Post-weaning management strategies and impacts on ewe subclinical mastitis and antimicrobial susceptibility

Ryan M. Knuth, Kelly L. Woodruff, Gwendolynn L. Hummel, Jordan D. Williams, Whitney C. Stewart, Hannah C. Cunningham-Hollinger, and Bledar Bisha

Department of Animal Science, University of Wyoming, Laramie, WY 82071, USA

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INTRODUCTION

Mastitis, defined as the inflammation of mammary tissue, often results from microbial infection (Kahn and Line, 2010) and is prevalent from parturition to weaning. Subclinical mastitis is prevalent (Ahmad et al., 1992a), although on-farm diagnosis is difficult, especially in non-dairy animals, and bacterial culture remains the standard diagnostic tool. Through managing environmental pathogens, animal and facility management practices may reduce the prevalence of subclinical mastitis. For example, withholding feed from ewes for 48 h following weaning with an additional 2 wk of low-energy, low-protein feed is recommended (American Sheep Industry Association Inc., 2015). The use of antimicrobials to control mastitis and other diseases in the livestock industry (Sawant et al., 2005; Raymond et al., 2006) raises concerns of antibiotic resistant strains affecting human consumers (Skovgaard, 2007). Studies evaluating antimicrobial resistance in dairy sheep report variability in resistance, though bacterial strains most commonly display resistance to penicillin and ampicillin (Concepción Porrero et al., 2012; Azara et al., 2017).

Therefore, the objectives of this study were to quantify the prevalence and etiological agents of subclinical mastitis at weaning, analyze the antimicrobial susceptibility in ewe milk, and determine the effectiveness of weaning treatments to mitigate subclinical mastitis. We hypothesized that most ewes would present subclinical mastitis around weaning and antimicrobial resistance of isolates would be low in the absence of prolonged treatment with antimicrobials.

MATERIALS AND METHODS

The experimental protocol was approved by the University of Wyoming Institutional Animal Care and Use Committee (20200214HC00408-01).

Animals and Management

Multiparous commercial Rambouillet ewes (n = 22) were managed by standard Laramie Research and Extension Center practices and lambed between March and April. Prior to weaning, ewes were assigned to 1 of 3 treatment groups: 1) intramuscular injection of penicillin (PENN; n = 8; Durvet; penicillin G procaine injectable suspension; 300,000 units mL⁻¹; 1 mL per 45 kg BW) at weaning, 2) restricted feed access 48 h prior to and 72 h post-weaning (FAST; n = 6), and 3) a combination of penicillin at weaning and restricted feed access (COMBO; n = 8).

For FAST and COMBO rations, ewes consumed 1.57% to 1.72% BW grass hay per day, whereas PENN continued to consume 2.85% and 2.47% BW grass and alfalfa hays per day, respectively.

Milk Collection

Milk samples were collected at weaning (68.31 ± 5.47 d) and post-weaning (71.31 ± 5.47 d). Teats were scrubbed with 70% ethanol and
the first streams of residual milk were discarded. Approximately 20 mL of milk was obtained from the two udder halves. All aliquots were frozen at −80 °C until sample preparation.

**Microbial Culturing and Identification**

Thawed milk samples were streaked onto plates containing microbiological media (MacConkey agar; trypticase soy agar (TSA); and TSA + 5% sheep blood agar). Plates were incubated (37 °C for 24 h) and those that exhibited no growth were incubated an additional 24 h. Colony counts and morphologies were recorded, and culture-positive samples were considered to have subclinical mastitis (Dohoo et al., 2011) and then were sub-cultured for isolation and used to make a preserved freezer stock.

Cultures were re-activated on TSA plates for identification via matrix-assisted laser desorption/ionization spectrometry (MALDI-TOF MS). The extended direct colony transfer method was used by transferring colonies onto a steel-target plate where 70% formic acid (1 µL) and HCCA matrix (1 µL; α-cyano-4-hydroxycinnamic acid) were added to each well. Bruker Biotyper RTC software (Version 3.1) was used for MALDI-Biotyping (Proteomics and Metabolomics Facility; Colorado State University; Fort Collins, CO).

**Antimicrobial Susceptibility Testing**

Bacteria with a high-quality score for a MALDI-TOF MS identification match were subjected to antimicrobial susceptibility testing. After re-activation of cultures using above methods, colonies were added to demineralized water to achieve a 0.5 McFarland standard before 10 µL of suspension (30 µL for staphylococci) was added to Mueller-Hinton broth. The antibiotic plate (Sensititre Vet Mastitis CMV1AMAF; Thermo Scientific; Waltham, MA) was inoculated with 50 µL per well of suspension to test isolates against ampicillin, penicillin, erythromycin, oxacillin +2% NaCl, pirlamycin, penicillin/novobiocin, tetracycline, cephalothin, ceftiofur, and sulphadimethoxine. The plate was analyzed via a Sensititre Vizion instrument.

**Data Analyses**

Subclinical mastitis prevalence and MALDI-TOF MS identifications were analyzed within and across weaning treatment and day. Binomial

| Item                      | Treatment       | Overall       |
|---------------------------|-----------------|---------------|
| No. of samples            | 8 (88)          | 6 (83)        | 8 (100)   | 22 (91) |
| No. of positive (%)       | 7 (88)          | 5 (83)        | 8 (100)   | 20 (91) |
| Species                   |                 |               |           |         |
| Unidentified1             | 0.14 [0.00, 0.58] | 0.20 [0.01, 0.72] | 0.38 [0.09, 0.76] | 0.25 [0.09, 0.49] |
| All Bacillus spp.         | 0.29 [0.04, 0.71] | 1.00 [0.48, 1.00] | 0.62 [0.24, 0.91] | 0.60 [0.36, 0.81] |
| Bacillus circulans        | –               | –             | 0.12 [0.00, 0.53] | 0.05 [0.00, 0.25] |
| Bacillus clausii          | –               | 0.40 [0.05, 0.85] | –          | 0.10 [0.01, 0.32] |
| Bacillus licheniformis    | –               | 0.20 [0.01, 0.72] | –          | 0.05 [0.00, 0.25] |
| Bacillus megaterium       | –               | –             | 0.12 [0.00, 0.53] | 0.05 [0.00, 0.25] |
| Bacillus pumilus          | 0.29 [0.04, 0.71] | 1.00 [0.48, 1.00] | 0.38 [0.09, 0.76] | 0.50 [0.27, 0.73] |
| Bacillus subtilis         | 0.14 [0.00, 0.58] | –             | –          | 0.05 [0.00, 0.25] |
| Bacillus thuringiensis    | 0.14 [0.00, 0.58] | –             | –          | 0.05 [0.00, 0.25] |
| Brachybacterium paraconglomeratum | 0.14 [0.00, 0.58] | –             | –          | 0.05 [0.00, 0.25] |
| Mannheimia haemolytica    | –               | 0.20 [0.01, 0.72] | 0.12 [0.00, 0.53] | 0.10 [0.01, 0.32] |
| Moraxella ovis            | –               | –             | 0.12 [0.00, 0.53] | 0.05 [0.00, 0.25] |
| All Staphylococcus spp.   | 0.29 [0.04, 0.71] | 0.40 [0.05, 0.85] | 0.12 [0.00, 0.53] | 0.25 [0.09, 0.49] |
| Staphylococcus aureus     | 0.14 [0.00, 0.58] | –             | –          | 0.05 [0.00, 0.25] |
| Staphylococcus capitis    | –               | –             | 0.12 [0.00, 0.53] | 0.05 [0.00, 0.25] |
| Staphylococcus equorum    | 0.14 [0.00, 0.58] | 0.40 [0.05, 0.85] | –          | 0.15 [0.03, 0.38] |
| Streptococcus pyogenes     | 0.14 [0.00, 0.58] | –             | –          | 0.05 [0.00, 0.25] |

1Treatment: PENN = penicillin-treated ewes; FAST = feed-restricted ewes; COMBO = feed-restricted ewes treated with penicillin.

2Unidentified = culture-positive samples with at least one bacterial isolate without a successful species match.

Table 1. Number of milk samples collected at weaning (68.31 ± 5.47 d postpartum) within treatment and estimated species frequency [95% confidence interval] within culture-positive samples.
proportions and 95% confidence intervals of taxa identified within culture-positive samples were estimated using the binom package of R (Dorai-Raj, 2014; RStudio Team, 2020). The survival package of R (Therneau, 2021) was used for a survival analysis of isolates based on penicillin usage at weaning (PENN+ = PENN or COMBO; PENN− = FAST), where antimicrobial concentrations present in plate wells were used as “time” and “event” was inhibition of bacterial growth.

Table 2. Number of milk samples collected at 3 d post-weaning (71.31 ± 5.47 d postpartum) within treatment¹ and estimated species frequency [95% confidence interval] within culture-positive samples

| Item               | Treatment | Overall |
|--------------------|-----------|---------|
| No. of samples     | PENN 6    | FAST 8  | COMBO 8 | Overall 22 |
| No. of positive (%)| 6 (75%)   | 4 (67%) | 7 (88%) | 17         |
| Species            |           |         |         |            |
| Unidentified²      | –         | –       | –       | 0.14 [0.00, 0.58] | 0.06 [0.00, 0.29] |
| Bacillus spp.      | 0.50 [0.12, 0.88] | 0.25 [0.01, 0.81] | – | 0.71 [0.29, 0.96] | 0.53 [0.28, 0.77] |
| Bacillus clausii   | 0.33 [0.04, 0.78] | 0.25 [0.01, 0.81] | – | 0.14 [0.00, 0.58] | 0.24 [0.07, 0.50] |
| Bacillus licheniformis | –     | –       | –       | 0.43 [0.10, 0.82] | 0.18 [0.04, 0.43] |
| Bacillus pumilus    | 0.33 [0.04, 0.78] | –       | –       | 0.43 [0.10, 0.82] | 0.29 [0.10, 0.56] |
| Bacillus vietnamensis | –      | –       | –       | 0.25 [0.01, 0.81] | – |
| Mannheimia haemolytica | –    | –       | –       | 0.14 [0.00, 0.58] | 0.06 [0.00, 0.29] |
| Moraxella ovis      | –         | 0.25 [0.01, 0.81] | –       | – | 0.06 [0.00, 0.29] |
| All Staphylococcus spp. | 0.33 [0.04, 0.78] | 0.25 [0.01, 0.81] | 0.29 [0.04, 0.71] | 0.29 [0.10, 0.56] |
| Staphylococcus aureus | 0.17 [0.00, 0.64] | –       | –       | – | 0.06 [0.00, 0.29] |
| Staphylococcus epidermidis | –   | –       | 0.14 [0.00, 0.58] | 0.06 [0.00, 0.29] |
| Staphylococcus equorum | 0.33 [0.04, 0.78] | –       | 0.14 [0.00, 0.58] | 0.12 [0.01, 0.36] |
| Staphylococcus vitulinus | –      | 0.25 [0.01, 0.81] | –       | 0.06 [0.00, 0.29] |

¹Treatment: PENN = penicillin-treated ewes; FAST = feed-restricted ewes; COMBO = feed-restricted ewes treated with penicillin.

²Unidentified = culture-positive samples with at least one bacterial isolate without a successful species match.

Table 3. Minimum inhibitory concentration (MIC) of selected ewe milk bacterial isolates obtained from ewes administered penicillin at weaning and collected at weaning and 3 d post-weaning

| Bacterium                  | Antimicrobial agent | Penicillin treatment² | n   | Percentages of isolates at each indicated MIC (µg/mL)¹ |
|----------------------------|---------------------|-----------------------|-----|-------------------------------------------------------|
| B. clausii                 | Ampicillin          | PENN+ 11              | 91  | 0.12 0.25 0.50 1 2 4 8                                |
|                            |                     | PENN- 2               | 100 |                                         |
| Erythromycin               | PENN+ 11            | 100                   |     |                                         |
|                            | PENN- 2             | 50 50                 |     |                                         |
| Penicillin                 | PENN+ 11            | 91 9                  |     |                                         |
|                            | PENN- 2             | 100                   |     |                                         |
| Tetracycline               | PENN+ 11            | 82 18                 |     |                                         |
|                            | PENN- 2             | 100                   |     |                                         |
| Coagulase negative staphylococci⁴ | Erythromycin     | PENN+ 3               | 33 33 33 | 67 – |
|                            | PENN- 3             | 33 33 33 | 67 – |
| Tetracycline               | PENN+ 3             | 100                   |     |                                         |
|                            | PENN- 3             | 100                   |     |                                         |
| Staphylococcus aureus      | Erythromycin        | PENN+ 10              | 10 90 | – |
|                            | PENN- 0             | –                     |     |                                         |
| Tetracycline               | PENN+ 10            | –                     |     |                                         |
|                            | PENN- 0             | 100                   |     |                                         |

¹Blank spaces represent concentrations not tested for the indicated antimicrobial drug; empty cells represent 0% of isolates were inhibited at that concentration.

²Penicillin treatment: PENN+ = ewes administered penicillin; PENN- = no penicillin administered.

³Bacillus spp. = B. clausii, B. licheniformis, B. pumilus, B. subtilis, and B. thuringiensis.

⁴Coagulase negative staphylococci = S. epidermidis, S. equorum, and S. vitulinus.
RESULTS AND DISCUSSION

Previous investigations of antimicrobial therapy at dry-off on subclinical mastitis revealed low to moderate frequencies (15%–45%) and high cure rates in dry-treated ewes (Ahmad et al., 1992a; Chaffer et al., 2003). Data from the present study illustrate a bacterial presence in most samples (84%; n = 37/44) and a slight numerical decrease (P = 0.23) in subclinical mastitis post-weaning (Tables 1 and 2). Subclinical mastitis frequencies for PENN, FAST, and COMBO were 88%, 83%,

Figure 1. Kaplan–Meier survival estimates of selected bacterial isolates against various antimicrobial agents.
and 100% at weaning (Table 1), which decreased to 75%, 66%, and 88% 3 d post-weaning (Table 2), respectively. There was no evidence of a treatment effect on subclinical mastitis ($P = 0.68$).

Upon culturing and confirmation, 17 unique bacterial species were identified as belonging to the genera *Bacillus*, *Brachybacterium*, *Mannheimia*, *Moraxella*, *Staphylococcus*, and *Streptococcus* (Tables 1 and 2). Many of these bacteria are common isolates of subclinical mastitis cases in extensive/semi-extensive systems (*Bacillus* spp., *Staphylococcus* spp., *Mannheimia haemolytica*, *Streptococcus* spp.; Arsenault et al., 2008; Knuth et al., 2019) and intensive systems (*Staphylococcus* spp. and *Streptococcus* spp.; Ahmad et al., 1992b). Although these preliminary data do not report statistical differences in prophylactic management strategies, further research is warranted to identify efficacy of weaning treatments to reduce subclinical mastitis.

Antimicrobial resistance levels for common agents of ovine mastitis have been estimated to range between 14% and 43% against penicillin, 41% and 43% against ampicillin, 7% and 50% against tetracycline, 5% and 6% against erythromycin, and 7% and 22% against streptomycin (Concepción Ergün et al., 2012; Porrero et al., 2012; Azara et al., 2017). In the present study, all *S. aureus* isolates were resistant to tetracycline (Table 3; Figure 1). Ampicillin- and penicillin-resistant isolates were observed among both coagulase negative *Staphylococci* (17%; $n = 1/6$) and bacilli (8%; $n = 1/13$). Furthermore, a difference in antimicrobial resistance to erythromycin was suggested ($P = 0.02$) between PENN+ and PENN− bacilli isolates, but no other antimicrobial agent-bacteria combination showed penicillin treatment differences ($P \geq 0.40$).

**IMPLICATIONS**

Subclinical mastitis is likely more prevalent than previously thought, and production impacts on ewe productivity and animal welfare are not well understood in wool-type ewes. The present data are preliminary results of a broader investigation of lambing and weaning management practices on subclinical mastitis and the milk microbiome. These data show many potentially pathogenic bacteria isolated from subclinically infected ewes, where some isolates showed resistance to antimicrobial agents that are not extensively administered. Future research is warranted to judiciously employ antimicrobial agents in the livestock industry and ensure animal health while maximizing efficient and effective production.

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Conflict of interest statement. Mention of commercial products was solely stated to provide specific information and does not imply recommendation. The authors confirm that the research was conducted in the absence of commercial or financial relationships that could have influenced the outcome of the study.

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