Novel sequence types and low levels of antimicrobial resistance associated with clinical mastitis in sheep flocks across Scotland

Keith T. Ballingall, Riccardo Tassi, Jane Gordon, Carol Currie, Kath Dun Nigel Miller and Nuno Silva

Supplementary File

Supplementary Table S1. Details of the survey questionnaire

Sample Profile	Flock profile
Sample date	Name
Farm Identity	Flock 1
Hogg, Yes/No	Flock 2
Gimmer, Yes/No	Area
Ewe (age)	Type, Hill, Lowland
Condition Score	Type
Signs	Ewe no
Udder condition	Breed 1
Stiff/lame, Yes/No	Breed 2
Dull, Yes/No	Breed 3
Recumbent, Yes/No	Tup a
Lamb x1	Tup b
Lamb x2	Scan 17/18
Lamb x3	Lambing start date
Sample Yes/No	Lambing system
Treat Yes/No	Feed supplement pre
	Feed supplement post
Antimicrobial type	Triplet
Dose/days	
Intra-mammary tube Yes/No	
Treatment other	
Outcome	
Recover, Yes/No	
Part recovered, Yes/No	
Died, Yes/No	
Mastitis record - lambing period	
Quarantined Yes/No	
Days post lambing	

Supplementary Information. Hogg, defined as a female sheep between weaning and first shearing. Gimmer, defined as a female yearling.

Supplementary Table S2. Origin of each sample, corresponding isolate number with species identification.

Farm code*	Milk Sample	Isolate	Bacterial identification
01	9	MRI-SI-010	*Mannheimia haemolytica*
01	23	MRI-SI-024	*Staphylococcus aureus*
01	23	MRI-SI-025	*Mannheimia haemolytica*
02	29	MRI-SI-030	*Staphylococcus aureus*
02	29	MRI-SI-033	*Mannheimia haemolytica*
02	30	MRI-SI-035	*Staphylococcus simulans*
02	31	MRI-SI-034[2]	*Staphylococcus aureus*
03	7	MRI-SI-008	*Staphylococcus aureus*
04	21	MRI-SI-022[2]	*Staphylococcus aureus*
04	22	MRI-SI-023	*Staphylococcus aureus*
04	26	MRI-SI-029	*Staphylococcus aureus*
07	24	MRI-SI-027	Bacillus spp.
08	8	MRI-SI-009	*Mannheimia haemolytica*
08	12	MRI-SI-013	*Mannheimia haemolytica*
09	4	MRI-SI-004[2]	Stock contaminated
09	5	MRI-SI-005	*Staphylococcus aureus*
09	6	MRI-SI-006	*Mannheimia haemolytica*
09	6	MRI-SI-007	*Streptococcus* spp.
10	11	MRI-SI-012	*Mannheimia haemolytica*
11	10	MRI-SI-011	*Mannheimia haemolytica*
11	19	MRI-SI-020[3]	*Staphylococcus aureus*
11	28	MRI-SI-032	*Staphylococcus aureus*
12	3	MRI-SI-003	*Staphylococcus aureus*
13	1	MRI-SI-001	*Mannheimia haemolytica*
13	2	MRI-SI-002	*Staphylococcus warneri*
14	14	MRI-SI-015	*Staphylococcus aureus*
14	15	MRI-SI-016	*Staphylococcus aureus*
14	16	MRI-SI-017	*Staphylococcus aureus*
15	13	MRI-SI-014	*Staphylococcus aureus*
15	32	MRI-SI-037	*Staphylococcus aureus*
15	33	MRI-SI-036	*Staphylococcus aureus*

16	17	MRI-SI-018[1]	*Staphylococcus simulans*
16	27	MRI-SI-031	*Staphylococcus aureus*
17	18	MRI-SI-019	*Histophilus somni*
17	25	MRI-SI-028[2]	*Staphylococcus aureus*
18[1]	20	MRI-SI-021	*Staphylococcus aureus*
19	38	MRI-T1-020	*Staphylococcus aureus*
19	39	MRI-T1-026	*Staphylococcus aureus*
19	40	MRI-T1-204	*Staphylococcus aureus*

[1]. Isolate MRI-S1-018 was resistant to oxytetracycline and positive for the *tetK* resistance gene.

[2]. *S. aureus* isolates MRI-SI-004; MRI-SI-22; MRI-SI-028; and MRI-S1-034 were intermediate in resistance to Oxytetrocycline.

[3]. *S. aureus* isolate MRI-SI-020 was intermediate in resistance to both Oxytetrocycline and Kanamycin

Supplementary Information

Sampling instructions

Ewe sampling method

The method described here is for milk sampling from an ewe with evidence of mastitis

Each sampling kit is designed for a single sample. If sampling more than one animal use a different kit and change gloves each time to avoid cross contamination of the sample or passing an infection from one animal to another.

It is important that the sample is not contaminated with bacteria from your hands or from the skin on the outside of the teat.

1. Hold the ewe in a corner of a pen or within a crush. It's easier to sample with the ewe on its feet rather than on its back.
2. Open the sampling box and put on the gloves.
3. Remove the yellow toped tube containing cotton soaked in alcohol from the Zip locked bag and remove one piece of cotton. Wipe your gloves with the cotton making sure to completely cover between the fingers.

4. Remove a second piece of alcohol-soaked cotton and thoroughly wipe the end of the teat until no dirt can be seen on the cotton or the teat end, making sure it is well soaked and rubbed with the alcohol.
5. Hand milk the teat avoiding touching the teat end. Discard the first two lots of milk.
6. Open the red toped tube making sure that the lid is kept clean and place the third milk sample into the red topped sample tube making sure **not to touch the inside of the tube with the end of the teat.**
7. We only require a few mls /drops for bacteriology so don't more than half fill the tube. Quickly close the lid.
8. Write the farm name, date and animal id on the sample tube label and replace the tube in the zip locked bag with the absorbent towel.
9. Return the sample in the sample box and post as quickly as possible to the address provided. If you can't return immediately, keep the sample box cool in a fridge.
10. Remove your gloves and place in the other ziplocked bag for disposal. Wash your hands with plenty of soap and water.
Note, some ewes with clinical mastitis may be dry and you will not be able to obtain a sample. Other samples will be heavily clotted, watery, or flecked with blood. These types of samples are useful.

Supplementary Methods

1. **Antimicrobial susceptibility testing**

Each bacterial isolate was tested for antimicrobial susceptibility using the Kirby- Bauer disk diffusion method and minimum inhibitory concentration (MIC), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2018) and Clinical and Laboratory Standards Institute (CLSI 2018a, CLSI 2018b). Individual colonies were suspended in 3 ml of saline solution and inoculated onto *Mueller-Hinton* agar (EO LABS, Burnhouse, UK). Antimicrobials in impregnated paper disks (Abtek Biologicals Limited, UK) were added and the plates, incubated at 37°C for 18 to 20 h before measuring inhibition zone diameters (IZD). For MIC, broth micro dilution testing was performed using cation-adjusted Mueller-Hinton broth (CAMHB, Sigma-Aldrich, UK). For *Mannheimia* isolates, in microtiter plates,

tetracycline and penicillin G were prepared in serial 2-fold dilutions ranging from 0.0625 to 32.0 mg/L respectively and 0.008 to 2.0 mg/L respectively in CAMHB supplemented with 5% lysed horse blood and 20 mg/L of β-nicotinamide adenine dinucleotide (Sigma-Aldrich, UK). The final test concentration of bacteria was approximately 5×10^5 colony-forming units/ml, using the McFarland standard and MICs were read after 18±2 h incubation at 37°C. The *M haemolytica* American Type Culture Collection (ATCC) 33396, *E coli* ATCC 25922 and *S aureus* ATCC 29213 were used as internal controls.

For staphylococcus isolates, breakpoints were taken from EUCAST standards when available (EUCAST, 2020), or from Clinical and Laboratory Standards Institute, (CLSI) standards if EUCAST guidelines did not exist (CLSI 2018a, CLSI 2018b). The antimicrobial panel included benzylpenicillin (1 U), cloxacillin (5 µg), ampicillin (10 µg), amoxicillin-clavulanic acid (20-10 µg), oxacillin (1 µg), ceftiofur (30 µg), cefquinome (30 µg), cefoxitin (30 µg), erythromycin (15 µg), kanamycin (30 µg), oxytetracycline (30 µg), sulfamethoxazole-trimethoprim (23.75-1.25 µg), enrofloxacin (5 µg), and ciprofloxacin (5 µg). The breakpoint interpretation for tetracycline was applied to oxytetracycline. For *Mannheimia* isolates, breakpoints were taken from CLSI guidelines (CLSI 2018b) and the antimicrobial panel included ceftiofur (30 µg), spectinomycin (100 µg), enrofloxacin (5 µg), tilmicosin (15 µg), florfenicol (30 µg) and MIC to tetracycline (Alfa Aesar, UK) and penicillin G (Sigma-Aldrich, UK). The reference strains *S aureus* ATCC® 29213 and *S aureus* ATCC® 25923 were used as internal controls.