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Abstract

The aim of this research was to compare two sampling methods quantifying microbial load on teat ends, especially mastitis pathogens originating from the cows' surroundings. Methods were compared using a split udder design, including 132 teat pairs in the study. For the first method, the wet/dry swab technique, a moistened swab was rotated 360° around the teat end, followed by a dry swab in the same manner. For the second and new method, the dipping technique, teat ends were immersed in a cup filled with Ringer's solution and were removed after five seconds. Microbial load per milliliter as well as per teat end was calculated by determining the number of total aerobic mesophilic bacteria as well as environmental pathogenic bacteria, including coliform bacteria and esculin-positive streptococci. The concordance correlation coefficient (**CCC**) was used to quantify the agreement between two series of measurements and revealed the following coefficients: 0.112 for total aerobic mesophilic bacteria; 0.008 for coliform bacteria and 0.001 for esculin positive streptococci. The results of this study point out that under field conditions, the new method does not provide similar results when compared with the wet/dry swab technique for determining teat end microbial load.

Keywords: Teat end colonization, mastitis pathogens, wet/dry swab technique, dipping technique, microbial load

Introduction

Bovine mastitis, the inflammation of the mammary gland, is a complex disease considering its etiology, pathogenesis and therapy. Due to its enormous importance for the individual cow as well as for the economic losses caused by the disease, it is necessary to further characterize causative pathogens in order to develop control strategies [1,2].
A wide variety of microorganisms are discussed as being responsible for the development of mastitis and these can be epidemiologically categorized into contagious, originating from infected quarters, or environmental, located in the surroundings of dairy cows [3,4,5,6]. While the prevalence of contagious mastitis has been reduced by control programs in recent years, environmental pathogens are becoming increasingly important [4]. Most prevalent environmental microorganisms isolated in milk samples of clinical mastitis cases are esculin-positive streptococci, *Escherichia coli* and *Klebsiella* spp. [7]. In recent years, many authors have shown that teat end bacterial load can affect udder health [8,9,10]. Microorganisms found on the teat surface, especially coliforms and streptococci, are chemotrophic organisms requiring organic material to use for their metabolism. If these bacteria are present in large populations on the teat skin, this reflects a transient rather than a resident flora [11]. Furthermore, Paduch et al., 2012 [12] pointed out that environmental bacterial load of the teat canal increases with highly calloused teat ends. Furthermore, some studies revealed a lower microbial load on teat skin in primiparous cows, which might be caused by less contact with litter due to a lower udder depth or smoother surface [6]. Thus, it can be assumed that the teat skin serves as a reservoir for pathogens, posing a risk for udder health.
As can be seen, it is necessary to gain more information concerning the variation in the bacterial load on teat epithelia. For this purpose, some researchers described methods quantifying teat end bacterial load. Most authors used only one cotton or gauze swab, either dry [13] or moistened [14,15]. Teats were sampled by rotating [13,16] or by wiping one side of the teat barrel from top to bottom, passing over the teat end and wiping the other side of the teat barrel from top to bottom [14]. Paduch & Krömker [17] modified the wet/dry swab technique (DIN 10113-1; 1997-07) used in a previous study by Pfannenschmidt, 2003 [18] for determining the bacterial content in milking equipment. Furthermore, the technique was used in other investigations dealing with teat end bacterial load [10,12,19]. Nevertheless, personal influences, particularly the pressure exerted on the swab or the speed of swabbing, together with the amount of work invested in sample collection and preparation (two swabs) need to be reduced to provide reliable results [18]. To make the procedure faster and more objective, we evaluated a new method where teats were dipped in a sample vessel filled with Ringer's solution for a constant period of time. The aim of the study at hand was to compare the analytical performance of two methods, the wet/dry swab technique and a new dipping technique, for quantifying the microbial load of environmental mastitis pathogens on dairy cows' teat skin.

Material and Methods

The study took place from June to July 2018 and was conducted on two commercial dairy farms in Lower Saxony, northwestern Germany. Two

Table 1: Calculations of log10 cfu/mL and log$_{10}$ cfu/teat end of 132 teat skin pairs classified by wet/dry swab technique and dipping technique. Concordance correlations coefficient (CCC) is given to evaluate the inter-rater reliability

Pathogen group	Wet/dry swab technique		Dipping technique		CCC[1]
	Median [log$_{10}$ cfu/mL] (Minimum - Maximum)	Median [log$_{10}$ cfu/teat end] (Minimum - Maximum)	Median [log$_{10}$ cfu/mL] (Minimum - Maximum)	Median [log10 cfu/teat end] (Minimum - Maximum)	
Total aerobic mesophilic bacteria	5.008 (3.107 - 6.477)	5.309 (3.407 - 6.778)	3.374 (1.653 - 6.233)	4.975 (3.149 - 7.835)	0.112 (CI: 0.057 - 0.165)
Coliform bacteria	1.301 (1.000 - 3.138)	1.477 (1.000 - 3.438)	1.000 (1.000 - 2.952)	1.000 (1.000 - 4.549)	0.008 (CI: 0.005 - 0.010)
Esculin positive streptococci	2.249 (1.000 - 4.477)	2.538 (1.000 - 4.778)	1.000 (1.000 - 2.975)	1.000 (1.000 - 4.573)	0.001 (CI: -0.001 - 0.003)

[1] Strength-of-agreement criteria for Lin's concordance correlation coefficient by McBride [24]:
> 0.99   almost perfect agreement
0.95 - 0.99   substantial agreement
0.90 - 0.95   moderate agreement
< 0.90   poor agreement

herds participated: One herd of 35 cows mainly included German Holstein Red cows, with an average milk yield of 8,095 kg (Dairy Herd Improvement Association, **DHIA**) and mean bulk milk somatic cell count of 269,000 cells/mL; the second herd of 65 animals mainly consisted of German Holstein Black cows, with an average milk yield of 10,856 kg (DHIA) and a mean bulk milk somatic cell count of 218,000 cells/mL. All cows were housed in free-stall barns, either sawdust-bedded or bedded in dried horse manure mixed with shredded straw and alkalized with lime. Healthy lactating primiparous and multiparous cows were selected as described by Paduch & Krömker [17], according to the following criteria: Four functional quarters without udder infections or signs of clinical mastitis (i.e., no clotting or discoloration of milk, no swelling or udder redness and no heat upon udder palpation), apparently clean udders (teat skin without splashing or plaques of manure), no visible udder lesions or trauma, teat tissue and skin that appeared normal.

The study included the results of 66 cows, so that a total of 132 teat pairs were sampled. A split-udder design was used to compare the methods at udder half level. A teat was matched with its contralateral teat to eliminate individual influences. In the following, each method was used once per udder half and could be compared on the basis of this. Two teat pairs were tested per cow. Two contralateral teats (e.g., left front, right rear or right front, left rear) were sampled with the modified wet/dry swab technique after the pre-cleaning and pre-milking routine before milking as described by Paduch and Krömker [17]. The first swab (ultrafine, Dry Swab, Check Diagnostics GmbH, Westerau, Germany) was moistened with ¼ Ringer's solution (Merck, Darmstadt, Germany) and rotated 360° around the teat canal orifice at a distance of 1 cm from the teat apex. The same procedure was carried out with the dry swab. Immediately after sampling, the tips of both swabs were transferred to one tube containing 2 mL of sterile Ringer's solution. The remaining contralateral teats were prepared in the same way. These were dipped in a cup filled with 40 mL of ¼ Ringer's solution (Merck, Darmstadt, Germany) until the lower 1.5 cm of the teat had been moistened. After five seconds, the teat was removed from the dip solution. All samples were taken during the morning milking by one researcher and then transported at 5 °C to the microbiology laboratory at the University of Applied Sciences and Arts Hannover (Germany) within 8 h. Samples were discarded directly at the time of sampling when any obvious contamination took place or liquid was spilled. Therefore, a new pair of teats was sampled on another cow in a similar manner. In the laboratory, both swab samples and dipping samples were vortexed (Vortex Genie2, Scientific Industries Inc., Bohemia, NY, USA) each for 20 seconds and swabs were then removed from the swab samples with sterile tweezers. Serial 1:10 dilutions were prepared with ¼ Ringer's solution and a volume of 0.1 mL was spread in duplicate over the whole of a pre-dried 9 cm diameter agar plate with a Drigalski spatula. The total number of aerobic mesophilic bacteria was determined with Plate Count agar (Merck, Darmstadt, Germany) and incubated at 30 °C for 72 h. ChromoCult Coliform agar (Merck, Darmstadt, Germany) was used for detecting coliform bacteria, while esculin positive streptococci (e.g., *Streptococcus* (*S.*) uberis, *Lactococcus lactis*, *Enterococcus* spp.) were determined with Kanamycin Esculin Azide agar (Merck, Darmstadt, Germany). The latter two were incubated at 37 °C for 24 h. Plates with 1-300 colonies were used to calculate bacterial counts in swab and dipping solution [17]. The weighted arithmetic mean was calculated for each pathogen group and results were indicated in colony-forming units per milliliter (cfu/mL) as well as colony-forming units per teat end. A teat end represents the lower 1 cm (wet/dry swab technique) or 1.5 cm (dipping technique) of the teat and is assumed to have a diameter of 19.6 mm [20], giving an estimated teat skin area of around 9.2 cm² or 12.3 cm², respectively.

As bacterial counts were not normally distributed, results were log$_{10}$-transformed prior to further analysis. To calculate the reliability between bivariate pairs of observations, we used the Lin's Concordance Correlation Coefficient, as suggested by Watson & Petrie and Koch & Spoerl [21,22,23]. The CCC provides a measure of reliability based on correspondence. For measuring the agreement between two continuous variables, values from -1 to +1 occur, whereby 1 indicates strong concordance [24]. The CCC was computed by SPSS 25.0 (IBM Inc., Chicago, IL, USA). As there was no normal distribution of bacterial counts, we used the Kruskal-Wallis test to determine whether the teat end bacterial load showed a tendency towards a specific lactation number, related to the results of Rowbotham & Ruegg [6]. In accordance with Rowbotham & Ruegg [6] and because the wet/dry swab technique represents our reference method, results thereof were used for this purpose.

Results

The calculations included bacterial loads of 132 teat pairs (Table 1). In teat skin swabs, the median total mesophilic count was 5.008 log$_{10}$ cfu/mL Ringer's solution or rather 5.309 log$_{10}$ cfu/teat end. In

Table 2: Ranking means of teat end microbial loads of 132 teats examined by wet/dry swab technique, classified by lactation number and pathogen group. The Kruskal-Wallis test was performed to test for lactation-dependent differences in the microbial load of teat skin

No. Lactation	N (132)	Aerobic mesophilic bacteria [cfu/mL] ranking means	Coliform bacteria [cfu/mL] ranking means	Esculin-positive streptococci [cfu/mL] ranking means
1	54	63.558	60.417	64.278
2	22	56.750	83.614	73.955
3	28	71.036	63.446	65.786
4	28	70.446	67.839	65.643
p-value[1]		0.491	0.089	0.791

[1] $p < 0.05$, results of the Kruskal-Wallis test

dipping solution, the median was 3.374 $\log_{10}$ cfu/mL and 4.975 $\log_{10}$ cfu/teat end for total mesophilic counts. Calculation of concordance yielded the following results for total aerobic mesophilic counts: CCC = 0.112 (CI: 0.057 - 0.165). A closer evaluation of the group of environmental pathogens revealed the following for coliform bacteria: Teat swabs: median = 1.301 $\log_{10}$ cfu/mL or in relation to the sampled area: median = 1.477 $\log_{10}$ cfu/teat end. In the dipping solution, the median was 1.000 $\log_{10}$ cfu/mL and 1.000 $\log_{10}$ cfu/teat end. The CCC result was 0.008 (CI: 0.005 - 0.01) for $\log_{10}$-transformed counts. The other main group of environmental bacteria, esculin-positive streptococci, were found in wet/dry swabs with a median of 2.249 $\log_{10}$ cfu/mL or rather 2.538 $\log_{10}$ cfu/teat end. For the new dipping technique method, a median of 1.000 $\log_{10}$ cfu/mL was found. Extrapolating the results to the teat apex provided a median of 1.000 $\log_{10}$ cfu/teat end. Nevertheless, the CCC was 0.001 (CI: - 0.001 - 0.003). We could not detect any differences in the bacterial load of the teat ends between the lactation numbers. Results of the Kruskal-Wallis test are presented in Table 2.

Discussion

Swabbing surfaces to determine their bacterial load is one of the oldest methods for this purpose. This sampling procedure is easy to handle for researchers and can be applied in places which are difficult to access as well as requiring low expenditure in terms of equipment and time. Referring to the above-mentioned points, the swabbing method has become a widely used method in many studies [11,17,18,26]. On the other hand, the high work-load involved in preparing and processing the swabs and the deficiencies of a standardized procedure are regarded as disadvantages. Above all, the inconsistency of the pressure applied on the swabs for removing bacteria from surfaces is to be mentioned [18]. In order to standardize the method for determining the microbial load on the teat, we tried to verify a comparative method, which could lead to similar results as the more complicated method described above.

With regard to the microbial load on teat skin, Paduch & Krömker [17] reported similar results for wet/dry skin swabs as we did. They found that the largest populations on teat skin were S. uberis (maximum: 6.48 $\log_{10}$ cfu/mL) and coliforms (maximum: 6.48 $\log_{10}$ cfu/mL). These results were comparable with those of our study, whereby the largest populations of environmental pathogens were esculin-positive streptococci (maximum: 4.477 $\log_{10}$ cfu/mL) found in wet/dry teat skin swabs. It can therefore be suspected that esculin-positive streptococci mainly originated from the environment which the cows' teats were exposed to for most of the day or that esculin-positive streptococci are a part of the teat skin flora [6,14]. The latter disagrees with findings of other researchers examining the microbial teat skin flora, by showing that

esculin-positive streptococci can be influenced by bedding and milking hygiene. [19,25]. On the contrary, the median amount of these streptococci in our study (median: 2.249 $\log_{10}$ cfu/mL) was above those values published by Paduch & Krömker (median: 1.71 $\log_{10}$ cfu/mL) [17] as we considered all streptococci, hydrolyzing esculin (S. uberis, Lactococcus spp., Enterococcus spp.), while Paduch & Krömker only referred to S. uberis, subcultivated on modified Rambach agar. The median values for coliforms differed slightly in both studies. The different results for esculin-positive streptococci and coliforms can be explained by the larger sample size examined by Paduch & Krömker [17] (n = 839 teat skin swabs from 32 herds), therefore leading to a wider variation in teat skin bacterial load. These differences reveal that the udder skin is not a uniform system and that the bacterial population differs from udder to udder [27]. Reasons for this might be physiological or environmental selective processes, possible preferences of microorganisms for particular udder sites or any bacterial antagonism mechanisms. [26]. Furthermore, the above listed individual influence of the sampling itself might be responsible for differing results. Cullen & Herbert [27] observed fluctuations in bacterial load of staphylococci on teat skin throughout the year, considering seasonal changes and changing stages of lactation. Furthermore, antagonistic organisms on teat skin, particularly Staphylococcus chromogenes, are suspected to affect the presence of other organisms [28].

Another source of differences to the above-named study of Paduch & Krömker [17] could be that cows from different numbers of lactation were sampled in different proportions in the studies. It is not mentioned in which ratio the primiparous and multiparous cows were sampled. Rowbotham & Ruegg, 2016 [6] concluded that primiparous cows have less bacterial teat load than multiparous cows, which, in turn, leads to different study results depending on whether more primiparous or multiparous cows are tested. Although the amount of cows per number of lactation is low in the present study, the number of lactations is not decisive because both methods were tested on the same cow in a split-udder design. To determine the extent of influence of parity on teat skin bacterial load, we used the findings of the wet/dry swab technique. However, remarkable differences could not be found for all pathogen groups, which means that teat end bacterial load does not depend on the number of lactations in the current study, as reflected in our results for wet/dry swabs. With regard to teat skin bacterial load, varying correlations clarify that it is affected by several factors.

Referring to the CCC described by Lin, 1989 [21], comparing data of the new dipping technique with the wet/dry swab technique for investigating teat end bacterial load showed large differences between the two methods for esculin-positive streptococci as well as for coliform counts. Highest concordance between the results of the two measurement series existed with the total aerobic mesophilic bacteria, where

the CCC was 0.112, which, however, describes a poor agreement [24] . In addition, the bacterial loads per teat end determined by the dip technique were basically lower than those values determined with the wet/dry swab technique. This probably results from the fact that the dipping solution cannot remove a sufficient number of bacteria from the teat skin in the short duration of time. Prolonging the dipping time could cause the results of the samplings to converge. In a pilot study, different immersion times were tested, wherein five seconds turned out to be the longest span accepted by the cows. However, the dipping method actually sampled a greater teat area, as the swabs only dabbed the side walls of the lower 1 cm of the teat, whereas the dipping solution also covered the teat apex with the teat canal orifice. On the other hand, the dipping technique does not involve the mechanical action of wiping, as with the wet/dry swab technique, so that a lower yield could be expected for the dipping technique. Thus, the reasons for the poor concordance of the methods could be the differences in location, surface sizes and the mechanical impacts.

Determining bacterial load is a destructive method, one sampling excluding a subsequent one, since the bacterial load after the first sampling would of course be reduced regardless of which sampling method is carried out first [29]. Therefore, we used the split udder design, although we presumed that different teats would also differ in their teat skin bacterial load. Furthermore, lower bacterial counts produced by the dipping method were suspected to be a result of the higher volume and the higher dilution of Ringer's solution. While establishing the method, we tried to keep the volume as low as possible but the limiting factor was the sampling vessel, which had to be large enough for the teats to fit in and to dip them in the solution.

For both techniques, time and effort in the laboratory was the same. Without having measured the time for sampling, the time required for the dipping technique seems to be less, as the two swabs have to be taken out of their sterile packages one by one. However, after some repetitions, the wet/dry swab technique with two swabs is a practicable method as well. It also has to be noted that the Ringer's solution for the dipping technique should be at room temperature. Otherwise, there was a massive defense reaction by the cows.

Neither for esculin-positive streptococci nor for coliforms was the CCC greater than 0.112, which was calculated for total mesophilic counts. This means that the dipping method only produces results compared to those of the wet/dry swab technique with poor concordance. The median values of each method for each pathogenic group differ with inconsistently wide ranges, which might be unacceptable for standardized measurements.

Conclusion

In general, teat skin bacterial load is affected by many factors and alongside many other influences it can affect udder health. Our investigations showed that determining teat skin bacterial load with the tested dipping technique does not produce similar results to those when using the wet/dry swab technique. Dipping the teats in the test medium does not produce equivalent results or results that correlate sufficiently well with those gained from swabbing the teats. In addition, the procedure appears neither faster nor easier to handle.

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