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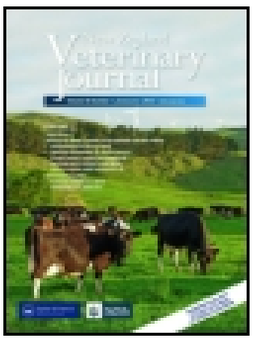

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Scientific Article

The ability of four strains of *Streptococcus uberis* to induce clinical mastitis after intramammary inoculation in lactating cows

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Abstract

AIM: To compare the ability of four strains of *Streptococcus uberis* at two doses to induce clinical mastitis in lactating dairy cows after intramammary inoculation in order to evaluate their usefulness for future experimental infection models.

MATERIALS AND METHODS: Four field strains of *Streptococcus uberis* (26LB, S418, and S523 and SR115) were obtained from cows with clinical mastitis in the Wairarapa and Waikato regions of New Zealand. Twenty-four crossbred lactating cows, with no history of mastitis and absence of major pathogens following culture of milk samples, were randomly allocated to four groups (one per strain) of six cows. Each cow was infused (Day 0) in one quarter with approximately 10^4 cfu and in the contralateral quarter with approximately 10^6 cfu of the same strain. The other two quarters remained unchallenged. All four quarters were then inspected for signs of clinical mastitis, by palpation and observation of the foremilk, twice daily from Days 0–9, and composite milk samples were collected from Days 0–8 for analysis of somatic cell counts (SCC). Quarters were treated with penicillin when clinical mastitis was observed. Duplicate milk samples were collected and cultured on presentation of each clinical case and on Day 4 from challenged quarters with no clinical signs.

RESULTS: Clinical mastitis was diagnosed in 26/48 (54%) challenged quarters. Challenge with strain S418 resulted in more cases of mastitis (12/12 quarters) than strains SR115 (7/12), 26LB (6/12) or S523 (1/12), and the mean interval from challenge to first diagnosis of mastitis was shorter for S418 than the other strains ($p<0.001$). The proportion of quarters from which *S. uberis* could be isolated after challenge was less for strain 26LB (1/6) than SR115 (6/7) ($p<0.05$), and SCC following challenge was lower for strain S523 than the other strains ($p<0.05$).

CONCLUSIONS: There were significant differences between the strains in the proportion of quarters developing clinical mastitis, the interval to mastitis onset, SCC following challenge and the proportion of clinical cases from which *S. uberis* could be isolated. These results illustrate the difference in the ability of *S. uberis* strains to cause mastitis and the severity of the infections caused.

CLINICAL RELEVANCE: Experimental challenge models can be used to compare infectivity and pathogenicity of different strains of mastitis-causing bacteria, the efficacy of pharmaceutical products and host-responses in a cost-effective manner.

KEY WORDS: *Mastitis, Streptococcus uberis, challenge model, bovine, bacterial strains*

PBS Phosphate buffered saline

SCC Somatic cell count,

SCS Somatic cell score

Introduction

Streptococcus uberis has been reported as the predominant environmental mastitis-causing organism in New Zealand (Lopez-Benavides *et al.* 2007; McDougall *et al.* 2007; Petrovski *et al.* 2009), and overseas (Jayarao *et al.* 1999; Zadoks *et al.* 2005). *S. uberis* strains differ genetically and consequently their virulence factors are expected to vary. For instance, the presence or absence of a genetically coded capsule, plasmin activator or *S. uberis* adherence molecule was shown to determine the ability of strains to colonise the mammary gland (Hill 1988a; Leigh and Lincoln 1997; Almeida *et al.* 2006).

In vivo hypothesis-testing research on bovine mastitis is generally based on comparisons between naturally infected cases, or the use of experimentally infected animals. Field studies using naturally

infected animals can be costly and require the monitoring and use of a large number of animals to identify enough cases to produce adequate statistical power. This is due to the variability which exists between farms, infectious doses, bacterial strains and environments. Longitudinal studies based on the natural incidence of mastitis can require years to recruit a sufficient number of cases (Deluyker *et al.* 1999; Zadoks *et al.* 2001).

Experimental infection or challenge studies require less time to acquire cases and are therefore quicker to conduct than field studies and should require fewer animals (Pedersen *et al.* 2003; Rambeaud *et al.* 2003). They also have advantages from an animal welfare perspective, as closer veterinary monitoring can be provided, and treatment of clinical infections can thus start earlier than in field studies. Many challenge models have been developed using different strains of *S. uberis* (Oliver *et al.* 2003; Petrovski *et al.* 2011), *Staphylococcus aureus* (Boddie and Nickerson 1996), *Escherichia coli* (Heyneman *et al.* 1990; Kutila *et al.* 2004), and other major mastitis pathogens. Different aspects of infection have been evaluated including the incidence of clinical mastitis after challenge, the incubation time to detection of clinical mastitis, the dose required to induce infection and the immune response (Moyes *et al.* 2009, 2010; Simojoki *et al.* 2009). *S. uberis* challenge models comparing the effects of different doses of the same strain introduced in quarters of different cows have also been described (McDougall *et al.* 2004), as well as those assessing between-strain variability (Hill 1988b; Pryor *et al.* 2009; Tassi *et al.* 2013; Khazandi *et al.* 2015).

The aim of the present study was to develop a *S. uberis* intramammary infection model suitable for future studies. For this, four strains isolated from natural cases of mastitis in New Zealand were compared for their ability to cause clinical mastitis in lactating dairy cows at two different doses. The outcomes assessed were the proportion of quarters diagnosed with clinical mastitis following challenge, the interval to the onset of clinical signs and the effects of infection on somatic cell counts (SCC).

Materials and methods

Animal selection, allocation to treatments and husbandry

The study was performed at the Massey University Nº1 Dairy farm (Palmerston North, New Zealand) and was approved by the AgResearch Grasslands Animal Ethics Committee (authorisation Nº 12463). The farm had a herd of approximately 170 crossbreed autumn-calving cows. All cows with the following parameters were eligible for enrolment in the study: four functional quarters, no history of mastitis in the current lactation, no antimicrobial treatments received in the 14 days prior to the commencement of the study and a composite milk SCC <250,000 cells/mL at a herd test carried out 6 days prior to challenge. On the day of the screening, all cows were 2–5 years old and

the mean days in milk was 174 (SE 49). Thirty-five cows met the pre-selection criteria and quarter milk samples were collected from these cows for bacteriological analysis. Eleven cows were eliminated due to contaminated samples or infection with major pathogens (*S. aureus*), leaving 24 cows for enrolment. Cows with minor pathogens present (e.g. coagulase-negative staphylococci or *Corynebacterium* spp.) were included.

Cows were allocated to one of four groups (one per strain) of six cows, using blocked randomisation based on milk yield at the pre-screen test. This was done by ranking the 24 cows according to descending milk yield and placing them into six blocks. Block 1 consisted of cows with the highest yields (11.5–14 L/day); Block 2 cows with milk yields between 10–11.5 L/day, cows in Block 3 had milk yields of 9.5 L/day, in Block 4 had yields of 9.0 L/day, in Block 5 had yields between 8.5–9.0 L/day, and in Block 6 between 7.5–8.0 L/day. One replicate from each block was randomly assigned to each of the four groups. Cows were also randomly allocated to be challenged in either the front right/rear left or front left/rear right contralateral quarters with either high or low concentrations of the challenge suspension. Another level of blocking ensured an even distribution of doses among front and rear quarters, so that for each strain, three cows were inoculated with the high dose in the front quarters.

The challenged cows were managed as a single group, were milked twice daily and were fed by grazing predominantly perennial rye grass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture. Water was available *ad libitum*.

Selection of strains and preparation of the challenge suspension

The four *S. uberis* strains used for the challenge originated from cows with clinical mastitis from the Wairarapa and Waikato regions of New Zealand. Initially, seven strains were taken at random from a collection maintained in glycerol at −80ºC at the Institute of Veterinary, Animal and Biomedical Sciences microbiology laboratory (Massey University, Palmerston North, NZ). The strains were re-identified phenotypically as *S. uberis* according to the following criteria: positive Gram stain, negative catalase reaction, positive aesculin and inulin reactions and negative growth in buffered azide glucose glycerol broth. The results of a standard *in vitro* disc diffusion antimicrobial susceptibility test (Wikler *et al.* 2006) indicated all these isolates were susceptible to penicillin and the three strains showing the widest inhibition zone diameters were selected for the study (strains 26LB, S418, and S523). This selection criterion was established to safeguard animal welfare and to increase the likelihood of cure after antimicrobial treatment of the induced infections. The fourth strain used for the challenge (SR115) was also isolated from an infected cow and was provided by

Dr S McDougall (Cognosco, Anexa FVC, Morrinsville, New Zealand). Antimicrobial susceptibility testing indicated this strain was also susceptible to penicillin.

Preparation of the challenge suspension of *S. uberis* strains was carried out as follows. Four days before the challenge, an aliquot of each frozen strain was streaked onto a Columbia agar plate supplemented with 5% defibrinated sheep blood (Fort Richard, Auckland, NZ). The plate was incubated at 37ºC for 48 hours in 5–10% CO_2-enriched environment. The plates were observed for purity, and a number of colonies were suspended in 10 mL phosphate buffered saline (PBS), pH 7.3. Bacterial suspensions were mixed and 10 sheep blood agar plates were flooded with 100 µL of suspension and incubated for 48 hours as above. On the day of the challenge (Day 0), bacteria were harvested using sterile cotton swabs and re-suspended in PBS to a visual turbidity corresponding to the 0.5 McFarland standard (Remel, Lenexa, KS, USA). Such a procedure was previously shown to generate bacterial suspensions containing approximately 10^7 cfu/mL (Petrovski *et al.* 2011). Following the turbidity adjustment, these suspensions were serially diluted in PBS to make 1:10 and 1:100 dilutions. These suspensions were expected to yield bacterial counts of approximately 10^6 cfu/mL (high dose) and 10^4 cfu/mL (low dose). The suspensions were prepared at volumes that were sufficient to challenge the quarters and for retrospective bacterial counting and purity testing. Four mL of the prepared suspensions were transferred into series of syringes for intramammary administration.

Retrospective purity testing and bacterial counting was performed on the same day using aliquots of the PBS that was used to suspend the bacterial colonies and syringes containing microbial suspensions. The serial dilution viable count method conducted in triplicate was used for bacterial counting. Briefly, 1 mL of the contents of each one of three syringes containing the final microbial suspension (high and low) was serially diluted ten-fold in PBS and 100 µL-aliquots of 1:100, 1:1,000 and 1:10,000 dilutions were spread onto blood agar plates and incubated as above. The arithmetic means of the number of colonies that grew on the three plates inoculated with these dilutions showing 30–300 bacterial colonies were used to estimate the number of cfu/mL of the inoculum in each challenge suspension, for each of the four strains used for the challenge.

Challenge procedure

Immediately after the morning milking, each cow was experimentally challenged once in two contralateral quarters as described above. Before inoculation, teat-ends were thoroughly cleaned using cotton swabs moistened with 70% ethanol. The entire contents of the syringe (4 mL) was inoculated into a quarter after full insertion of the syringe nozzle through the teat canal. The two unchallenged quarters served as negative controls.

Clinical assessments

Clinical examinations were conducted twice daily following the challenge, for 10 consecutive days (Days 0–9) during each milking. Prior to milking, the foremilk of each quarter was examined for presence of milk clots or colour changes. Following milking, individual quarters were inspected and palpated for clinical signs of mastitis: heat, swelling, redness and/or pain. A clinical score (0–5) was determined for each quarter based on the signs observed, as previously described (Petrovski *et al.* 2011). Quarters were considered to have clinical mastitis if the clinical score was ≥3, when evidence of irritation and/or pain of a moderate intensity or moderate redness were observed, swelling and/or heat of the quarter was detected and/or the secretion contained small clots or flecks. Scores 0–1 indicated absence of clinical mastitis.

Quarters diagnosed with clinical mastitis were treated immediately after the milking at which the diagnosis was made with a penicillin-based intramammary product (Lactapen G, 26.7% w/v penicillin procaine, Bomac Laboratories, Ltd., Auckland, NZ) three or six times as necessary for recovery. A systemic penicillin-based antibiotic (Penethaject, 33% w/v penthemate hydriodide, Bomac Laboratories Ltd.) was used in addition to the intramammary treatment for cows which had quarters with clinical scores ≥4.

Collection of milk samples

Composite milk samples from the four quarters were collected using in-line milk samplers each morning from Days 0–8. These samples were used for SCC determination at a private laboratory (MilkTestNZ, Hamilton, New Zealand). Logistics prevented the collection of quarter milk samples for SCC analysis. Duplicate milk samples were collected aseptically from each quarter diagnosed with clinical mastitis and cultured. The second sample was cultured only if the first sample was considered contaminated. In order to estimate the total number of intramammary infections and identify non-clinical mastitis, milk samples were also collected from all the challenged quarters not affected with clinical mastitis on the morning of Day 4.

Culturing of milk samples

The culture procedures and pathogen identification followed established guidelines (Hogan *et al.* 1999). Briefly, 10 µL of aseptically collected single-quarter milk from each cow was deposited as a drop and spread onto one quarter of a Columbia agar plate supplemented with 5% defibrinated sheep blood (Fort Richard). Plates were incubated at 37°C in an aerobic environment and bacterial growth was observed after 24 and 48 hours incubation. A sample was considered contaminated if more than two colony types were visible after 48 hours and was not further analysed.

Statistical analysis

The cumulative proportions of quarters diagnosed with clinical mastitis were calculated for each strain, and the relative risk for developing mastitis, with 95% CI, was calculated between strains using Episheet 2004 (http://www.epinet.se/Epidemiologicaltools.htm; Rothman 2002). The mean interval to first diagnosis of clinical mastitis for the strains (expressed as days after challenge) was compared using Kaplan-Meier survival analysis using XL-Stats package (XL-Stats 2012, Addinsoft SARL, Paris, France). The proportion of quarters with clinical mastitis from which *S. uberis* was isolated after culture was compared between strains using a two-tailed Fisher's exact test.

Statistical comparison of SCC was performed at the cow level, as only composite milk samples were collected daily. Individual cow SCC were not normally distributed and were therefore transformed into somatic cell scores (SCS), calculated as:

$SCS=\log_2 (SCC/1000)$

The effect of strain on SCS after challenge was analysed using a repeated measures analysis of variance using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). The model included the fixed effect of strain and day of sampling, the interaction of strain and dose, and a random effect of cow to account for within-cow correlation between measures.

Results

Retrospective bacterial challenge count

The challenge suspensions of the four strains yielded pure *S. uberis* cultures and no bacterial growth was detected from the PBS alone. The results of the retrospective viable bacterial counting are given in Table 1.

Diagnosis of mastitis

Clinical mastitis was diagnosed in 26/48 (54%) challenged quarters. No unchallenged quarters developed clinical or subclinical mastitis, therefore no samples from unchallenged quarters were cultured. The proportion of quarters diagnosed with clinical mastitis for each strain and dose are presented in Table 1. Challenge with strain S418 resulted in more cases compared with any of the other strains; the RR compared with SR115 was 1.7 (95%CI=1.1–2.8); compared with 26LB was 2 (95%CI=1.1–3.5) and with S523 was 12 (95%CI=1.8–78.4). Challenge with strains SR115 and 26LB resulted in more cases than strain S523 (RR 7 (95%CI=0.9–52.0) and 6 (95%CI=0.8–40.3), respectively). The peak of clinical mastitis incidence was observed 48–72 hours after challenge. The Kaplan Meier analysis indicated a difference in the interval to onset of clinical mastitis between

the four strains ($p<0.001$), with the interval to diagnosis being shortest following challenge with strain S418 (Figure 1; Table 1).

Streptococcus uberis was isolated from 20/26 (73%) of the clinical cases identified between Days 1 and 4. No organisms other than *S. uberis* were isolated from milk samples from the clinical cases. Seven cases (27%) yielded no growth. Fewer clinical cases diagnosed following challenge with strain 26LB resulted in isolation of *S. uberis,* compared with strain SR115 ($p<0.05$; Table 1). No bacteria were isolated from any of the milk samples collected from the challenged quarters which had no clinical mastitis.

Somatic cell count

Mean SCS increased on Day 1 in all treatment groups and remained elevated until the end of the study (Figure 2). Overall SCS differed between strains ($p<0.05$), and the rise in SCS was less evident in the cows challenged with strain S523.

Discussion

This study compared the ability of four strains of *S. uberis* to induce clinical mastitis in healthy quarters after intramammary inoculation of two different bacterial concentrations and resulted in the identification of one suitable strain for subsequent clinical trials.

The overall percentage of quarters diagnosed with clinical mastitis was 54% with variability observed between the four different strains. The percentage of cases observed was low compared with other studies carried out using different strains of *S. uberis*. Oliver *et al.* (2003) and Rambeaud *et al.* (2003) reported approximately 80% of quarters developing clinical mastitis following challenge using a single *S. uberis* strain UT888, and 90–100% being reported using other strains (Hillerton and Bramley 1989; Pryor *et al.* 2009).

The RR for development of mastitis was significant for strain S418 compared with the other strains. There were also differences in the SCS measured in composite milk samples following challenge, even if only one or two infected quarters per cow were present. Logistic limitations of the study meant that quarter milk samples could not be obtained, so analysis of the direct effect of challenge with the four strains on SCS was not possible. The other limitation of this study was the modest number of cows challenged compared to other studies.

It has been postulated that survival of different *S. uberis* strains in the mammary gland might be multifactorial and related to individual strain virulence factors and adaptability to the host (Pryor *et al.* 2009; Tassi *et al.* 2013). This suggestion is consistent with the results in the current study, where

the prevalence of mastitis differed between the strains despite the strains being exposed to similar *in vitro* and *in vivo* conditions; i.e. similar inoculation method, cows were from a single farm and treatments were balanced for milk yield, and of comparable ages.

The challenge dose for strain S523 were greater than most of the other strains but this strain resulted in the lowest number of clinical cases and the increase in SCS was relatively moderate in comparison with the other strains. Conversely, strain 26LB had the lowest viable bacterial counts, but produced more clinical cases than strain S523. It is therefore likely that there were strain-related virulence mechanisms responsible for these differences, which overwhelmed any possible dose effect.

The peak of clinical mastitis incidence was observed 48–72 hours after challenge. Rambeaud *et al.* (2003) observed a peak in the number of new clinical cases later, at 144 hours after challenge, when using a challenge suspension at a dose of 3.3 x 10^4 cfu for Jersey cows and 5.2 x 10^4 cfu for Holstein cows, slightly lower than the dose used in the present study. In another study, inoculation of 7.5 x 10^3 cfu/ challenge suspension generated the highest number of cases on 7 days after challenge (Oliver *et al.* 2003). It is possible that there is a relationship between the infective dose and the time of onset of clinical mastitis, but not necessarily between the infective dose and mastitis prevalence. Previous studies have demonstrated mastitis prevalence after challenge was associated with a strain's virulence rather than the infective dose used (Leigh 1999; Oliver *et al.* 2003; Rambeaud *et al.* 2003). The host immune system is another factor that is likely to contribute to differences in development of clinical mastitis following infection (Sordillo and Streicher 2002; Moyes *et al.* 2009).

It was important to isolate bacteria after culture of the milk samples in order to demonstrate that the pathogen inoculated was responsible for the clinical cases observed. Interestingly, only 1/6 cases diagnosed following challenge with strain 26LB resulted in isolation of *S. uberis* after culture. The milk culturing technique used in this study used a 10 µL aliquot of milk for culture on the agar plates. It is common to find approximately 10% of the samples to be negative at culture when inoculating such a small volume of milk (Milne *et al.* 2005). However, low inoculum volume alone does not explain such a low proportion of positive culture results in the case of this strain. Again, strain-related factors are likely to have played a role (Tassi *et al.* 2013). The lack of genetic typing data for the strains used in this study was a limitation.

This paper describes an experimental challenge model of clinical mastitis using four field strains of *S. uberis* s. Such models may be a useful tool to test the efficacy of new pharmaceutical products in

a safe and cost-effective manner, and could have additional scientific value for the study of the host response and further characterisation of the pathogenicity of the organisms.

The differences observed between the strains in some of the outcomes measured appeared to be related to the characteristics of the strains, rather than the number of bacteria inoculated. Challenge with strain S418 resulted in the highest prevalence of clinical mastitis whereas strain S523 resulted in the lowest prevalence. The proportion of cases of mastitis from which *S. uberis* could be isolated after challenge with strain 26LB was lower than the other strains. Based on its moderate ability to produce clinical mastitis in lactating cows and isolation after culture, *S. uberis* strain SR115 was selected for use in further experiments using challenge models. Taken together these results also predict a significant variability in the pathogenicity of *S. uberis* strains under field conditions.

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Table 1. Results of a challenge study to examine differences between two concentrations of four strains of *Streptococcus uberis* infused into the quarters of lactating dairy cows (n=24), showing the dose of bacteria given, the proportion of quarters diagnosed with clinical mastitis, the median interval to diagnosis of the first case of mastitis, and the proportion of quarters with mastitis from which *S. uberis* was cultured.

			Clinical mastitis		
Strain	**Concentration**	**Dose (cfu)**	**Quarters**	**Mean survival (95%CI) [a]**	***S. uberis* isolated**
S418	Low	9.84×10^4	6/6		
	High	4.26×10^6	6/6		
	All		12/12	2.42 (2.02–2.81)	12/12
SR115	Low	5.92×10^4	2/6		
	High	2.56×10^6	5/6		
	All		7/12	3.17 (2.62–3.72)	6/7
26LB	Low	2.4×10^3	4/6		
	High	1.58×10^5	2/6		
	All		6/12	3.83 (3.31–4.36)	1/6
S523	Low	1.2×10^5	1/6		
	High	2.96×10^6	0/6		
	All		1/12	3	1/1

[a] Interval from challenge to first diagnosis of clinical mastitis (days), from Kaplan-Meier survival analysis

Figure 1. Cumulative percentage of quarters of lactating dairy cows (n=24) diagnosed with clinical mastitis following intramammary inoculation (challenge) with one of four strains of *Streptococcus uberis* on Day 0; strain S418 (-■-), strain SR115 (-▲-), strain 26LB (-♦-) and strain S523 (-●-).

Figure 2. Mean (± SEM) somatic cell score (log_2 somatic cell count/1000) of lactating dairy cows (n=24) before and after intramammary inoculation (challenge) with one of four strains of *Streptococcus uberis* on Day 0; strain S418 (-■-), strain SR115 (-▲-), strain 26LB (-♦-) and strain S523 (-●-).