produced more milk in the subsequent lactation than those given a dry period under 40 days. In the present study it was found that dry periods between 60 and 70 days (which was the maximum dry period length observed in studied herds) increased the probability of isolation of *S.* non-*agalactiae* ($p<0.05$). Dry periods shorter than 45 days were less likely than the previous, to increase the probability of isolation. This finding should be cautiously regarded as a protective determinant factor, since the use of this dry period duration reflects the reality of only 9 farms. Furthermore, Church *et al.* (2008) and Pinedo *et al.* (2011) reported that no difference in somatic cell count and cases of mastitis were found for shorted dry periods (from 30 to 34) versus 45-, 55-, and 60-dry periods. In the present study, difference of duration of the dry period influenced the occurrence of mastitis by *S.* non-*agalactiae*, being the dry period of 45 to 60 days better to prevent infection by this agent.

Possible effects of teat disinfection practices were also investigated, although the composition of the dipping solution was not assessed. It was observed that the simultaneous use of pre- and post-milking teat dipping, was associated with a higher probability of isolating *Streptococcus* non-*agalactiae* ($p<0.05$). In contrast, absence of teat disinfection in milking was associated with decreasing isolation probability. Such result was equally found in the analysis of the relationship between isolation of environmental agents and use of teat disinfections ($p<0.01$). Post-dipping is used as a control measure of contagious mastitis for several years, whereas pre-dipping, more recent, is used as a control measure in the case of environmental mastitis (Morn, 2009). Teat dipping in solutions composed by, for instance sodium hypochlorite, iodine, chlorhexidine and chorine, decreases the population of microorganisms on the teat skin. At the end of milking, debris of dipping should be discarded and the container should be cleaned before the next milking to avoid contamination. In addition, components of the dipping solution may be inactivated with organic material and thus it is important that the dipping container does not become contaminated with feces (Blowery & Edmondson, 2010). The results obtained in this study, can be explain by bad dipping practices (can act as a source of infection if the product is contaminated at the time of administration when both teat disinfectants are used) or by the fact that dipping practices were being used in the most affected farms.

To assess the influence of the season, the year was divided into four trimesters, starting in January. Significant association ($p<0.05$) was found between the fourth trimester and increased frequency of *S. uberis* isolates, i.e. between October and December. The first trimester of the year (January-March) followed. The second trimester corresponding to the spring season was the one where fewer *S. uberis* isolates were found.

Similarly to *S. uberis* the frequency of isolates of *Enterococcus* and environmental agents, was significantly higher ($p<0.01$) in the fourth trimester (from October to December), followed by the first trimester (January until March). The summer season (third trimester) was the period of the year when the frequency of isolates of these agents was lower.

Described results of increased frequency of isolation during the fourth and first trimester can be explained by the recognized effects of temperature and humidity over mastitis (Morse *et al*., 1988). Indeed, the fourth and first trimester of the year correspond to the months of greater humidity in São Miguel. Although the rain is more or less constant throughout the year in the island, it is more intensive during winter, i.e. from October until March. Similarly, Lopez-Benavides *el al.* (2007) reported the lowest concentration of *S. uberis* in several ecological niches of a dairy farm of New Zealand during the summer season.

Finally, concerning the remaining factors, namely the type of milking machine, the existence of cowshed and the location of farms no significant statistical association was observed. The type of milking machine was used as a variable in the study because a large proportion of producers use mobile outdoor milking equipment's, given that the cows are at pasture. This is different from the reality observed in the farms of Portugal mainland.

The present study has some limitations, namely due to the impossibility of performing the questionnaires in all of the 133 holdings that provided milk samples to the AJAM laboratory throughout the year of 2013. It was further difficult to engage the producers to answer all of the parameters abridged by the questionnaire. Concerning the laboratory records, the major limitations were the absence of information regarding the patterns of resistance of isolated agents. Comparison with previous national and foreign studies was also hampered by the different ABs employed in the susceptibility testing assays.

The major outcomes of this study were: 1) higher percentage of environmental agents isolated in the analyzed milk samples; 2) isolation of a significant number of contagious agents with a good AB response; 3) association of teat dipping practices and internal teat sealant to a higher percentage of isolation of some agents, contrarily to what might be expected. The findings of the present study suggest that an improvement of the holdings hygiene along with better compliance with good milking practices might contribute to a decrease of mastitis occurrence.

Thus, further work is needed to characterize the features of the Azorean holdings and herds that can sustain the high frequency of occurrence of mastitis caused by environmental agents. It would also be important to determine the resistance patterns and identify the agents involved not only in clinical mastitis, as well as in subclinical mastitis. Other significant aspects that deserve further attention are determination of the risk factors for the occurrence of mastitis, based particularly on the characteristic management of the holdings of São Miguel, hygiene scores, compliance with good milking practices and the influence of different dry periods. By targeting these aspects, better and more effective mastitis control plans could be implemented with the aim to provide a higher quality and valorization of Azorean milk and its derivatives.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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ANNEX

Annex 1

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Annex 2

Estudo retrospectivo sobre agentes de mastites e a sua sensibilidade a antimicrobianos em explorações de bovinos de leite de S. Miguel (Açores)

Questionário

NOTAS PRÉVIAS

Os dados recolhidos por este questionário destinam4se exclusivamente a fins académicos, especificamente no âmbito da Tese de Mestrado em Medicina Veterinária da aluna da Escola Universitária Vasco da Gama Andreia Filipa Gomes Vieira, sendo que a confidencialidade dos dados recolhidos é assegurada.

Os dados são recolhidos após o consentimento expresso dos respectivos proprietários, previamente informados sobre a natureza e objectivos do estudo.

IDENTIFICAÇÃO DA EXPLORAÇÃO

A. Nome do proprietário: ____

B. Local da exploração (Concelho): _______________________________________

C. Número de animais na exploração: ____________________________________

D. Número de vacas em ordenha: ____

CARACTERÍSTICAS DA EXPLORAÇÃO

E. Número de vacas com mais que um parto: _______

F. Tipo de exploração:

Vacas sempre em pastoreio ☐

Vacas secas em pastoreio e vacas em lactação estabuladas ☐

Vacas sempre estabuladas ☐

Estabulação no Inverno ☐

G. Realiza a recria:

Sim ☐ Não ☐

H. Exploração:

Aberta ☐ Fechada ☐

H.1. Se **ABERTA** adquiriu animais:

Nos últimos 6 meses ☐

No último ano ☐

Há mais de um ano ☐

MANEIO DAS VACAS SECAS

I. Duração em média do período de seca:

> que 60 dias e < que 70 dias ☐

> 70 dias ☐

> que 45 e < que 60 dias ☐

< 45 dias ☐

J. Realiza a separação de vacas secas das vacas em lactação:

Sim ☐ Não ☐

K. Utiliza antibióticos intraamamários à secagem:

Sempre ☐

Frequentemente ☐

Raramente ☐

Só em caso de mamites identificadas ☐

Nunca ☐

L. Utiliza selantes internos a secagem:

Sempre ☐

Frequentemente ☐

Raramente ☐

Nunca ☐

M. Realiza a testagem de animais a secagem:

Sempre ☐

Frequentemente ☐

Raramente ☐

Nunca ☐

12.1 Se **REALIZA** testagem, qual:

Isolamento e TSA ☐

TCM ☐

N. Local do parto:

Maternidade individual ☐

Parque das secas ☐

Parque das vacas em lactação ☐

Outro: ________________________________

HIGIENE NA ORDENHA

O. Utiliza luvas durante a ordenha:

Sim ☐ Não ☐

P. Utiliza:

Pré4dipping ☐

Pós4dipping ☐

Ambos ☐

Nenhum ☐

Q. Realiza a limpeza do úbere com toalhetes individuais secos:

Sim ☐ Não ☐

R. Desinfecção de tetinas entre ordenhas:

Sim ☐ Não ☐

S. Ordenha as vacas com problemas de mastites no fim da ordenha:

Sim ☐ Não ☐

T. Tipo de ordenha:

Móvel ☐ Fixa ☐

U. Nº de pontos de ordenha: _____________________

MANEIO ALIMENTAR VACAS SECAS, PRODUÇÃO, NOVILHAS

V. Utiliza correctores vitamínicos e minerais nas vacas em lactação:

Sim ☐ Não ☐

W. E nas vacas secas:

Sim ☐ Não ☐