Prevalence of bovine milk pathogens in Azorean pastures: mobile versus fixed milking machines

C. Azevedo,1 D. Pacheco,2 L. Soares,3 M. Moitoso,3 J. Maldonado,1 R. Guix,1 J. Simões4

ABSTRACT

The aims of the present study were (1) to evaluate the influence of using mobile (n=47) or fixed (n=45) milking machines in Azorean herds on the apparent prevalence of several milk pathogens in bulk tank milk (BTM) and (2) to determine whether separated subclinical mastitic cows can serve, in real time, as predictors of milk pathogen prevalence for the remaining animals at the herd level. The use of a mobile or fixed milking machine influenced (P≤0.05) the prevalence of Staphylococcus aureus (72.3 per cent; n=34 v 51.1 per cent; n=23, respectively) and Klebsiella species (46.8 per cent; n=22 v 26.7 per cent; n=12, respectively). S. aureus (95 per cent CI OR 1.0 to 6.0) and Klebsiella species (95 per cent CI OR 1.0 to 5.8) were 2.5 times more likely to increase in the BTM of herds using mobile milking machines. The prevalence of coagulase-negative staphylococci (100 per cent; n=92), Escherichia coli (75.0 per cent), Enterococcus species (55.4 per cent), Streptococcus dysgalactiae (51.1 per cent), Streptococcus uberis (41.3 per cent), Actinomycetes pyogenes or Peptostreptococcus indolicus (41.3 per cent) and Streptococcus agalactiae (32.6 per cent) in BTM remained similar among the herds. κ coefficients were always <0.70, indicating intra-herd disagreement of the prevalence of milk pathogens between BTM and separated milking cows. Milking hygiene should be improved in pastures, focusing specifically on herds that use a mobile milking machine. The segregated cows at milking time are not good predictors of milk pathogens in BTM.

INTRODUCTION

Microbiological milk quality on herd bulk tanks is primarily dependent on the subclinical mastitic milk produced by infected cows, which remains a major problem in dairy herds that use different production systems (Piepers 2014), and on bacterial contamination during milking procedures (Berry and others 2006, Bava and others 2011).

In many dairy herds on San Miguel Island (Azores), mobile milking machines and mobile bulk tank milk (BTM) were largely used by farmers to support the transhumance of dairy cows.

Cow and stall hygiene, milking procedures, the cleaning of milk handling equipment and milking machine maintenance all represent significant risk factors of pathogen presence and bacterial increment counts in BTM (Elmoslemany and others 2009, 2010).

Segregation practices (Wilson and others 1995), or milking at the end, were well implemented in Azorean herds as well as for cows presenting high individual somatic cell count levels in order to minimise somatic cells presence in BTM, mainly due to economic and legal constraints.

The objectives of the present study were (1) to evaluate the influence of using mobile or fixed milking machines in Azorean herds on the apparent prevalence of several milk pathogens from BTM and (2) to determine whether separated subclinical mastitic cows can serve, in real time, as predictors of milk pathogen prevalence for the remaining animals at the herd level.

MATERIALS AND METHODS

Samples

From all regions of San Miguel-Azores, 92 dairy herds for a total of 5520 adult cows were used (total population=1833 herds and 51,684 females; SDASM 2014). From each one of the eight regions (Table 1), at least 25 per cent of the herds in each region, on dependence of the local Young Farmers’ Association (http://www.ajamcja.com/), were preselected for a previous study (Azevedo and others 2016) by a researcher under local veterinarian assessment. From 100 preselected herds, 92 also presented segregated subclinical mastitic cows and were used for the present study.

The authors considered subclinical mastitis in agreement with the selection of segregated cows by farmers due to persistent high
individual somatic cell count levels, normally more than one week, without apparent clinical signs, other than the chronic inflammatory consequences, such as alterations of quarter conformation.

Pooled milk samples were collected from all quarters of each one of the segregated cows (average=3) in each farm. Simultaneously, after milking all cows, a sample was collected from the BTM.

Finally, the criteria for inclusion were large herd size (from 20 to 260 lactating cows), farmer permission, the presence of segregated milking cows with subclinical mastitis and use of mobile (n=47; Fig 1) or fixed (n=45) milking machines. A lower mean number of lactating cows was observed in herds using mobile (39.4±4.8; ±SEM) than fixed (70.0±4.7; P≤0.001) milking machines.

All final samples were collected using the Startcheck sampling kit (HIPRA, Spain) to test the presence of coagulase-negative staphylococci (CNS), Escherichia coli, Staphylococcus aureus, Corynebacterium bovis, Enterococcus species, Streptococcus dysgalactiae, Streptococcus uberis, Actinomyces pyogenes/Pepstostreptococcus indolicus, Klebsiella species, Streptococcus agalactiae and Serratia marcescens. A total of 250 μl of milk were taken from each tank or pool samples, immediately after milking, and transferred on to a fast technology for analysis (FTA) card that was dried (GE Healthcare, Barcelona, Spain), according to the instructions of the manufacturer, and posted to Diagnos Laboratory (HIPRA).

**DNA extraction and (qPCR) amplification**

DNA was extracted and amplified using the PathoProof Mastitis Complete-12 Kit (Thermo Fisher Scientific, Massachusetts, USA) as described by Koskinen and others (2009). All procedures concerning the collection and preparation of samples, DNA extraction, and PCR and qPCR amplification for this study were reported by Azevedo and others (2016).

For each milk pathogen, a positive qPCR result, that is, the presence of bacteria in the milk samples, was recorded when the cycle threshold value was ≤37.

**Statistical analysis**

Descriptive statistics were used for the apparent prevalence of milk bacteria and 95 per cent CI determination.

The effect of herds using fixed or mobile milk machines on the presence or absence of milk bacteria was tested using univariate logistic regression models. For likelihood ratio tests at the 0.05 level, the respective ORs were also calculated.

Considering all herds, a Spearman correlation considering the apparent prevalence of each milk pathogen between milk from segregated cows and BTM was used.

<table>
<thead>
<tr>
<th>Region</th>
<th>Herds Total number</th>
<th>Selected</th>
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</thead>
<tbody>
<tr>
<td>Nordeste</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>Povoação</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>Vila Franca do Campo</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Lagoa</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>Ribeira Grande/Lomba da</td>
<td>39</td>
<td>10</td>
</tr>
<tr>
<td>Maia</td>
<td>79</td>
<td>17</td>
</tr>
<tr>
<td>Ponta Delgada</td>
<td>86</td>
<td>22</td>
</tr>
<tr>
<td>Arrifes</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

**TABLE 1:** Number of herds selected in each region of San Miguel Island (25° 30’ west longitude and 37° 50’ north latitude) and on dependence of São Miguel Young Farmers’ Association

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**Fig 1:** Picture of milking practice in herds using mobile milking machines and mobile tank milk at San Miguel- Azores

The κ coefficient value and the McNemar-Bowker test were used to determine the intra-herd agreement of the apparent prevalence of milk pathogens between BTM and pooled samples from segregated mastitic cows.


RESULTS

Prevalence of milk pathogens

CNS were observed in 100 per cent of samples and only the prevalence of S aureus or Klebsiella species in milk sampled from bulk tanks was influenced (P ≤ 0.05) by milking machines types in Azorean herds. In fact, S aureus (95 per cent CI OR 1.1 to 6.0) and Klebsiella species (95 per cent CI OR 1.0 to 5.8) were 2.5 times more likely (P<0.05) to increase in the BTM of herds using mobile milking machines. The prevalence of all other milk pathogens remaining similar between herds is described in Table 2.

Agreement of milk pathogens between bulk tank and mastitic segregated cows

Overall, a high Spearman correlation of apparent prevalence from each pathogen across herds was observed between milk from segregated cows and BTM (r=0.90; n=11; P≤0.005). The agreement between intra-herd apparent prevalences (Table 3) was low (κ coefficient <0.70) or not significant.

DISCUSSION

Although probably the milk dilution of pooled and milk samples and their potential contamination during collect can affect the sensibility and specificity of the PCR technique, there are several studies indicating their advantages regarding the classical bacterial cultures methods (Taponen and others 2009, Katholm and others 2012, Mahmmod and others 2013a,b, Zanardi and others 2014), notwithstanding different Ct-value cut-offs can change the mastitis infection definition (Cederlöf and others 2012). Reyher and Dohoo (2011) estimate the sensitivity and specificity of bacterial cultures techniques using composite milk samples, that is, from cow’s four mammary quarters, for several milk pathogens or group pathogens (CNS, S aureus, E coli, S dysgalactiae, S iberis, Enterococcus species and Corynebacterium species). In general, these researchers observed that the composite samples have very high specificity (≥86.7) but not high sensitivity, which ranged from 24.5 (Corynebacterium species) to 77.1 (S aureus). Probably an agreement in the same direction can also observed using BTM samples at level herd. On the other hand, the sampling technique effect on PCR-based

<table>
<thead>
<tr>
<th>TABLE 2: Apparent prevalence (95% CI) of milk pathogens in Azorean herds using a mobile or fixed milking machine</th>
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<tbody>
<tr>
<td><strong>Bacteria species</strong></td>
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<tr>
<td></td>
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<tr>
<td>Coagulase-negative staphylococci</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<td></td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
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<tr>
<td><em>Corynebacterium bovis</em></td>
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<tr>
<td><em>Enterococcus species</em></td>
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<tr>
<td><em>Streptococcus dysgalactiae</em></td>
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<td></td>
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<td><em>Streptococcus iberis</em></td>
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<tr>
<td><em>Actinomyces pyogenes/Peptostreptococcus indolicus</em></td>
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<tr>
<td><em>Klebsiella species</em></td>
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<td></td>
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<tr>
<td><em>Streptococcus agalactiae</em></td>
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<td></td>
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<tr>
<td><em>Serratia marcescens</em></td>
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</tbody>
</table>

Differences between prevalence values in bold are statistically significant for the same line.

Coagulase-negative staphylococci: *Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus, Staphylococcus simulans, Staphylococcus warneri and Staphylococcus xylosus

Klebsiella species: *Klebsiella oxytoca and Klebsiella pneumoniae

*Likelihood ratio test (1 d.f.) for logistic regression

BTM, bulk tank milk; NS, not significant

bacteriological results was well evidenced regarding the bacterial contamination in teat or extra-mammary sites (Hiiitö and others 2016) mainly for non-obligate intrabacterial contamination, resulting in false-positive intra-mammary infections. Consequently, the interpretation of the PCR results alone from BTM and composite milk should be done with caution and other records such as somatic cell counts and clinical observations are important tools for intramammary infection diagnosis.

CNS were observed in all samples of this study, which is in agreement with Danish dairy herds (Katholm and others 2012). In the last decade, these minor pathogens have been considered by some as emerging pathogens (Pyörälä and Taponen 2009).

*S aureus* is a major agent of contagious bovine mastitis associated with high economic losses (Hogeveen and others 2011), and the study’s findings (up to 72.3 per cent) are in agreement with the prevalence estimates ranging from 31 per cent to 100 per cent in Europe and North America (Richard and others 2006), the cumulative prevalence of 74 per cent in Prince Edward Island, Canada (Riekerink and others 2009), and the prevalence of 57 per cent in New Zealand (Howard 2006). The transmission of this milk-contagious pathogen between cows is well studied and many tasks related with milking process represent significant risk factors. In fact, *S aureus* can be isolated from several utensils used during milking as well as from animal body surfaces and the hands of the milking operator (Benić and others 2012). In a recent study (Azevedo and others 2016), the authors observed, using a six-point scale, that the poor hygiene during milking is an important risk factor for Azorean herds. Unfortunately, the milking machine maintenance and their vacuum levels and even the teat’s condition of dairy cows were not assessed, and further studies were needed in order to evaluate the impact of these factors in the incidence of *S aureus*.

The prevalence of *Klebsiella* species (up to 46.8 per cent) observed in this study was higher than the 13 per cent observed by Katholm and others (2012), who also used a PCR technique. However, milk contamination with *Klebsiella* species occurs from numerous sources, including sampling techniques (Zadoks and others 2011), and care must be taken in comparing the prevalence values of different studies. These environmental bacteria are normally shed only for a short time by cows with clinical mastitis, and their presence in BTM is suggestive of poor stall management, udder hygiene and milking practices, resulting in the contamination of milk (Hogan and Smith 2012).

The higher likelihood of *S aureus* or *Klebsiella* species observed in BTM from herds with mobile than fixed machines can be probably attributed to some poor milking practices. In another study, the authors observed
that the absence of practices concerning the gloves used during milking procedures and the cleaning of the milking machine with hot water were significant risk factors for coliform presence, including Klebsiella species in milk from BTM (Azevedo and others 2016). These practices should be improved, especially in Azorean herds using mobile machines.

Concerning the other major contagious mastitis pathogen, the authors found that the prevalence of S agalactiae in the present study is higher than the prevalence found in Denmark, which ranged between 3 per cent and 7 per cent (Rynasek and others 2009, Katholm and others 2012), and the 7.7 per cent prevalence found in Québec (Francoz and others 2012), but this result is similar to the 26 per cent of positive BTM samples found in Australian herds (Phuektes and others 2003). Being an obligate intramammary pathogen, the bovine udder is recognised as the only reasonable source of S agalactiae, and consequently, the specificity in BTM approaches 100 per cent (Keefe 2012, De Carvalho and others 2015).

In countries that have had long-term control plans for the control and eradication of S agalactiae, prevalence has declined, but in emerging dairy industries without effective milk control plans prevalence remains high (Francoz and others 2012, Keefe 2012). Efforts should be undertaken to achieve the goal of control and eradication.

The prevalence of S dysgalactiae observed in the Azorean BTM samples is a much lower result compared with the 86 per cent apparent prevalence found by Katholm and others (2012), but this result is very similar to the 55 per cent found by Phuektes and others (2003) in the BTM of 42 herds in Australia. The higher prevalence of this environmental bacteria might be related to the limited use of dry cow therapy in Danish herds due to restrictive legislation concerning the use of antimicrobials without microbiological diagnosis (Danish Veterinary and Food Administration 2010). The prevalence found in both these studies for S iberis (95 per cent and 83 per cent, respectively) was also much higher than the apparent prevalence found in this study for this mastitis pathogen.

In the same way, the apparent Enterococcus species prevalence reported in this study is much lower than the prevalence (78 per cent) reported by Katholm and others (2012).

The presence of environmental Streptococcus species has been exacerbated due to the increasing implementation of control strategies against contagious pathogens such as S aureus. These programmes have reduced the incidence of contagious mastitis, but they have had low impacts on mastitis caused by Streptococcus species (Ruegg 2012). Moreover, the dairy environment is a determinant factor for mastitis development due to Streptococcus species, and stabled dairies are at greater risk than those held in open pastures (NMC 1999).

Minor pathogens such as C bovis and A pyogenes or P indolicus were found in the BTM samples were much lower values than the 90 per cent and 63 per cent positive samples found in Danish farms (Katholm and others 2012). In fact, in herds using dry off therapy, a common practice in countries without antibiotic use restrictions, such as Portugal, levels of infection with minor pathogens such as Corynebacterium species are low (Honkanen-Buzalski and others 1984).

The high Spearman correlation of apparent prevalence from each pathogen across herds observed between milk from segregated cows and BTM suggested that mastitic cows in herds are in fact the reservoir of bacteria in BTM. However, the low χ coefficients observed in the present study indicated that separated mastitis cows cannot serve as predictors of milk pathogen prevalence for the remaining animals at the herd level.

The authors concluded that the likelihood of finding S aureus or Klebsiella species in BTM was higher in herds using mobile milking machines. The milk pathogens of segregated cows with subclinical mastitis at milking time are not good predictors for BTM at the herd level.

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Contributors Substantial contributions to the conception or design of the work: CA, DP and LS. Acquisition of data and milk samples: LS and MM. Laboratory milk samples analysis (responsibility): JM and RG. Statistical analysis: JS. Drafting and interpretation of data for the work: CA, JS, DP, MM and LS. Final approval of the version to be published: all authors.

Competing interests CA (Technical and Marketing Manager Ruminants for Portugal), RG (Corporate Marketing Manager Mastitis and Small Ruminants Vaccines) and JM (DIAGNOS Manager) are employed by HIPRA SA Laboratorios - Amer (Girona), Spain. These authors declare that this professional relationship did not affect the design, sample collection and analysis, results, discussion or conclusions of the present study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Blaz gene was also evaluated using the PathoProof Mastitis Complete-12 Kit (Thermo Fisher Scientific, Massachusetts, USA) and may be available at HIPRA for researchers who wish to deeply confirm the genotypic characterization of antimicrobial resistance in milk pathogens from Azorean dairy herds.

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