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Evaluation of diagnostic procedures for subclinical mastitis in meat-producing sheep

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Samples of foremilk were collected from 261 clinically normal glands of 150 ewes, and tested using the California mastitis test (CMT). Further samples were collected from 195 of these glands for determination of automated somatic cell counts (SCC), and from 60 of these glands for bacteriological assessment. The sensitivity and specificity of CMT for detecting samples with SCC above different threshold levels and for CMT and SCC in determining bacteriological status were evaluated using two-graph receiver operating characteristics (TG-ROC). Milk samples were obtained subsequently from ten CMT positive, and five CMT negative first- and second-lactation ewes. Samples were cultured using a variety of media, incubation temperatures and atmospheric conditions, immediately after collection, and 1 week after storage at 4 °C and −21 °C. Results suggested that CMT is best used as a diagnostic test for ovine subclinical mastitis (SCM) with a cut-off of 3 (distinct gel formation), and that automated SCC thresholds of $>1200 \times 10^3$ cells/ml are appropriate, especially where low prevalences are expected (e.g. <5%). Additionally, this study showed that routine bacteriological methods were appropriate for isolation of most species of pathogen responsible for ovine SCM, but storage of samples prior to culture, either at 4 °C or −21 °C, was detrimental to the isolation of several of these organisms.

Keywords: Subclinical mastitis, ovine, California mastitis test, somatic cell count, sensitivity, specificity.

Diagnosis of subclinical mastitis (SCM) in meat-producing sheep is important because this disease is known to cause decreased quantity and quality of milk production (Torres-Hernandez & Hohenboken, 1979; Fthenakis, 1988; McCarthy et al. 1988; Fthenakis & Jones, 1990a, b), resulting in decreased lamb growth rates and increased lamb mortality (Gross et al. 1978; Fthenakis, 1988; Fthenakis & Jones, 1990a) and may precede cases of clinical mastitis in ewes (Watkins et al. 1991). Diagnosis of SCM in sheep is based on demonstration of increased numbers of inflammatory cells and isolation of the causative bacteria from milk samples. Numbers of inflammatory cells have been measured either directly, via direct microscopic cell counts (DMSCC) (Watson et al. 1990; Keisler et al. 1992), or indirectly, via automated somatic cell counts (SCC) (Green, 1984; Fthenakis et al. 1991; Keisler et al. 1992; Burriel, 1997; McDougall et al. 2001; Sargeant et al. 2001) or ewe-side tests such as the Whiteside test (WST) (Murphy & Hanson, 1941; Watkins et al. 1991), the California mastitis test (CMT) (Hueston et al. 1986; Keisler et al. 1992; McDougall et al. 2001; Sargeant et al. 2001) and electrical impedance (McDougall et al. 2001). In udders affected with mastitis the neutrophil is the predominant leucocyte, although the proportions of different types of leucocyte vary depending on the duration of infection and the aetiological agent (Leitner et al. 2000; Riollet et al. 2001). The number of inflammatory cells that are either normal or optimal for the uninfected ovine mammary gland is debatable (Fthenakis, 1988; Fthenakis et al. 1991). No consensus has, therefore, been reached on what cut-off levels are indicative of SCM for any of the previously mentioned tests.

The bacteriological techniques used for culturing and isolating bacteria tend to be consistent in published surveys of ovine SCM although the significance of isolation of different bacterial species has been a topic of debate (Hueston et al. 1986; Watson et al. 1990; Watkins et al. 1991; Burriel, 1997). Various culture and storage conditions have been examined for their influence on isolating a variety of mastitis pathogens from bovine milk samples,

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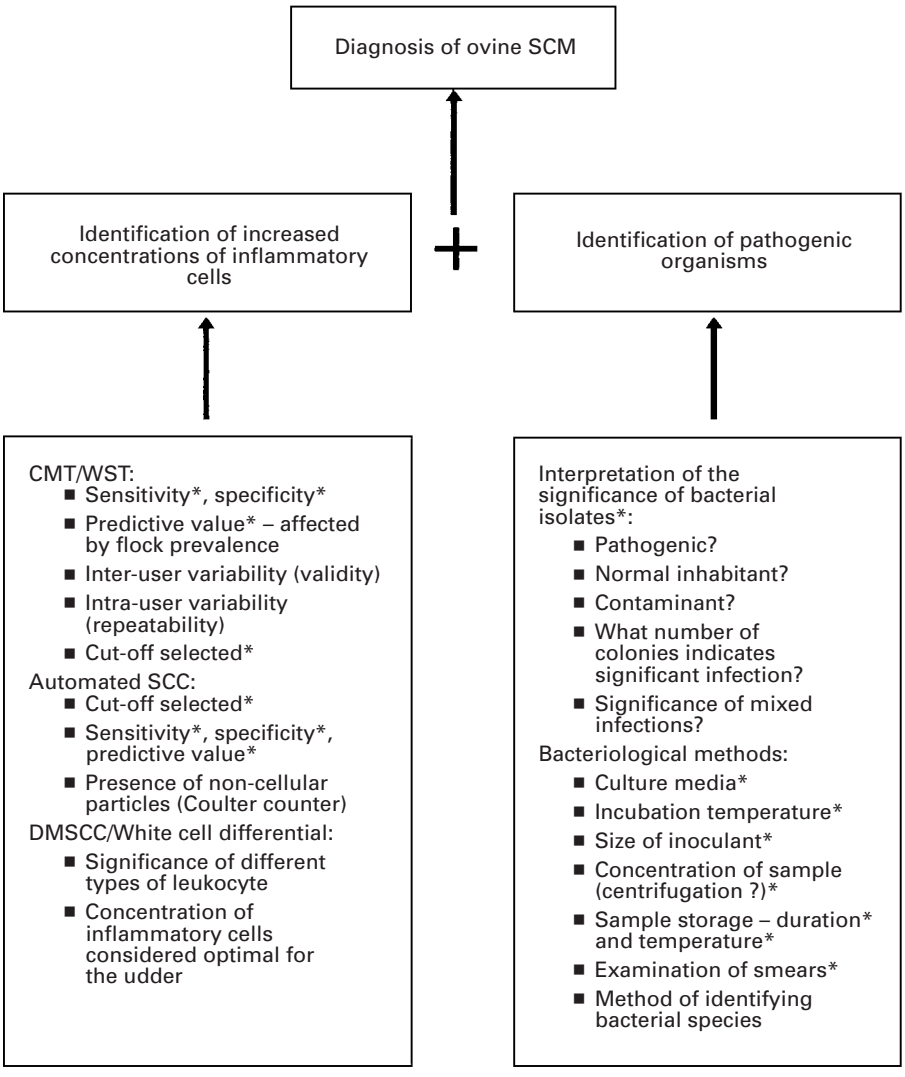

*Main factors examined in the current study

Key: SCM = Subclinical mastitis, CMT = California mastitis test, WST = Whiteside test, SCC = Somantic cell count, DMSCC = Direct microscopic somatic cell count.

Fig. 1. Concept map of the factors affecting the validity of diagnoses of ovine subclinical mastitis.

such as freezing samples prior to plating and centrifugation. Centrifugation, for example, was found to increase the number of bovine milk samples from which *Staphylococcus aureus* was isolated (Zecconi et al. 1997). There is very little published information, however, on the effects of different sample storage conditions and culture techniques in relation to diagnosing cases of ovine SCM.

The potential causes of variability in diagnosing cases of ovine SCM are presented in Fig. 1. The current study was undertaken to investigate a number of these potential causes of variability, with the following specific aims: 1) to measure the efficiency of CMT as a field-based estimate of SCC, and thus as an indicator of the concentration of inflammatory cells in ovine milk samples; 2) to measure the efficiency of CMT and SCC as predictors of bacteriological status; 3) to evaluate the effectiveness of standard bacteriological techniques and sample storage conditions in isolating pathogens responsible for ovine SCM, and 4) to develop recommendations on the use of diagnostic tests for ovine SCM for managers of commercial flocks of meat-producing sheep.

Materials and Methods

The present study was conducted in three phases. In phase 1, the efficiency of CMT was investigated in terms of its ability to estimate the concentration of somatic cells. The automated SCC using a Fossomatic counter is an accurate indicator of somatic cell concentrations (Gonzalo et al.

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1993) and was therefore considered a suitable gold standard for this assessment. As no definitive cut-off SCC has been determined, CMT was compared with SCC using a number of cut-offs. In phase 2, the efficiency of CMT and SCC in determining cases of ovine SCM was assessed using bacteriological status as the gold standard. Phase 3 was undertaken to assess the value of bacteriological status, derived using standard bacteriological techniques, as a gold standard for determining cases of ovine SCM.

Phases 1 and 2

CMT and SCC. The sheep flock used for this investigation was in southwest Scotland, and consisted of 500 spring-lambing Scottish Mule ewes grazing upland pasture (intermediate altitude, often improved pasture grazed with a medium stocking density). One-hundred-and-fifty ewes, selected at random from all ewes lambing over the initial 2 weeks of the lambing period, were examined clinically 2–5 d after parturition. CMT was performed on 3–5 ml of foremilk from 261 clinically normal glands as the remaining 39 glands were not clinically normal and were therefore excluded from the study. The degree of precipitation and gel formation was assessed subjectively and graded according to Schalm & Noorlander (1957). The grades were: 1) no precipitate; 2) trace precipitate; 3) distinct precipitate/weak gel formation; 4) distinct gel formation; 5) strong gel formation. A further 10–20 ml were collected for determination of automated SCC using a Fossomatic counter (Foss UK Ltd, York, UK). Sixty-six glands yielded insufficient milk to undertake SCC, giving a total of 195 SCC performed. Finally, after vigorous rubbing of the teat end with cotton wool soaked in surgical spirit, 1–5 ml of milk were collected aseptically from all 261 clinically normal glands. Heat-fixed smears were made from 138 of these samples (numbers were constrained by time and resources) within 3 h of collection, and all 261 samples were placed in a commercial freezer for storage at approximately −21 °C.

Bacteriological examinations. Sixty samples, selected to ensure that all CMT-grades were represented (randomly within each CMT category), were removed from the freezer and thawed at room temperature. Samples were plated on 7% sheep blood agar base number II (Oxoid, Basingstoke, UK) at 37 °C for 24 h and then re-incubated for a further 24 h if no bacterial colonies were detected. Presumptive determination was based on colony morphology and reaction to Gram-stain. Bacterial isolates were subsequently identified to species using the API systems (BioMerieux, Basingstoke, UK). All 138 heat-fixed smears were Gram-stained, and examined by light microscopy for the presence of bacteria.

Statistical analyses. Two-graph receiver operating characteristics (TG-ROC) analyses, which establish cut-off values (denoted d_0) that realize equal values for sensitivity and specificity (denoted θ_0), were performed using CMDT software (Freie Universität Berlin) (Greiner et al. 1995).

Phase 3

CMT was performed on foremilk samples from a selection of ewes (a convenience sample of first and second lactation individuals) to identify ten glands with CMT grades 3 or greater, and five glands with CMT grades 1 or 2. Immediately following collection of the CMT sample, additional milk samples were collected aseptically from each of the 15 glands, and were labelled according to the ear tag number of the ewe, and the corresponding CMT result. Within 3 h of collection, two heat-fixed smears were made from each sample. One heat-fixed smear from each sample was defatted by bathing the slide in acetone for 30 s and rinsing with tap water. The defatted and non-defatted slides were Gram-stained and were examined later under a light microscope. Samples were divided into three separate aliquots. One aliquot from each gland was placed in a commercial refrigerator, at 4 °C and a second aliquot was placed in a commercial freezer at −21 °C. The third aliquot was further divided into three sub-samples. One sub-sample was submitted for DMSCC and differential white blood cell counts at the University of Glasgow Veterinary School in-house cytology laboratory. These were performed using standard manual light microscopic techniques. A second sub-sample was placed in a centrifuge and spun at 15 000 ***g*** for 1–2 min. Centrifuged and non-centrifuged samples were plated, within 6 h of collection, on 7% sheep blood agar, 7% horse blood agar, chocolate agar, MacConkey's agar and sheep milk agar plates. The sheep milk agar was prepared using aseptically collected sheep milk from CMT negative first lactation ewes, pasteurized at 72 °C for 30 min. Sheep blood agar, horse blood agar, MacConkey's agar and sheep milk agar plates from each centrifuged and non-centrifuged sample were incubated aerobically at 42, 37 and 28 °C, and at room temperature (approximately 23 °C). Chocolate agar plates from each centrifuged and non-centrifuged sample were incubated in microaerophilic conditions (5% carbon dioxide/5% oxygen) at 37 °C. Sheep blood agar, horse blood agar, chocolate agar, MacConkey's agar and sheep milk agar plates from each centrifuged and non-centrifuged sample were incubated in an anaerobic cabinet (Don Whitley Mk III) at 37 °C. All plates were checked after 24-h incubation, and were incubated for a further 24 h if no bacterial growth was detected. One week after storage, the refrigerated and frozen samples were retrieved, and the same process was repeated, except that refrigerated and frozen samples were not plated on sheep milk agar, as the sheep milk agar had been found to be contaminated with *Bacillus* sp. following the plating of fresh samples, and no samples were centrifuged prior to plating. Presumptive determination was based on colony morphology and Gram-staining potential. All morphologically distinct bacterial colonies were sub-plated on sheep blood agar and

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were incubated at 37 °C under the same atmospheric conditions as the primary plate. Isolates were identified to species using the API systems (BioMerieux, Basingstoke, UK). Strains of bacterial isolates within species were not identified.

Results

Phase 1

The proportion of glands considered positive for different CMT thresholds varied considerably. One-hundred-and-thirty-three samples tested (51·0%) were CMT grade 2 or greater, whereas 81 (31·0%) were CMT grade 3 or greater and 30 (11·5%) were CMT grade 4 or greater. Similarly, the proportions of glands considered positive for different automated SCC varied, depending on the threshold selected. If a threshold of 200×10^3 cells/ml was selected, 125 samples (64·4%) were positive, if a threshold of 600×10^3 cells/ml was selected, 52 samples (26·8%) were positive and if a threshold of 1000×10^3 cells/ml was selected, 35 samples (18·0%) were positive. The sensitivity and specificity of CMT scores for predicting automated SCC varied depending on the threshold SCC selected. If threshold automated SCC of 200×10^3 cells/ml or 400×10^3 cells/ml were selected to define a true positive, the TG-ROC analysis showed that the sensitivity of the CMT was optimized (d_0) below a cut-off of 2 (1·5), with θ_0 of 0·73 and 0·74 respectively, suggesting that the CMT was too crude a measure to define the status of ovine milk samples where these thresholds are selected. If SCC thresholds of 600 or 1000×10^3 cells/ml were selected, d_0 occurred between CMT cut-offs of 2 and 3 (2·5), suggesting that identification of a trace level of gel formation or distinct precipitation provided the optimal sensitivity and specificity. For each of these cut-offs θ_0 was 0·80.

Phase 2

The date of lambing was recorded for 131/150 ewes. The number of CMT that were performed on ewes that were known to have lambed 2, 3 and 4/5 d prior to the test were: 141; 44 and 40. There was no significant difference in the CMT results between the three groups (Kruskal–Wallis test, $P=0·09$). The number of automated SCC that were performed on ewes that had lambed 2, 3 and 4/5 d prior to the test were: 118; 35 and 27. There was no significant difference in the SCC results between the three groups (one way ANOVA, $P=0·93$). No significant difference was found in the proportion of samples that were bacteriologically positive when comparing samples collected 2 d post-lambing ($n=38$) and those collected 3+ days post-lambing ($n=16$) (chi-square, $P=0·98$). This indicated that the number of days post-lambing when samples were collected did not significantly affect the results in the current study and the results are presented for all sampling days combined.

Table 1. The number of samples from which each species of bacteria was isolated in Phase 1

Species of isolate	Number of samples
Coagulase-negative Staphylococci†	15
Staphylococcus sp.	1
Streptococcus suis and *Enterococcus faecalis*	1
Streptococcus sp.	1
Mannheimia haemolytica	2
Actinomycete	1
No isolate	39
Total	60

† *Staph. cohnii* (4), *Staph. sciuri* (3), *Staph. epidermidis* (2), *Staph. warnerii* (2), Other Coagulase-negative Staphylococci (4)

The bacterial species isolated from these samples are listed in Table 1. Coagulase-negative staphylococci (CNS) were the predominant isolates (15/21, 71·4% of isolates). Twelve of the 138 Gram-stained smears contained visible Gram-positive cocci, ten of which were bacteriologically negative on culture. This might suggest that the routine techniques used for isolating bacteria were inadequate in some instances. However, as all but two of the bacteriologically positive samples did not show visible bacteria on Gram-stained smears, it is more likely that assessment of the smears was not an efficient method for identifying samples that contained bacteria.

The sensitivity and specificity for different CMT cut-offs and automated SCC thresholds in predicting a positive bacteriological result are plotted in Figs. 2 and 3. θ_0 was very similar for both tests, 0·68 for the CMT and 0·67 for the SCC. θ_0 for the CMT occurred at a cut-off of CMT grade 3, and for the automated SCC at a threshold of 1284×10^3 cells/ml. The predictive values for positive and negative tests at various CMT and SCC cut-offs for different prevalences of ovine SCM are presented in Table 2. The negative predictive values for all presented cut-offs at prevalences of 5, 10 and 20% were greater than or equal to 0·83, and increased with lower cut-offs and lower prevalences. The positive predictive values varied considerably for both tests at different prevalences. The positive predictive value of the CMT increased from 0·07 for a cut-off of 2 where prevalence is 5%, to 0·44 for a cut-off of 4, where prevalence is 20%. The positive predictive value of the automated SCC increased from 0·05 for a cut-off of 200×10^3 cells/ml where prevalence is 5%, to 0·35 for a cut-off of 1400×10^3 cells/ml, where prevalence is 20%.

Phase 3

CMT and DMSCC. CMT results, DMSCC and white cell differential counts are given in Table 3. The ranges of DMSCC for each CMT score were: CMT grade 1, 220–2200×10^3 cells/ml; CMT grade 3, 315–7581×10^3 cells/ml; CMT grades 4 and 5, 336–9279×10^3 cells/ml. In all

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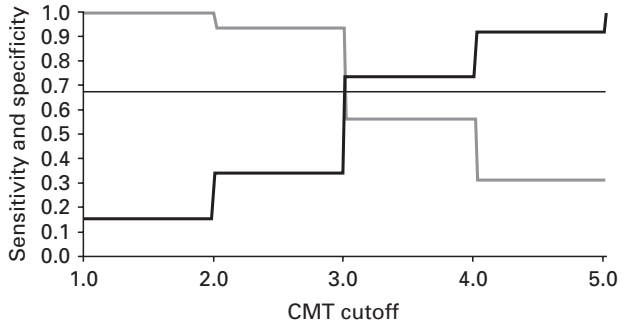

Fig. 2. Two Graph Receiver Operating Characteristics (TG-ROC) for CMT in determining a positive bacteriological result. —— Sensitivity, —— specificity, —— $\theta_0 = 0{\cdot}68$.

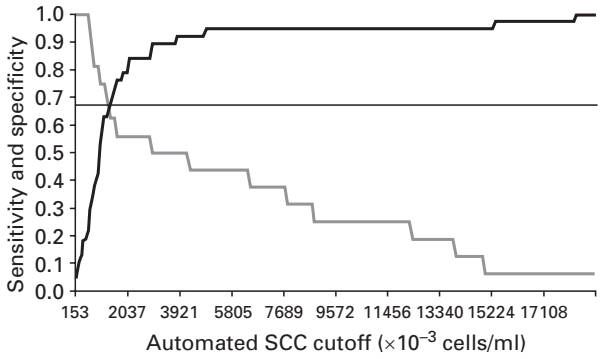

Fig. 3. Two Graph Receiver Operating Characteristics (TG-ROC) for automated SCC in determining a positive bacteriological result. —— Sensitivity, —— specificity, —— $\theta_0 = 0{\cdot}68$.

CMT grade 3, 4 and 5 samples, the predominant leucocyte was the neutrophil, except in sample Y105L, where lymphocytes and macrophages predominated. Although samples W66R and G24L were CMT grade 1, they had DMSCC $>900 \times 10^3$ cells/ml, with neutrophils predominating.

Bacterial species isolated. *Staphylococcus aureus* was isolated in small numbers from samples W48L and NT01L, and *Mannheimia haemolytica* was isolated from samples Y146R and NT01L, in small numbers from the former, and in moderate numbers from the latter (Table 4). Profuse cultures of the coagulase-negative staphylococci *Staph. hominis* and *Staph. saprophyticus* were obtained from samples Y37L and W54R respectively. *Staph. xylosus*, *Staph. capitis* and *Staph. sciuri* were isolated in small numbers from samples G157R, W66R, G16R, W48L, NT01L, W58L, Y4L, Y105L, W125L and W102L, and in moderate numbers from samples W118R, Y37L and Y146R. A number of bacteria of unknown pathogenicity for the ovine mammary gland were isolated in moderate numbers from samples G16R, W118R, Y37L, NT01L, Y146R and W58L. No bacteria were isolated from sample G24L. The samples and culture conditions from which common and unusual bacterial species were isolated are provided in Tables 4 and 5.

Effects of sample storage. All species of bacteria were isolated from freshly plated samples, with the exception of *Pseudomonas aureofaciens* and *Ps. fluorescens*, which were isolated from one sample only after refrigeration for 1 week; *Staph. saprophyticus*, which was isolated from one sample only after freezing for 1 week and thawing, and *Micrococcus* sp., which was isolated from one sample after both refrigeration and freezing/thawing. All species were isolated following refrigeration for 1 week except *Staph. capitis*, *Staph. saprophyticus*, *Staph. aureus* and *Pasteurella* sp. The only bacterial species isolated after freezing/thawing were *Staph. xylosus*, *Staph. saprophyticus*, *Aerococcus viridans*, and, on one occasion, *Micrococcus* sp. *Staph.* saprophyticus and *Micrococcus* sp. were the only species that grew prolifically following freezing/thawing. *Staph. xylosus* and *Aec. viridans* were the only bacterial species isolated from freshly plated, refrigerated and frozen/thawed samples.

Effects of centrifugation of samples. All bacterial species were isolated from non-centrifuged samples, although more prolific growth of *Staph. xylosus*, *Staph. saprophyticus*, *Acinetobacter lwoffii* and *Pasteurella* sp. occurred following centrifugation. *Staph. hominis* was the only bacterial species isolated from non-centrifuged samples that was not isolated from centrifuged samples.

Effects of culture media. Many sheep milk agar plates were contaminated with *Bacillus* sp. colonies, particularly when incubated at 42 °C. The use of Horse Blood Agar or Sheep Milk Agar did not confer an advantage in isolating any bacterial species when compared with the use of routine Sheep Blood Agar, as all bacterial species were isolated using the latter. All bacterial species were isolated using Horse Blood Agar except *Staph. saprophyticus* and *Ps. aureofaciens*, and all bacterial species were isolated using Sheep Milk Agar except *Staph. saprophyticus*, *Micrococcus* sp., *Ps. aureofaciens* and *Ps. fluorescens*.

Effects of incubation temperature. All bacterial species were isolated when cultured at room temperature except *Staph. saprophyticus, Staph. aureus, Man. haemolytica, Aec. viridans*, and *Ps. fluorescens*. All bacteria were isolated when cultured at 28 °C, except for *Staph. saprophyticus* and *Ps. aureofaciens*, and all species were isolated when cultured at 37 °C except *Ps. aureofaciens*. The following species were not isolated at 42 °C: *Staph. saprophyticus*, *Staph. aureus, Corynebacterium propinquum, Pasteurella* sp., *Ps. aureofaciens* and *Ps. fluorescens*.

Effects of atmospheric conditions. All bacterial species were isolated in aerobic conditions, and all bacterial species

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Table 2. Predictive values of positive and negative tests for various CMT and SCC cut-offs at flock prevalences of 5, 10 and 20%

			Predictive value positive test			Predictive value negative test		
Test	Cut-off	Prevalence	5%	10%	20%	5%	10%	20%
CMT	2		0·07	0·14	0·26	0·99	0·98	0·97
CMT	3		0·11	0·21	0·37	0·98	0·95	0·90
CMT	4		0·14	0·26	0·44	0·96	0·92	0·83
SCC	200 000		0·05	0·11	0·21	1·00	1·00	1·00
SCC	600 000		0·06	0·12	0·24	0·98	0·95	0·90
SCC	1 000 000		0·08	0·16	0·30	0·98	0·95	0·90
SCC	1 400 000		0·10	0·19	0·35	0·97	0·94	0·88

Table 3. Results of CMT, DMSCC and white cell differentials from the 15 ovine mammary glands in Phase 3

Sample number	CMT grade	DMSCC	% neutrophils	% lymphocytes	% macrophages
Y4L	1	220	20	20	60
W58L	1	795	7	66	27
G16R	1	381	—	—	—
W66R	1	2200	67	19	14
G24L	1	931	70	20	10
Y37L	3	369	69	23	8
W102L	3	565	51	22	27
W54R	3	315	44	37	19
W118R†	3	2300	91	7	1
Y105L	3	693	23	39	38
G157R	3	598	73	12	15
W48L	3	7581	71	15	14
W125L	4	503	50	40	10
Y146R	4	336	72	15	13
NT01L	5	9279	97	0	3

† Eosinophils comprised 1% of leucocytes

were isolated in microaerophilic conditions except for *Staph. hominis*, *Staph. saprophyticus*, *Pasteurella* sp., *Ps. aureofaciens* and *Ps. fluorescens*. The only bacterial species isolated in anaerobic conditions was *Aec. viridans*.

Gram stained smears. Non-defatted Gram-stained smears contained visible Gram-positive cocci in only one sample, and defatted Gram-stained smears contained visible Gram-positive cocci in a single, different sample.

Discussion

In making recommendations about a diagnostic test, it is important to consider the consequences of a false negative result (imperfect sensitivity) *v.* the consequences of a false positive result (imperfect specificity). If the aim is to identify every case of a condition in order to eradicate a disease, and if the cost of treating or culling false-positives is relatively low relative to the cost of having the disease in a population, then sensitivity has a higher priority than specificity. If, on the other hand, the costs of treatment or culling of test positive animals are important factors, and if the aim is to reduce overall prevalence of infection without necessarily eliminating the disease, then specificity takes priority. In the case of ovine SCM, it can be argued that the latter generally applies, and therefore it would be reasonable to give greater weighting to the specificity of the CMT or SCC for selecting cases for treatment. The aim of selecting a CMT or SCC cut-off would therefore be to maximize specificity while maintaining a reasonable sensitivity so that the majority of cases of SCM are identified. Verification would be required from an analysis of the economic consequences of diagnosing and treating cases of ovine SCM.

Keisler et al. (1992) reported that CMT was a poor means of estimating the SCC of ewe milk compared with automated methods and DMSCC. In the current investigation it was demonstrated that for SCC thresholds of 600 or of 1000×10^3 cells/ml, sensitivity and specificity of the CMT test were relatively high (0·8) mid-way between cut-offs of grades 2 and 3. In the current study, CMT was found to be of little use in predicting SCC status if SCC thresholds of 200×10^3 cells/ml or 400×10^3 cells/ml were selected. The question then arises: which threshold SCC or CMT should be selected to best determine a case of SCM? Burriel (1997) suggested that a threshold SCC of 400×10^3 cells/ml was indicative of mild SCM, and that a threshold of 600×10^3 cells/ml was indicative of more severe SCM. A number of reports have investigated the effect of prevalence of SCM on the predictive value of CMT (Hueston

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Table 4. Common ovine SCM pathogens isolated from milk samples, and the culture conditions under which more than one colony was isolated

Bacterial species	Isolated from: (Sample)	Culture conditions†‡
Staph. xylosus	G157R, W66R, W48L, **Y37L**, **Y146R**, W58L, W125L, NT01L, **W118R**, Y105L, Y4L, W102L	F(U(RoSb, RoHb, RoSm, 28Sb, 28Hb, 37Sb, 37Sm, 42Sm, CoCh), Sp(**RoSb**, **RoHb**, **RoSm**, **28Sb**, **28Hb**, **28Sm**, **37Sb**, **37Hb**, **37Sm**, **CoCh**)), R(**RoSb**, RoHb, **37Sb**, 37Hb, **28Sb**, 28Hb, **CoCh**), T(RoSb, RoHb, 28Sb, 28Hb, CoCh)
Staph. sciuri	G157R, G16R, W118R, **Y146R**	F(U(RoSb, RoHb, RoSm, 28Sb, 28Hb, 28Sm, 37Sb, 37Hb, 37Sm, 42Sb), Sp(**RoSb**, **RoHb**, **28Sb**, **28Hb**, **28Sm**, **37Sb**, **37Hb**, **37Sm**)), R(37Hb, 42Sb, 42Hb, 28Sb, 28Hb, CoCh)
Staph. capitis	**W118R**, Y105L, Y4L, W102L	F(U(RoSb, RoSm, **28Sb**, **28Hb**, **28Sm**, **37Sb**, **37Hb**, **37Sm**, **42Sb**, **CoCh**), Sp(RoSb, RoHb, RoSm, **28Sb**, **28Hb**, **28Sm**, **37Sb**, **37Hb**, **37Sm**, **42Sb**, **42Hb**, **42Sm**, **CoCh**))
Staph. hominis	**Y37L**	FU(RoSb, RoHb, RoSm, 28Sb, 28Sm, 37Sb, 37Hb, 37Sm, **42Hb**)
Staph. saprophyticus	**W54R**	**T37Sb**
Staph. aureus	W48L, NT01L	F(U(28Sb, 28Hb, 37Sb, 37Hb, CoCh), Sp(28Sb, 28Hb, 37Sb, 37Hb, 37Sm, CoCh))
Micrococcus sp.	**W118R**, Y146R	R(**37Sb**, **37Hb**), T(RoSb, RoHb, **37Sb**, **37Hb**, **42Sb**, **42Hb**, 28Sb, 28Hb, CoCh)
Mannheimia haemolytica	**NT01L**, Y146R	F(U(**28Sb**, **28Hb**, 28Sm, **37Sb**, **37Hb**, 37Sm, **42Sb**, **42Hb**, **42Sm**, CoCh), Sp(**28Sb**, **28Hb**, 28Sm, **37Sb**, **37Hb**, **37Sm**, 42Sm, CoCh)), R(**37Sb**, **37Hb**, **28Sb**, **28Hb**)

† Bold Print = >10 colonies isolated. Normal print = 2–10 colonies isolated
‡ Key: F = fresh; Sb = sheep blood agar; 42 = 42 °C
R = refrigerated; Hb = horse blood agar; 37 = 37 °C
T = frozen/thawed; Ch = chocolate agar; 28 = 28 °C
Sp = centrifuged; Sm = sheep milk agar; Ro = room temperature
U = non-centrifuged; Co = microaerophilic (CO_2); An = anaerobic

et al. 1986; McDougall et al. 2001) and automated SCC (Fthenakis et al. 1991; McDougall et al. 2001) in determining positive bacteriological results. Fthenakis et al. (1991) suggested that for SCM prevalences of 5%, 10–25% and 50%, SCC thresholds of 1500, 1000 and 750×10^3 cells/ml were most accurate. Hueston et al. (1986), who used a CMT cut-off of grade 3 to predict a positive bacteriological result, obtaining a sensitivity of 69·3% and specificity 76·5%, which were similar to the results of the current investigation, concluded that the use of CMT as a diagnostic test for SCM was questionable in flocks where the prevalence was low. McDougall et al. (2001) also reported similar results to those in the current study in relation to predictive values at different cut-offs for various prevalences of ovine SCM.

The results of the current investigation suggest that the use of a CMT grade 3, or a SCC threshold of 1200×10^3 cells/ml in determining a positive bacteriological result, allows for optimization of sensitivity and specificity, but that the sensitivities achieved are only 67% and 69% respectively and specificities are 72% and 63% respectively. If a higher CMT cut-off is selected to achieve sufficient specificity to avoid a high proportion of false positives, sensitivity would drop to an unacceptably low level (24% for a cut-off of 4), making the test of little use. A cut-off of 3 should therefore be adopted, accepting that the predictive value of a positive test will be low where prevalence is low, and that a high proportion of false positives might diminish the economic advantages of testing and treating for ovine SCM. To reduce the proportion of false positives where samples are tested using automated SCC, thresholds higher than 1200×10^3 cells/ml should be selected, especially where prevalence is expected to be low. These findings suggest it would be reasonable to adopt the recommendations of Fthenakis et al. (1991) mentioned above.

It should be noted that in the current study repeatability of the CMT was not assessed by measuring samples from the same gland more than once. Additionally, between-observer variation of CMT was not assessed. Such undertakings would have proved useful in determining the influence of user error and interpretation of gel formation on the efficiency of the CMT. Some glands were excluded from SCC because they did not yield sufficient milk for the test and it may be argued that bias could arise because the excluded glands were more (or less) likely to be infected. It was not possible to determine the influence of this bias on calculations of flock prevalence derived from automated SCC results. This bias should not, however, have significantly affected measures of sensitivity or specificity of the CMT in determining a high SCC, or of the CMT and automated SCC in determining bacteriological status, because sensitivity and specificity are features of the test rather than the population.

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Table 5. Bacterial species of unknown pathogenicity for the ovine mammary gland, isolated from milk samples in significant numbers (>10 cfu in at least one sample), and the culture conditions under which more than one colony was isolated

Bacterial Species	Isolated from: (Sample)	Culture conditions†‡
Acinetobacter lwoffii	**Y146R**, W58L	F(U(RoSb, RoHb, RoSm, 28Hb, 37Sb, 37Hb, 37Sm, 42Hb, 42Sm), Sp(**RoSb**, **RoHb**, **RoSm**, **28Sb**, **28Hb**, **28Sm**, **37Sb**, **37Hb**, **37Sm**, CoCh)), RRoSb
Aerococcus viridans	**NT01L**, Y37L	F(U(28Sb, 28Hb, 37Sb, 37Hb, **42Sb**, **42Hb**, **42Sm**, CoCh), Sp(28Sb, 28Hb, 37Sb, 37Hb, 37Sm, CoCh)) R(37Sb, 37Hb, 42Hb, 28Sb, 28Hb, CoCh, AnHb) T37Sb
Corynebacterium propinquum	**Y37L**, W58L	F(U(RoSb, RoHb, RoSm, 28Sb, 37Hb), Sp(RoSb, RoHb, RoSm) R(**RoSb**, **37Sb**, **28Sb**, 28Hb, **CoCh**)
Pasteurella sp.	**Y146R**	F(U(RoSb, RoSm, 37Sb, 37Sm), Sp(**RoSb**, **RoHb**, **28Sb**, **28Hb**, **28Sm**, **37Sb**, **37Hb**, **37Sm**))
Pseudomonas aureofaciens	**G16R**	R**RoSb**
Pseudomonas fluorescens	**G16R**	R(**37Sb**, **37Hb**, **28Sb**, **28Hb**)

† Bold Print = >10 colonies isolated. Normal print = 2–10 colonies isolated
‡ Key: as for Table 4

Many samples in phases 1 and 2 had elevated SCC, but no bacteria were isolated when the samples were cultured. A number of possible explanations have been suggested in the literature, including that elevated SCC result from infections by Gram-negative bacteria that have been rapidly eliminated by the host, or that some organisms, known to cause mastitis, can only be isolated using special media and incubation temperatures (Jones, 2000; Leitner et al. 2001). The high SCC associated with CNS infection in some samples were consistent with the findings of Leitner et al. (2001), who suggested that ovine udders have an augmented cellular response to CNS compared with bovine udders.

There was poor association between CMT score and DMSCC in phase 3. The significance of different bacterial isolates was difficult to assess – some bacteria may have been present in the teat canal, without replicating or causing significant infection, and others may have been present as contaminants. DMSCC ($>2000 \times 10^3$) and differential leucocyte counts confirmed acute subclinical mastitis in samples W66R, W118R, W48L and NT01L, where the aetiological agents were likely to have been: CNS (*Staph. xylosus*); *Micrococcus* sp. and/or a CNS (*Staph. xylosus, Staph. capitis*); *Staph. aureus* and *Man. haemolytica* respectively. *Aec. viridans* was isolated in large numbers from sample NT01L, and may have been present as an environmental contaminant, although Devriese et al. (1999) reported isolating *Aec. viridans* from cases of bovine SCM. In samples W58L, G24L, W102L, Y105L, G157R and W125L, DMSCC was $500–1000 \times 10^3$ cells/ml. Sample G24L was bacteriologically negative and in the other four samples small numbers of CNS, *Acinetobacter* sp. and *Corynebacterium* sp. were found. It seems unlikely that these bacteria were involved in significant infection, although the CNS may have initiated a mild inflammatory response. In samples Y37L and Y146R, DMSCC was $300–400 \times 10^3$ cells/ml. Neutrophils predominated, which may suggest an inflammatory response, although Leitner et al. (2000) found neutrophils to be the predominant leucocyte in normal bovine milk samples. Large numbers of CNS, *Corynebacterium* sp., *Acinetobacter* sp. and *Pasteurella* sp. were isolated. It is possible that the CNS were causing a mild inflammatory response, although bacterial isolates may have been present as normal flora or environmental contaminants. Isolation of *Acinetobacter* sp. in large numbers from sample Y146R is interesting given that Ndegwa et al. (2001) isolated *Acinetobacter* sp. from mastitic caprine glands. In the remaining samples (Y4L, G16R, and W54R), DMSCC was $<400 \times 10^3$ cells/ml and only small numbers of CNS were found, suggesting that they were not responsible for significant infection. Large numbers of *Pseudomonas* sp. were isolated from G16R and although they may have been present as contaminants, Kumari et al. (2002) have isolated *Pseudomonas* sp. organisms from cases of bovine SCM.

Pre-culture incubation and increased plate inoculation volumes were not tested in the current investigation, although they can increase the frequency of isolation of clinical mastitis pathogens in bovine milk (Dinsmore et al. 1992). In the current study, refrigeration or freezing of samples for 1 week had a detrimental effect on the isolation of a number of bacterial species, including *Staph. aureus* and *Man. haemolytica*. These findings contradict those of Villanueva et al. (1991) and McDougall (1999) who found that pre-culture freezing of bovine and caprine milk samples respectively, at −20 °C, increased the frequency of *Staph. aureus* isolation. Murdough et al. (1996) and McDougall (1999) found that pre-culture freezing at −20 °C had no effect on the viability of a range of bacterial species isolated from subclinically affected bovine and caprine milk samples. Schukken et al. (1989) found that that pre-culture freezing at −20 °C, and increased duration of freezing, decreased the number of bovine milk samples from which *Esch. coli* and *Arcanobacterium pyogenes*

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were isolated, had no effect on the number of samples from which *Streptococcus* spp. and *Staph. aureus* were isolated and increased the number of samples from which CNS were isolated. The affect of freezing on isolation of individual species of CNS was not reported. Bradley et al. (2002) reported that inclusion of a cryopreservant such as glycerol improved the survival of bovine mastitis pathogens in frozen samples. In the current study, isolation was augmented by storage (refrigerated or frozen) in only a few bacterial species, including *Staph. saprophyticus*, *Pseudomonas* sp. and *Micrococcus* sp. It is concluded that bacteriological investigations are best performed on fresh milk samples. The potential benefits of using cryopreservants to enhance the isolation of ovine SCM pathogens from frozen samples warrant further investigation.

Incubation of cultures at room temperature was shown to be detrimental to the isolation of *Staph. aureus* and *Man. haemolytica*, whereas there was little difference in the likelihood of isolating the known SCM pathogens when incubation temperatures of 28 and 37 °C were used. Incubation at 42 °C appeared to be detrimental to the isolation of a number of species, including *Staph. aureus*. These results suggest that the routinely used incubation temperature of 37 °C is appropriate for the isolation of most pathogens responsible for ovine SCM. Additionally, the results of this investigation suggest that centrifugation of samples prior to plating, the use of sample media other than the routinely used sheep blood agar, incubation in anaerobic and microaerophilic conditions, and examination of defatted or non-defatted Gram-stained smears, do not confer an advantage over other materials and methods when attempting to identify pathogens responsible for ovine SCM.

In making recommendations for sheep producers, economic data are needed to determine whether diagnosis of ovine SCM is beneficial at the flock level. Costs, including the expense of the CMT reagent or automated SCC and laboratory costs for bacteriology, as well as the cost of training in the use of CMT and the labour required to undertake the testing, must be considered along with the potential benefits of increased lamb growth rates, reduced lamb mortality and reduced incidence of clinical mastitis, leading to a reduction in ewe mortality and premature culling rates. The main advantage of diagnosis would be to allow for targeted 'dry-ewe' therapy for cases that are identified early in lactation. Additionally, lambs of ewes affected by SCM could receive supplemental feeding or be removed and placed with a foster ewe, allowing immediate treatment of the affected dam.

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