

# Efficacy of recombinant bovine epidermal growth factor in the treatment of experimental subclinical *Staphylococcus aureus* mastitis in a ewe model

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## ABSTRACT

*Staphylococcus aureus* is the most common contagious mastitis pathogen of dairy cattle. Antimicrobial treatment of infected cattle results in variable cure rates. Epidermal growth factor (EGF) plays an important role in the modulation of host innate immune responses and the regulation of mammary epithelial regeneration, indicating that EGF may be useful as a treatment for mastitis. A pilot study was conducted to evaluate the efficacy of recombinant bovine EGF (rbEGF) for the treatment of *S aureus* intramammary infection (IMI) using an ovine model. Each ewe was experimentally infected with *S aureus* in both udder halves. One udder half of each ewe received one of two treatments: EGF (n=13) or pirlimycin (n=13). The contralateral udder half of each ewe received sterile saline as a control. The bacteriological cure rate following rbEGF was significantly lower (15 per cent) than that attained with pirlimycin hydrochloride (61 per cent) and did not differ from that following treatment with sterile saline. Cure rates following treatment with rbEGF were not significantly different to those following sterile saline. Given that EGF is associated with modulation of host immunity and wound healing, future studies into EGF should not focus on whether EGF increases cure rates of *S aureus* IMI.

## INTRODUCTION

Mastitis is a highly economically significant disease. In Canada, the cost of a case of clinical mastitis has been estimated at approximately \$345 per case of clinical mastitis (Carrier 2009). A study that analysed records from 22 herds in the USA revealed that the cost of subclinical mastitis is about \$285 based on loss of 718 kg of milk during the first 210 days of lactation (Kirkpatrick and Olson 2015). *Staphylococcus aureus* is the most commonly isolated contagious mastitis pathogen in Canada (Olde Riekerink and others 2006, Olde Riekerink and others 2008). *Staphylococcus aureus* intramammary infection (IMI) causes persistent subclinical disease with clinical flare-ups. Several different intramammary

antimicrobial treatments have been used to treat *S aureus* IMI with highly variable cure rates. The cure rate of *S aureus* IMI is influenced by host, pathogen and treatment factors (Barkema and others 2006). Cure rates of subclinical *S aureus* mastitis during lactation are influenced by parity, stage of lactation, somatic cell count and quarter (Sol and others 1997). A systematic review determined that cure rate of *S aureus* mastitis following antimicrobial treatment is less than 50 per cent (Roy and Keefe 2012). Poor cure rates of *S aureus* IMI following antibiotic treatment demonstrate the importance of novel treatment development for *S aureus* IMI.

Epidermal growth factor (EGF) is a 53-amino acid polypeptide with biological roles including embryonic organ development, mammary morphogenesis (Vonderhaar 1987, Silberstein and others 1990), mammary cell proliferation and mammary development (Troyer and Lee 2001). Epidermal growth factor receptor (EGFR) ligands have been identified as important components in the modulation of innate immunity. These ligands are produced by inflammatory cells including neutrophils, eosinophils, monocytes, epithelial cells and fibroblasts (Calafat and others 1997, Bannerman and others 2006). These ligands are involved in the upregulation of prostaglandin production and the production of interleukin 1 (IL-1), interleukin 8 (IL-8), tumour necrosis factor and antimicrobial peptides in the mammary gland of dairy ruminants (Ezzat Alnakip and others 2014). Both IL-1 and IL-8 enhance neutrophil influx and upregulation of oxygen radical formation during the course of *S aureus* mastitis in ruminants, thus combating bacterial invasion (Daley and others 1991, Barber and others 1999). Antimicrobial peptides are



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important components of the innate immune system, and there is a direct association between EGFR activation and secretion of these peptides in various tissues such as the mammary gland and skin (Malmsten and others 2007, Tomasinsig and others 2010, Simanski and others 2016). For example, treatment of cancer patients with EGFR inhibitors results in acneiform skin toxicities due to the dysregulation of normal inflammation, disruption of normal defence, impaired antimicrobial peptide secretion (Lichtenberger and others 2013) and failure of *S aureus*-induced IL-1 secretion (Simanski and others 2016).

EGF is also an important factor in wound healing alongside tissue repair and regeneration. For example, EGF and related peptides are secreted by cells associated with wound healing such as platelets, activated macrophages, keratinocytes, fibroblasts and vascular endothelial cells. Local application of EGF on wounds results in enhanced growth, healing and tensile strength (Schultz and others 1991) suggesting that EGF is useful in tissue repair and healing. Treatment of bovine mammary epithelial cell cultures with EGF results in increased cell proliferation, enhanced mesenchymal epithelial transition factor expression and enhanced mammary epithelial cell motility, suggesting that EGF can play a role in healing of mammary epithelia (Matthay and others 1993, Accornero and others 2009). Furthermore, experimentally induced *Streptococcus agalactiae* mastitis resulted in significantly higher EGF levels in infected quarters (Sheffield 1997). This evidence suggests that EGF plays an important role in the modulation of host innate immune responses and the regulation of mammary epithelial regeneration, indicating that EGF may be useful as a treatment for mastitis. However, no evidence exists that EGF has direct antimicrobial activity. Our null hypothesis was that administration of recombinant bovine EGF (rbEGF) did not increase cure rate of *S aureus* IMI when compared with administration of sterile saline.

## MATERIALS AND METHODS

### Study animals

Fifty-seven crossbred ewes were purchased from a commercial sheep sale. These ewes were primarily of meat breed origin. All sheep had body condition scores greater than 2 on a five-point scale. The mouth of each animal was examined for broken or missing teeth. All of the selected animals had eight intact incisors. All of the sheep were bright, alert and responsive with no lameness. The whole body, including external lymph nodes and mammary gland, was palpated for possible masses, abscesses or joint swelling. Sheep were monitored for their activity and feed intake three times daily during a two-week acclimatisation period immediately before experimental procedures. Examination and selection of these animals was performed by two veterinarians. These ewes are not milking as their lambs were weaned, but all selected animals for the final trial had active udders with

presence of milk. Lacteal secretions from each udder half were cultured three times at one-week intervals, and 37 culture-negative ewes were selected for experimental inoculation. Animals were housed in accordance with good animal care and husbandry practises as defined by the Canadian Council on Animal Care (Tennessen and others 2009) and were provided with full veterinary care. Experimental protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply (protocol no. 20110139).

### Experimental bacterial inoculation

An *S aureus* isolate that had been previously used in ovine mastitis models was obtained from the Vaccine and Infectious Disease Organization, University of Saskatchewan culture collection (Luby 2010). The organism was initially isolated from a subclinical bovine IMI. One colony of *S aureus* was inoculated into 5-ml sterile tryptone soy broth (TSB) and incubated at 37°C overnight in a rotating shaker. These culture media were added to sterile TSB at a 1:100 concentration and incubated at 37°C with shaking until the optical density reached 0.35 at 600 nm. Cultures were centrifuged at 3000 g at 4°C for 10 minutes, and the supernatant was discarded. Bacterial pellets were suspended in 25 ml of 1M PBS. Bacterial suspensions were centrifuged again at 3000 g at 4°C for 10 minutes, and the supernatant was discarded. Washing steps were performed to remove culture supernatant. Bacterial pellets were resuspended in 10-ml PBS. One millilitre of the final suspension was serially diluted 10-fold, and each dilution was cultured on trypticase soy agar (TSA) with 5 per cent sheep blood agar at 37°C overnight while all remaining dilution samples were kept refrigerated at 4°C. Bacterial suspensions of 50 colony-forming units (cfu) in 2 ml sterile 1M PBS were made based on colony counts. This dose of bacterial suspension was successfully produced subclinical *S aureus* infection in sheep in previous trials (Luby 2010). Ewes were challenged immediately following inoculum preparation, and two quality control cultures were performed immediately before and after inoculation to ensure that bacterial load did not change with time. Each udder half was inoculated aseptically using a plastic teat cannula (Jorgensen Laboratories, Colorado, USA). Milk samples were cultured according to National Mastitis Council guidelines (Hogan and others 1999). Briefly, 10 µl of secretion from each udder half was plated on half a sterile TSA plate with 5 per cent sheep blood and incubated at 37°C. Bacterial growth was determined at 24 and 48 hours, and bacterial shedding was quantified. Colonies were presumptively identified according to colony morphology, haemolysis pattern and catalase test result. *S aureus* isolates were confirmed by the tube coagulase test. All of the inoculated udder halves were cultured three times during the 17-day postinoculation period. The first two samples were taken at a weekly interval, and last the sample was taken just before commencing treatment on day 17. Udder halves were defined as infected if the last sample was culture positive for *S aureus*.

### Experimental treatment groups and bacterial cultures

Thirty-one sheep with confirmed *S aureus* IMI were randomly assigned initially to two experimental groups by drawing lots. All of the ewes were housed together, and all of the individuals that were involved in treatment, sample collection and sample processing were blinded with regard to group. Experimental treatments were prepared in the laboratory by a separate individual and labelled in a coded manner. The blinding was removed following statistical analysis of the data. One group (EGF; n=13) was treated with 1-mg rbEGF (Zyme Fast, Winnipeg, Manitoba, Canada) in 2-ml sterile endotoxin-free PBS in a single udder half at weekly intervals for three weeks (Daley and others 1993). The other group (pirlimycin; n=13) was treated with 5-mg pirlimycin hydrochloride (Pirsue sterile solution; Zoetis, Kirkland, Quebec, Canada) in a single udder half daily for three consecutive days. Time from infection to first treatment was 17 days. One udder half from each animal was treated with 1-ml sterile endotoxin-free PBS at the same time as experimental treatment to serve as a control. Bacteriological cure was defined as secretions from an udder half being culture negative for three consecutive samples collected at one-week intervals following the final treatment. Milk samples that were negative were confirmed as such by inoculation of the sample in 5 ml of brain–heart infusion (BHI) broth for 24 hours at 37°C and plating on TSA with 5 per cent sheep blood.

Data were analysed using statistical analysis software (Stata V.13.1). Association between cure rates and each treatment with its control was compared using a McNemar test ( $P<0.05$ ). The comparison between EGF and pirlimycin groups was performed using Fisher's exact test ( $P<0.05$ ).

### RESULTS

Each animal received a challenge dose of 50 cfu of *S aureus* in 2-ml sterile 1M PBS. Thirty-one sheep were confirmed as infected with *S aureus*, and five sheep were removed from the final analysis due to dry udder halves. Of the samples that were initially culture negative, three were reclassified as culture positive following BHI incubation.

The cure rate in the rbEGF-treated udder halves (2/13, 15 per cent) was not significantly different to the cure rate in the saline-treated control halves of the same animals (2/13, 15 per cent) ( $P=1.000$ ). The cure rate in the pirlimycin-treated udder halves (8/13, 61 per cent) was significantly higher than the cure rate in the saline-treated control halves of the same animals (1/13, 7 per cent) ( $P=0.015$ ). Cure rate was significantly higher in the pirlimycin-treated udder halves when compared with the rbEGF-treated udder halves ( $P=0.040$ ).

### DISCUSSION

This is the first study that has investigated the efficacy of rbEGF for the treatment of *S aureus* IMI. Our findings indicate that the efficacy of rbEGF was no better than

that of sterile saline and significantly worse than that of pirlimycin hydrochloride. Based on a systemic review of published studies, pirlimycin hydrochloride has the greatest efficacy among lactating cow products labelled in Canada for *S aureus* IMI (Roy and Keefe 2012). Thus, rbEGF does not appear to be a promising treatment for *S aureus* IMI.

The lack of efficacy of rbEGF in the current study could be due to inadequate dose and interval of rbEGF treatment. Our rbEGF dose was selected based on a previous safety trial carried out in cull dairy cows (C. Luby, unpublished data). In that study, 10 mg rbEGF resulted in significant systemic clinical signs in treated cattle, whereas 5 mg did not. Given the relative body mass of cattle and sheep, we anticipated that 1 mg rbEGF would be the maximum safe dose to use in this trial.

We selected a pirlimycin dose of 5 mg by comparing the body mass of our ewes with those of adult cattle. Given that the cure rate following pirlimycin treatment is consistent with those reported by other studies, we believe that 5 mg pirlimycin was appropriate for our treatment model. For example, Deluyker and others (2005) reported *S aureus* cure rates of 56 per cent and 86 per cent after two and eight consecutive days of intramammary pirlimycin treatment in lactating cows, respectively. Other studies have observed lower cure rates for pirlimycin. Owens and others (1997) reported a cure rate for naturally occurring *S aureus* IMI of approximately 35 per cent following pirlimycin treatment. The duration of infection in our study was 17 days before treatment. Chronic infection can cause severe pathological udder changes that result in reduced bacteriological cure rate (Sol and others 1997, Barkema and others 2006). If the time between infection and treatment had been longer in our study or the study was carried out using naturally infected lactating animals, we would have likely observed decreased cure rates following pirlimycin treatment.

Cyclical and variable shedding patterns of *S aureus* from infected mammary glands have been reported (Sears and others 1990, Walker and others 2011, Britten 2012). During traditional culture methods (Hogan and others 1999), only 10 µl of milk is used. With this sample volume, for a sample to be culture positive, the original sample should contain a bacterial concentration of  $\geq 100$  cfu/ml (Britten 2012). Animals that are shedding bacteria below this detection limit would invariably be missed when traditional culture methods are used. To improve the detection of such low-level shedders, strategies such as repeated culturing, use of enrichment media for reculturing, culturing a larger volume of sample or PCR could be performed (Britten 2012). In this study, culturing milk weekly for 3 weeks post-treatment was performed to reduce false-negatives due to cyclical shedding. Sears and others (1990) found that the sensitivity of milk cultures was 75 per cent on the first milk sample, increasing to 94 per cent and 98 per cent with two and three consecutive samples, respectively. In this study, BHI enrichment step resulted in 7 per cent (3/41) of these

negative samples being identified as truly positive for *S aureus*. Similarly, Sol and others (2002), who used a broth incubation technique, showed a 9.5 per cent increase in the isolation percentage than standard culture.

Culturing a larger volume of milk was not attempted due to the low volume of milk that could be recovered from study animals. Studies have shown PCR to be a very useful technique in identifying low levels of *S aureus* shedding (Britten 2012, Botaro and others 2013). A major advantage of PCR is the high speed of detection that typically takes around 4 hours in comparison with 1–2 days required for the standard culture technique. The high specificity of PCR tests due to the amplifying of minute amounts of microbial DNA ensures the early detection of individuals that are shedding low levels of bacteria. However, the inability to differentiate DNA of live bacteria from that of dead bacteria remains a major problem in the use of PCR in that a false-positive result may occur in an individual that has resolved a past infection (Britten 2012). Therefore, PCR was not attempted in the current study.

Initially, there were 57 ewes were selected for screening, and only 37 of them were enrolled for *S aureus* challenge. Finally, 26 animals were available for final analysis in this pilot study. Given that this was a pilot study, that sample size calculation was not performed. However, based on our observed cure rates for pirlimycin and rbEGF, a larger sample would be highly unlikely to show a positive effect of rbEGF on cure rate of *S aureus* IMI.

*S aureus* causes subclinical mastitis in both cattle and sheep (Mørk and others 2005, Pugh and Baird 2011). Although the mammary gland of cows and ewes have similar morphogenesis, the milk secretory mechanism is different in both species. Ewe milk is considered an apocrine secretion, while cow milk is predominantly a merocrine secretion (Ruegg 2011, Capuco and Ellis 2013). Ewe milk consists of higher fat, proteins and total solids comparatively to cow milk (Hadjipanayiotou 1995), and these selected meat purpose ewes were not actively lactating. Furthermore, dairy cows have been more aggressively selected for high milk production. These differences might influence the immunology and behaviour of pathogens in the mammary gland of these two species.

The ewes that were used for this experiment were not being milked. In ruminants, while milk volume is minimal during the dry period, the concentration of leucocytes and other immune components such as immunoglobulin and lactoferrin are present at high concentrations (Snedeker and others 1991, Kenney and others 1995, Tatarczuch and others 1997). Increased activities of EGF and its ligands have been reported during mammary involution in mice (Capuco and Akers 1999, Darcy and others 1999, Andreotti and others 2014). The cure rate following dry cow therapy is higher for *S aureus* mastitis than cure rate following therapy during lactation (Keefe 2012). Higher therapeutic drug concentrations can be achieved during the dry period due to absence of

dilution. Furthermore, mammary tissue is exposed to the drug for a longer time than during lactation. Also, the risk of exposing cows to bacteria during milking procedures is minimal, and the udder has more time to regenerate damaged tissues (Royster and Wagner 2015). If rbEGF is ineffective as a treatment of *S aureus* IMI in ewes that are not being milked, it will most likely be not effective as a treatment during lactation.

In conclusion, treatment with rbEGF did not increase cure rates of *S aureus* IMI when compared with sterile saline. As such, studies into the cure of *S aureus* IMI following treatment with rbEGF are unwarranted. Future studies may be warranted that focus on whether rbEGF is effective at healing damaged mammary epithelia following cure of IMI with antimicrobials.

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**Data sharing statement** All raw data from the study are available by contacting the corresponding author.

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