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Recurrent mastitis–persistent or new infections?

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ABSTRACT

Recurrent clinical mastitis contributes to around half of all infections having an economic impact in the dairy industry. It leads to milk yield reduction, increased risk of mortality, and culling, and may be caused by new infections or a persistent infection after previous treatment. Disease management is dependent on the infecting species, necessitating accurate identification of the pathogen in the range of persistent and reinfection cases among recurrent infections using culture and molecular biological analysis. Milk samples from diagnosed clinical mastitis cases were collected from three Northern German dairy farms between 2011 and 2015. Totally, 2043 diagnosed mastitis cases were examined at quarter level (1598 (78.2 %) first and 445 (21.8 %) recurrent mastitis cases in lactation). Among the recurrent cases, 145 (32.6 %) cases were confirmed to harbor the same pathogenic species as previous infections. RAPD PCR confirmed the same species strain in 49 (11 %) of the recurrent infections. The contribution of new infections as compared to persistent infections in cases of clinical mastitis is clear from the data. Future studies in recurrent clinical mastitis control should be focused on influencing factors to prevent new infections in addition to therapeutic intervention and bacteriological cure.

1. Introduction

Udder health in subclinical mastitis has increased in the modern era, bringing more focus to clinical mastitis. Scrutiny is warranted in these cases in contexts of animal welfare, reduction of antimicrobial usage and interrupting the farm routine. German dairy herds report an almost 50 % rate of recurrent clinical mastitis (Picker, 2012; Zoche-Golob and Spilke, 2013), leading to a reductive influence on the herd life of animals (Bar et al., 2008). Preestablished risk factors include parity, higher milk production, known pathogenic species (Jamali et al., 2018), a previous infection, and infection pressure (Grieger et al., 2014). Persistent or novel infections leading to recurrent mastitis cannot be clearly distinguished by standard diagnostic methods, although disease management strategies differ considerably based on the nature of the infection. Unsuccessful therapy is one of the causes of persistent infection, e.g., low concentration of antimicrobial agent at a site of action also favored by biofilms (Oliveira et al., 2006; Høiby et al., 2011). Intracellular occurrence or growth of the pathogen (Shinji et al., 2011) and protection by connective tissue are other risk factors. Responses may include resistance testing, follow-up treatment or increased dosage, additional therapy or even culling. New infections, in comparison, may stem from a different or same strain or species of the causative agent of the pathogenic microorganism after achieving a bacteriological cure of the previous mastitis. Hygienic animal husbandry should be implemented in order to avoid new infections in these cases since pathogenic elimination has been achieved. This discrimination is useful in understanding recurrent mastitis. In order to expand the scarce knowledge on this issue, this study aimed to analyze the distribution of pathogens in persistent and new infection cases. The rates of these microorganisms occurring in recurrent cases were assessed to identify the frequency of pathogens persisting in the udder quarter and causing recurrent mastitis. Microbial isolates were collected from the first and all following mastitis cases on both host and quarter level. If the bacterial species was found to be the same in the first and subsequent infections, a molecular biological typing method was applied to compare the genetic fingerprint of the strains.

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2. Material and methods

Trained researchers aseptically collected quarter foremilk samples from confirmed clinical mastitis cases from three Northern German dairy farms as per the German Veterinary Association protocol (2009) during a period from 2011 to 2015. Symptoms like milk clots, swelling of the udder tissue, pain, and increased temperature were used to diagnose clinical mastitis cases. The samples were transported in a boracic acid-based preservative to the laboratory of the University of Applied Sciences and Arts Hannover (Hanover, Germany) for microbiological analysis within two days. Microbiological analysis was performed as per the GVA (German Veterinary Association) (2009).10 µL of each milk sample were streaked on one quadrant of an esculin blood agar plate (Oxoid, Wesel, Germany). The plates were examined after 24 h and 48 h of incubation at 37 °C. The grown colonies were initially differentiated by their hemolysis patterns, ability to hydrolyze esculin, cell morphology and Gram status. Non-hemolytic Gram-positive catalase positive cocci (3 % H_2O_2, Merck, Darmstadt, Germany) were defined as non-aureus staphylococci (NAS), such with β hemolysis were further differentiated applying clumping factor test (DiaMondiaL Staph Plus Kit, Sekisui Virotech, Russelsheim, Germany). *Staphylococcus* (*Staph.*) *aureus* was clumping factor positive and NAS negative. Catalase negative Gram-positive cocci which hydrolyzed esculin were subcultivated on modified Rambach agar according to Watts et al. (1993) to differentiate between *Streptococcus* (*Strep.*) *uberis* and Enterococcus species. Esculin non-hydrolyzing Gram-positive, catalase negative cocci were further differentiated via Lancefield serotyping (DiaMondiaL Streptococcal Extraction Kit, Sekisui Virotech, Russelsheim, Germany) and referred as *Streptococcus* (*Strep.*) *agalactiae, Streptococcus* (*Strep.*) *dysgalactiae* and *Streptococcus* (*Strep.*) *canis*. Gram-positive irregular rods with Y-shaped cell configuration were identified as *Trueperella* (*T.*) *pyogenes*, if they were β-hemolytic and catalase and esculin negative. Conversely, Gram-positive, non-hemolytic catalase positive irregular rods were specified as coryneforms. Gram-negative rods were distinguished by their ability to catabolize glucose under aerobic and anaerobic conditions (glucose supplemented oxidation-fermentation test medium, Merck, Darmstadt, Germany) and cytochrome C oxidase production (Bactident Oxidase, Merck, Darmstadt, Germany). Cytochrome C oxidase negative rods fermenting glucose were subcultured on Chromocult® Coliform Agar (Merck, Darmstadt, Germany) to distinguish *Escherichia (E.) coli* and other coliforms. Non-motile other coliforms were reported as *Klebsiella* spp. Gram-negative, cytochrome C oxidase positive bacteria which metabolized glucose oxidatively, were defined as *Pseudomonas* spp. Yeasts and Prototheca were differentiated through microscopy according to their specific cell morphology.

The samples were contaminated if more than two different colonies were identified per plate, although *Staph. aureus*, *Strep. dysgalactiae,* and *T. pyogenes* isolates were taken into account. One isolate from each identified species per sample was stored at −80 °C in a medium comprising 80 % Brain Heart Infusion Broth (Merck, Darmstadt) and 20 % glycerol until molecular analysis.

Recurrent mastitis was classified as repeated infection within one lactation cycle at a minimum interval of 14 days, in the same host and quarter, after a previous mastitis diagnosis (Barkema et al., 1998; Döpfer et al., 1999; Schukken et al., 2010). If the same pathogen was confirmed in the subsequent mastitis case as in the previous infection, a Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD PCR) was carried out for the further discrimination of these selected isolates.

Bacterial DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) as per the manufacturer's instructions. RAPD PCR was carried out in a 25 µL reaction volume containing 12.5 µL ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, Munich, Germany), 20 pmol of primer (listed in Table 1), 5 µL of the template, and water to make up the volume. Amplification was performed in an Mx3005 P qPCR System (Agilent, Santa Clara, California, USA), using previously published methods as described in Table 1. RAPD PCR products were stained with MIDORI^{Green} Direct (NIPPON Genetics Europe GmbH, Düren, Germany) and separated on a 2 % agarose gel. Identical RAPD patterns of PCR products were defined as the same strain.

A Chi-square (χ^2) test was performed for statistical analyses, using SPSS 25.0 (IBM SPSS 25.0.0.0., Armonk, USA). The results were defined to be statistically significant below a p-value of 0.05.

3. Results and discussion

Of the 2043 mastitis cases recorded on a quarter level, 1598 were first cases and 445 were recurrent cases within the same lactation cycle. Among the recurrent cases, 145 had the same pathogenic species compared to previous infections. RAPD PCR was utilized to confirm 49 (11 %) of all 445 recurrent cases to be the same pathogen strain as in previous infection (Fig. 1). Since reinfection with the same strain is possible, the number of identified persistent cases (same strain as previous case) can be generally lower than 11 %.

Table 2 describes the frequencies of different pathogens isolated from mastitis milk samples; *Strep. uberis* was the most frequently isolated pathogen, followed by *E. coli*, *Staph. aureus*, NAS, *Strep. dysgalactiae*, coliform bacteria other than *E. coli*, and *T. pyogenes* as the least frequently isolated pathogen. The highest frequency of persistent infections was recorded for *T. pyogenes* (37.5 %), although this is subjected to bias owing to the small number of cases (two animals), followed by *Staph. aureus* (29.0 %), *E. coli* (28.9 %), *Strep. dysgalactiae* (25.0 %), while *Strep. uberis* had the lowest frequency of persistent infections at 14.8 %. The contaminated samples were 10.9 % while 23.4 % did not show any growth.

The distribution of recurrent and persistent infections was statistically significant between all species, as revealed by chi-square (χ^2) test ($p < 0.05$). Some species showed more recurrence at the species level compared to others, while some were more likely to cause persistent infections. Similar to previous reports, *Strep. uberis* (Swinkels et al., 2013) and *Staph. aureus* recorded the highest frequencies causing recurrent infections. The contagious *Staph. aureus*, as expected, showed high persistence (29 % of recurrent *Staph. aureus* cases) and also the highest recurrence (27 % out of all *Staph. aureus* cases). *Strep. uberis* infections accounted for 14.8 % of persistent cases, and a high recurrence rate similar to *Staph. aureus* (24 % of all *Strep. uberis* cases). Persistent infection was recorded for each third recurrent case with *Staph. aureus* (same species as the previous case), while it was the seventh case for *Strep. uberis* (persistent). This indicates that the study farms harbor a high number of different *Strep. uberis* strains showing low persistence in the hosts' udders but causing high recurrence, probably due to their environmental origin (Wente et al., 2019). Infection with these pathogens may increase the hosts' susceptibility to novel infections with other pathogens. Additionally, of note is the fact that some persistent cases belonged to the same host; for instance, five *Strep. uberis* infected cows (two in their second, two in third, and one in fourth lactation cycle) harbored a persistent infection with several clinical episodes, and one *E. coli* infected cow (in the third lactation) suffered from five persistent infections. Few animals in this study were found with successive infections with the same strain (Table 2), with most in a higher lactation cycle. Higher lactation cycles have been previously correlated with higher susceptibility to mastitis (Pinzón-Sánchez and Ruegg, 2011). In light of this fact, it may be necessary to scrutinize the mastitis history of these animals to decide whether this therapy is worthy or not. Bar et al. (2008) found nearly the half of all recurrent cases to be caused by the same pathogen as in the previous infection, while the current study puts this proportion closer to one-third. Several factors influence this proportion and may possibly increase if the herd suffers from contagious mastitis. Microbial growth was absent in 23.4 % of clinical mastitis cases. This may be due to insufficient sample volume. Although 10 µL of the milk sample—as

Table 1
Applied RAPD PCR –Primer.

	RAPD-Primer	Primer sequence 5`–3`	Source
Staph. aureus	Primer C	CGGGGGACTGTTGGGCGCCATCT	Damiani et al., 1996
NAS	Primer C	CGGGGGACTGTTGGGCGCCATCT	Damiani et al., 1996
Coliform bacteria	256	AACGCGCAAC	Pacheo et al., 1996
E. coli	256	AACGCGCAAC	Pacheo et al., 1996
Strep. uberis	OPE 04	GTGACATGCC	Gillespie et al., 1998
Strep. dysgalactiae	OPE 04	GTGACATGCC	Gillespie et al., 1998
T. pyogenes	Primer A	CTGGCGGCTTG	Hijazin et al., 2013

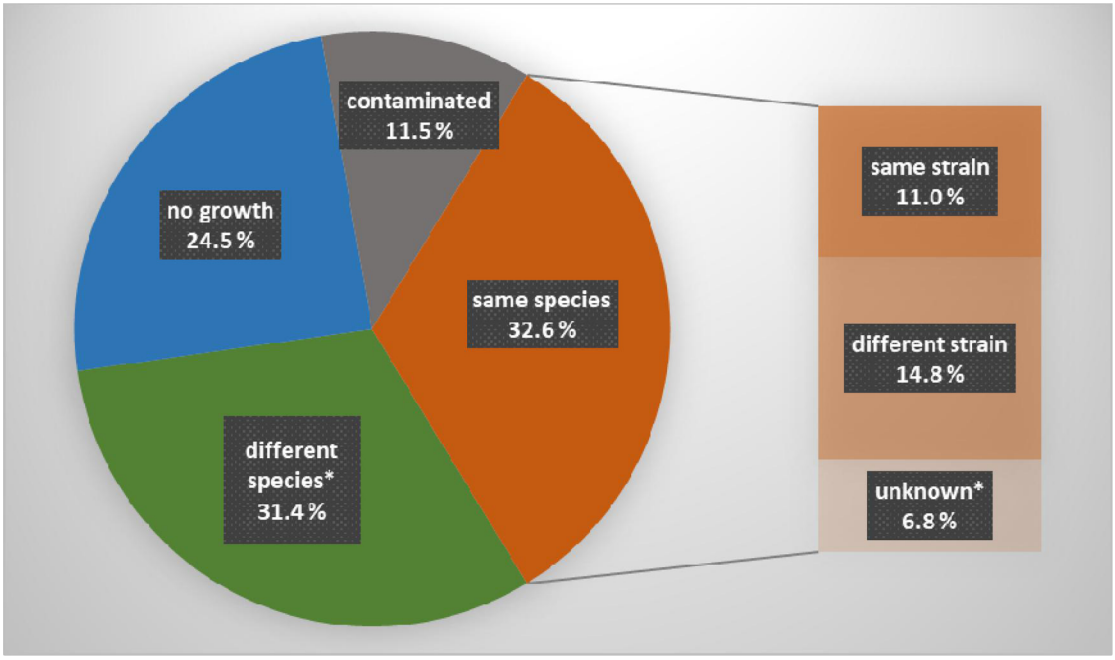

Fig. 1. Occurrence of 445 (100 %) recurrent clinical mastitis cases.
*strain typing not performed.

Table 2
Distribution of the cases by species.

Species	mastitis cases (% of all cases)	first cases (% of all cases)	recurrent cases (% of all cases)	Same species as in previous cases (% out of recurrent cases)	Same strain as in previous cases (% out of recurrent cases)	n.p.*** (% out of recurrent cases)
Strep. uberis	592 (100 %)	450 (76 %)	142 (24 %)	88/142 (62 %)	21/142[a] (14.8 %)	24/142 (16.9 %)
E. coli	306 (100 %)	261 (85.3 %)	45 (14.7 %)	25/45 (556 %)	13/45[b] (28.9 %)	2/45 (4.4 %)
Staph. aureus	115 (100 %)	84 (73 %)	31 (27 %)	23/31 (74.2 %)	9/31[c] (29 %)	3/31 (9.7 %)
non – *Staph. aureus* staphylococci	69 (100 %)	61 (88.4 %)	8 (11.6 %)	1/8 (12.5 %)	0	0
Strep. dysgalactiae	57 (100 %)	45 (79 %)	12 (21 %)	4/12 (33.3 %)	3/12 (25 %)	0
Coliform bacteria other than *E. coli*	54[K] (100 %)	44[Kf] (81.5 %)	10[Kr] (18.5 %)	3/10[Ks] (30 %)	0	0
T. pyogenes	36 (100 %)	28 (77.8 %)	8 (22.2 %)	4/8 (50 %)	3/8[d] (37.5 %)	1/8 (12.5 %)
no growth	479 (100 %)	370 (77.2 %)	109 (22.8 %)			
contaminated**	222 (100 %)	171 (77 %)	51 (23 %)			
others*	113 (100 %)	84 (74.3 %)	29 (25.7 %)			
Total	2043 (100 %)	1598 (78.2 %)	445 (21.8 %)			

**Bacillus* spp., coryneforms, *Enterococcus* spp., *Pseudomonas* spp., Prototheca, Yeasts, strain typing not performed; **more than two pathogens were isolated; ***n.p. = strain typing not performed; [K]*Klebsiella* spp. (n = 14); [Kf]*Klebsiella* spp. (n = 12); [Kr]*Klebsiella* spp. (n = 2); [Ks]*Klebsiella* spp. (n = 0); [a]five cows had two cases consecutively; [b]one cow had five cases consecutively, one cow had two cases; [c]two cows had two cases consecutively; [d]one cow had two cases consecutively.

standard—was used for the culture, this limits the pathogen detection in case of a low shedding (Krömker et al., 2010), additionally the reduction of the colony forming units during the transport is possible. Other pathogens, which do not grow under the chosen conditions (e. g. *Mycoplasma* spp.), can provide a no growth result. Since methodological choices can be important for the number of recurrent cases encountered, this study followed the 14 day-interval between distinct infections based on the udder quarters method. Time intervals of ≥3, 5, 7, or 14 days have been previously described in the literature (Barkema et al., 1998; Döpfer et al., 1999; Bradley and Green, 2001; Gröhn et al., 2004; Schukken et al., 2010). One isolate growth from milk samples was taken for strain typing since Oliver et al. (1998) showed that *Strep.*

uberis isolates from a single milk sample usually belong to the same strain. The RAPD PCR method is frequently chosen for its suitability as a molecular diagnostic tool for high throughput of isolates (Oliver et al., 1998;Döpfer et al., 1999; Zadoks and Schukken, 2006). This method does have a propensity for false results, since different amplified regions in different bacterial genomes may have the same length that would not be distinguishable from each other by RAPD PCR, and different species/strains may appear identical. However, the above results found more different amplification patterns than identical, suggesting this bias should not have severely influenced the final conclusions. In the case of identified identical strains, reinfection with the same strain cannot be ruled out, thus lowering the number of persistent cases than reported numbers, mentioned previously in the results.

4. Conclusion

Overall, about one-third of all recurrent cases (145 of 445) were caused by same species. The RAPD PCR results confirmed the frequency of persistent recurrent infections with the same strain at 11 % (49 of 445). Persistence and recurrence vary by pathogenic agents. Pathogens, such as *Strep. uberis*, may have high recurrence frequency but low persistence frequency. In conclusion, the enforcement of preventive methods to avoid new infections along with the improvement in treatment regimens is necessary for effective disease management of recurrent clinical mastitis.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108682.

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