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CLINICAL UTILITY OF TWO LEUKOCYTE ESTERASE REAGENT STRIPS FOR THE COW-SIDE DIAGNOSIS OF SUBCLINICAL MASTITIS IN LACTATING DAIRY CATTLE

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ABSTRACT

Mastitis increases the activity of more than 20 enzymes in the glandular secretions of dairy cattle, including esterase. We hypothesized that milk esterase activity provides an inexpensive, rapid, and practical cow-side method for diagnosing subclinical mastitis (SCM). Our objective was therefore to determine the clinical utility of measuring esterase activity in quarter milk samples using Multistix® and PeriScreen™ strips to predict SCM. Quarter foremilk samples were collected from 115 dairy cows at dry-off and 92 fresh cows within 4-7 days post calving. Quarter somatic cell count (SCC) was measured using Delaval® cell counter with SCC≥200,000 cells/mL as the reference method for diagnosing SCM. Milk esterase activity was measured using Multistix® and PeriScreen™ strips. The area under the receiver operating curve (AUC), kappa coefficient (κ), and positive likelihood ratio (+LR) were calculated and $P<0.05$ was considered significant. The Serim PeriScreen™ strips had a marginally better diagnostic performance than the Multistix® strips. At the optimal cut-point≥trace, the PeriScreen™ strip had an AUC=0.75, κ=0.32, and +LR=25.5 at dry-off, and AUC=0.66, κ=0.38, and +LR=∞ in fresh cows. At the optimal cut-point≥trace, the Multistix® strip had an AUC=0.71, κ=0.31, and +LR=4.2 at dry-off, and AUC=0.63, κ=0.31, and +LR=14.0 in fresh cows. The AUC,κ, and +LR values for the Multistix® and PeriScreen™ strips are considered suboptimal for a diagnostic test because clinically useful tests typically have an AUC >0.80,κ>0.6, or +LR>10. We therefore conclude that Multistix® and PeriScreen™ strips do not provide clinically useful cow-side tests for diagnosing SCM in lactating dairy cattle.

List of abbreviations:
SCC: Somatic cell count; IMI: Intramammary infection; SCM: Subclinical mastitis; PMNs: Polymorphonuclear cells; DCC: Delaval® cell counter; AUC: Area under the curve; ROC: Receiver operating characteristic curve; Se: Sensitivity; Sp: Specificity; K: kappa coefficient

Key words: Intramammary infection, Somatic cell count, Serim PeriScreen™ strips, Multistix® strips, Delaval® cell counter.

INTRODUCTION

Mastitis leads to a variety of compositional changes in milk due to local inflammation, damage to glandular tissue, increased leukocyte counts, and serum components entering the milk due to increased permeability of the blood-milk barrier (Kitchen, 1981; Pyörälä, 2003). As a result, mastitis is accompanied by change in the level of at least 20 enzymes in glandular secretions (Kitchen, 1981).

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These enzymes include N-acetyl-D-glucosaminidase (NAG'ase), beta-glucuronidase, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, plasmin, catalase, elastase, and esterase (Kitchen, 1981; Zhao and Lacasse, 2008). Measurement of milk enzyme activity therefore has potential as a screening test for subclinical mastitis (SCM; Viguier *et al.,* 2009).

Esterase is primarily found in leukocytes, especially granulocytes (neutrophils, eosinophis, basophils) and the azurophilic granules of monocytes. Esterase enzymatic activity is the basis of the leukocyte esterase reagent strips that have been developed and produced by several manufacturers and marketed under different commercial names worldwide to detect leukocytes in a variety of body fluids. The

Leukocyte esterase reagent strips were developed initially as a semi-quantitative test for leukocytes in urine (St. John *et al.,* 2006), and have been shown to be useful in other body fluids suc has peritoneal (Koulaouzidis *et al.,* 2008), synovial (McNabb *et al.,* 2017), cerebrospinal (Moosa*et al.,* 1995) and bronchoalveolar lavage fluid (Jacobs *et al.,* 2000) for detecting the presence of increased numbers of leukocytes (Mendler *et al.,* 2010). The World Health Organization has published guidelines for the development of diagnostic tests for infectious agents in resource-poor settings. The tests must be affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and delivered to those in need, providing the acronym "ASSURED" (Urdea *et al.,* 2006; Yetisen *et al.,* 2013). The esterase reagent strip has several advantages over other point-of care tests including ease of use, low cost, and short test time.

The Multistix® urine test strip pad contains a detergent and 3-hydroxy-5-phenyl-pyrole esterified with an amino acid that is used as an enzyme substrate (Mendler *et al.,* 2010). The detergent lyzes granulocytes, predominantly neutrophils, that have been absorbed into the pad and releases esterase that hydrolyses the ester compound, thereby releasing a pyrole compound that reacts with a diazonium salt to yield a violet/purple azo dye. The intensity of the violet color is correlated with the leukocyte count (Kutter *et al.,* 1987; Moosa *et al.,* 1995). The Peri Screen™ test strip is designed to provide a semi-quantitative indication of the leukocyte concentration in peritoneal dialysate effluent for the early identification of peritonitis. The reaction at the test strip pad is based on esterase catalyzing the hydrolysis of an indoxyl ester compound to indoxyl. The formed indoxyl reacts with a diazonium salt to produce a violet/purple color, and similar to the Multistix® strip the intensity of the color is correlated with the leukocyte count (Mendler *et al.,* 2010). The Porta SCC® test is a commercial adaptation of the esterase test specifically designed for detecting SCM in lactating dairy cattle. The Porta SCC® test utilizes a different dye substrate, 3-(N-tosyl-L-alanyloxy)-indol (Taloxin) that is hydrolyzed by esterasein milk to form an intense indigo blue colored dye. We have provided a preliminary report of the test performance of the PortaSCC® test elsewhere (Kandeel *et al.,* 2017) and the focus of this study is to characterize the diagnostic performance of the Multistix® and PeriScreen™ strips in milk.

Leukocyte esterase reagent strips have been evaluated as a method to diagnose reproductive infections in cattle. Our laboratory evaluated the clinical utility of the Multistix® stripin diagnosing seminal vesiculitis in 155 yearling beef bulls in 2011 (Hiew *et al.,* 2011). A Multistix® strip score of trace or greater had a sensitivity of 0.32 and specificity of 0.93 for detecting the presence of leukocytes in the semen sample. A 2012 study evaluated the clinical utility of the Multistix® stripin diagnosing endometritis in dairy cows at 40-60 days postpartum (Cheong *et al.,* 2012). A Multistix® strip score of moderate or greater had a sensitivity of 0.77 and specificity of 0.52 for detecting the presence of >10% neutrophils in cells obtained from uterine lavage fluid samples. Additional studies have been completed evaluating the clinical utility of the Multistix® strip in diagnosing endometritis in dairy cattle (Couto *et al.,* 2013; Hajibemani *et al.,* 2016) and beef cattle (Ricci *et al.,* 2017). Based on the above, we hypothesized that the semi-quantitative estimation of milk esterase activity using two leukocyte esterase reagent strips could provide an economic, rapid, and practical cow-side method for diagnosing SCM in lactating dairy cows. Our objective was therefore to characterize the ability of milk esterase activity measured by the Multistix® and PeriScreen™ strips to predict the presence of SCM and IMI in lactating dairy cattle at dry-off and freshening. These two time periods were selected for investigation because decisions are made at these time points as to whether intramammary antibiotics should be infused in order to treat SCM or IMI.

MATERIALS AND METHODS

All methods were evaluated and approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC). This study was part of a series of studies evaluating the diagnostic performance of a variety of tests for detecting SCM in lactating dairy cows.

1. Animals, housing, milking, and feeding

An observational study using a convenience sample of 115 dairy cows at dry-off, and 92 fresh cows during 4-7 days postpartum), was performed at the University of Illinois Dairy Research Farm (UIDRF) over a period of 13 months between July 1, 2015 and July 31, 2016.

Cows in late lactation were housed outside in a free stall barn. After calving, fresh cows were kept in a closed tie stall barn until they recovered from any postpartum health issues before being moved to a free stall barn. Cows were fed a dry cow ration, an acidogenic total mixed ration in the last 2-3 weeks of gestation, and a lactating cow total mixed ration based on formulations recommended by the National Research Council (NRC, 2001).

Late lactation cows were milked twice daily in a milking parlor at 04:00 and 16:00. Fresh cows were milked three times daily at 05:00, 14:00, 21:30. Data including breed, age, days in milk (DIM), daily milk production, and parity were retrieved from the farm records using the dairy's automatic recording software (PCDart, DRMS, Ames, IA).

2. Experimental methods

The dairy was visited at least once a week to collect foremilk samples from each quarter separately in the same week of drying-off and at 4 to 7 days postpartum. Cows in the free stallbarn were moved to a shaded area and restrained with the aid of a halter. A physical examination was performed on each cow in order to identify the presence of relevant systemic disorders. The teat end of each quarter was cleaned with alcohol 70% and 20 mL foremilk samples were collected separately within 50 seconds of first touching any teat using a sterilized plastic tube by hand stripping after discarding the first three squirts of milk.

Five mL duplicate milk samples for bacteriological examination were then collected aseptically from each quarter into sterile tubes. The teat end was scrubbed with 70% alcohol soaked cotton swabs until dirt no longer appeared on the swabs. The tube was held at a 45° angle from the teat end and a mid-stream milk sample was collected aseptically. The milk samples were stored in a cooler with iced water for transportation to the laboratory (National Mastitis Council, 1999) and cultured within 4 h of milk collection.

The udder and milk were examined by inspection and palpation in order to detect the presence of any abnormalities, including the cardinal signs of inflammation or abnormal milk.

3. Somatic cell count

Direct estimation of the SCC was performed electronically using a Delaval cell counter (DCC, DeLaval International AB, Tumba, Sweden). Approximately 1µL of foremilk was drawn into a single-use cassette and inserted into the DCC. The result in cells/µL of milk was displayed after 45 seconds; this number was multiplied by 1,000 to provide cells/mL of milk.

4. Esterase activity

Foremilk esterase activity was measured using two different colorimetric methods (Multistix® and Periscreen™ strips) within 4 h of milk collection.

Multistix® 10 SG urine test strips (Bayer HealthCare Inc., Elkhart, IN, USA) were used to measure milk esterase activity according to the manufacturer's instructions. The pad contained 0.4% w/w derivatizedpyrrole amino acid ester; 0.2% w/w diazonium salt; 40.9% w/w buffer; 58.5% w/w nonreactive ingredients. The stated lower limit of detection in urine was 10-20 leukocytes/µL, equivalent to 10,000-20,000 leukocytes/mL. One strip was removed from the bottle and the reagent pads of the strip were completely immersed in a well-mixed milk sample. The strip was removed from the sample within 1 second, gently shaken to remove excess milk, and left to stand on a flat clean surface with the indicator pad facing up for up to 60 minutes. The Multistix® strip reaction was visually scored using the five point chromatic scale provided by the manufacturer as follows; negative, trace, one positive (small), two positive (moderate), three positive (large).The manufacturer recommended that the strip be measured at 2 minutes when used to analyze urine. In preliminary studies we observed that the color of the esterase pad changed slowly over time after immersion in milk, and consequently we visually scored the test strip at 2, 30, and 60 minutes after immersion. Other investigators have determined that a 10 minute reading time was optimal for diagnosing endometritis in dairy cows (Hajibemani *et al.,* 2016).

Serim PeriScreen® test strips were then used to measure milk esterase activity according to the manufacturer's instructions. The developed color in the indicator pad was compared after 4 minutes of immersion in milk with the four point color chart provided by the manufacturer: negative (leukocytes ~ 20-30 cells/µL in dialysate), trace (leukocytes ~ 40-180 cells/µL in dialysate), small (leukocytes ~ 100-300 cells/µL in dialysate), and large (leukocytes ~ > 300 cells/µL in dialysate). The stated lower limit of detection in peritoneal fluid was therefore 40,000-180,000 leukocytes/mL.

5. Effect of sample temperature

The effect of sample temperature on the performance of the two esterase reagent strips was investigated using 15 randomly collected 20 mL composite milk samples obtained from Holstein-Friesian cows during milking in the parlor. The temperatures of the composite milk samples were equilibrated to approximately 4°C, 20°C, and 37°C by placing the samples in the refrigerator, at room temperature, or in a water bath at 37°C, respectively, for 30 minutes. The two esterase reagent strips were then used to test milk esterase activity as previously described.

6. Milk culturing for mastitis pathogens

Milk culturing was performed based on National Mastitis Council recommendations (NMC, 1999) except a 100 µL aliquot was used instead of the recommended 10 µL aliquot. A pipette and sterile pipette tips were used to aspirate 100 µL of each quarter milk sample and the aliquot was placed on one-half of Blood agar (TSA W/ 5% sheep blood agar; Remel, Lenexa, KS, USA) and MacConkey plates (Remel, Lenexa, KS, USA). The milk sample was then streaked using a sterile wire loop in a fashion that permitted the growth of isolated bacterial colonies. The plates were incubated in an inverted position at 37°C for 48 hours and the microbial growth and colony type recorded. The isolated pathogen was identified using colony morphology, hemolysis patterns, biochemical tests including catalase and coagulase test, and Gram staining reaction and cell morphology. Culture results were interpreted by applying published recommendations

where the isolation of single colony from 0.01 mL milk, equivalent to the isolation of 10 colonies from 100 µL milk, was categorized as an intramammary infection (IMI), (Dohoo *et al.,* 2011).

7. Statistical analysis

Data was expressed as median and interquartile range and $P < 0.05$ was considered significant. A statistical software program (SAS 9.4, SAS Inc, Cary NC) was used for all analyses. Subclinical mastitis was defined as SCC was >200,000 cell/mL using the DCC. Spearman's correlation coefficients (PROC CORR) were calculated to characterize the association between Delaval SCC and milk esterase activity measured by Multistix® and Periscreen™ strips.

Logistic regression (PROC LOGISTIC) was used to characterize the relationship between SCM as determined by the reference method (1 = SCC > 200,000 cells/mL; 0 = SCC ≤ 200,000 cells/mL) or IMI as determined by milk culture (1 = > 10 colonies; 0 = <10 colonies) and esterase score measured by the two methods at dry off and freshening. Receiver operating characteristic (ROC) curves were constructed for each logistic regression model. The area under the ROC curve (AUC) was calculated as a global index of test performance; AUC values for ROC curves > 0.9 typically indicate a highly accurate test, whereas AUC values of 0.7 to 0.9 indicates moderate accuracy, 0.5 to 0.7 low accuracy, and 0.5 represents a chance result (Swets, 1988). Sensitivity and specificity were calculated at the optimal cut-point of each ROC using the Youden index (the cut-point where the following expression has its maximum value: Se + Sp – 1). This equally weights the value of Se and Sp. The positive likelihood ratio (+LR) was calculated as: +LR = Se/(1-Sp); values >10 indicate that a positive test is good at ruling in a diagnosis, such as SCM or IMI (Grimes and Schulz, 2005). The Kappa coefficient (κ, PROC FREQ) was calculated using the optimal cut-point of the ROC to characterize the level of agreement between milk esterase activity and the two reference methods (SCM, IMI). Values for $\kappa < 0.2$ indicate poor agreement, whereas $0.2 < \kappa < 0.4$ indicates fair agreement, $0.4 < \kappa < 0.6$ indicates moderate agreement, $0.6 < \kappa < 0.8$ reflects good agreement, and $\kappa > 0.8$ indicates excellent agreement (Landis and Koch, 1977).

The effect of sample temperature on the test result was evaluated using Fisher's exact test (PROC FREQ) for Multistix® and Periscreen™ strips and mixed models analysis (PROC MIXED) for $\log_{10}$(SCC) using an unstructured correlation matrix.

RESULTS

Quarter milk samples (n = 459) were obtained at dry off from 115 cattle, comprising 102 Holstein-Friesian, 9 Jersey, 2 Ayrshire, 1 Brown Swiss, and 1 Milking Shorthorn. One cow had a blind quarter. Quarter milk samples (n = 364) were obtained from 92 cattle on day 4 to 7 of lactation, comprising 81 Holstein-Friesian, 8 Jersey, 1 Ayrshire, 1 Brown Swiss, and 1 Milking Shorthorn. Four cows had one blind quarter. The prevalence of SCC based on SCC > 200,000 cells/mL on a quarter basis was 69% at dry-off and 33% at freshening. Spearman correlation coefficients between variables of interest at dry-off and freshening are summarized in Table 1.

1. Multistix® stripscores

The number of quarters with a trace or higher reaction varied depending on whether the Multistix® strip reaction was visually scored at 2 min (0%, 0/820), 30 min (4%, 28/669), or 60 min (25%, 202/799). As we were more interested in a higher test sensitivity than a higher test specificity, we analyzed the data using a reading time of 60 min for the Multistix® stripreaction.

Only two quarters had a Multistix® strip score of large (both at dry off). Consequently, test strip scores of moderate and large were collapsed into a group named moderate/large. The median SCC measured at dry off was 244,500, 529,500, 1,249,000, and 1,818,000 cell/mL in quarters with Multistix® strip scores of negative, trace, small, and moderate/large, respectively (Fig. 1, top panel). For comparison, the median SCC measured at freshening was 100,000, 877,000, 4,000,000, and 4,000,000 cell/mL in quarters with Multistix® strip scores of negative, trace, small, and moderate/large, respectively (Fig. 1, bottom panel).

The distribution of Multistix® strip scores differed ($P < 0.0001$) for quarters with and without SCM or an IMI (Figs. 1, 2). Higher Multistix® strip scores in a quarter were positively associated with an increased probability of SCM and IMI using a cut-point of ≥ trace.

2. Periscreen™ stripscores

Only 2 quarters had a Periscreen™ strip score of large (one at dry off, one at freshening). Consequently, test strip scores of small and large were collapsed into a group named small/large. The median SCC measured at dry off was 258,500, 953,500,and 3,664,000 cell/mL in quarters with Periscreen™ test scores of negative, trace, and small/large, respectively, (Fig. 3, top panel). For comparison, the median SCC measured at freshening was 104,000, 901,000, and 4,000,000 cell/mL in quarters with Periscreen™ strip scores of negative, trace, and small/large, respectively (Fig. 3, bottom panel).

The distribution of PeriScreen™ strip scores differed ($P < 0.0001$) for quarters with and without SCM or an IMI (Figs. 3, 4). Higher PeriScreen™ strip scores in a quarter were positively associated with an increased

probability of IMI using a cut-point of ≥ trace for the identification of the infected quarters.

3. Logistic regression analysis

The AUC, optimal cut-point, Se, Sp, and +LR values identified from logistic regression analysis for diagnosing SCM are presented in Table 2. The AUC for the Multistix® strip at dry off and freshening were similar to that for the PeriScreen™ strip (P = 0.87 and 0.10, respectively). The κ values using the optimal cut-points for SCM for the Multistix® and Peri Screen™ strips were categorized as fair at dry off and freshening.

The AUC, optimal cut-point, Se, Sp, and +LR values identified from logistic regression analysis for diagnosing IMI are also presented in Table 2. The AUC for the Multistix® strip at dry off and freshening were similar to that PeriScreen™ strip (P = 0.97 and 0.055, respectively). The κ value using the optimal cut-points for SCM were only fair and similar for the Multistix® and Peri Screen™ strips at dry off and freshening (Table 2).

4. Effect of milk temperature on test performance

There was no effect of temperature on the SCC (P = 0.058) or the Multistix® and Periscreen™ strip score (P = 1.00).

Table 1: Spearman correlation coefficients among variables of interest for 115 dairy cows at dry-off and 92 cows at freshening. The information in parentheses are the number of quarters used for comparison and the P value. SCC = somatic cell count

Variable	Delaval SCC	Multistix® strip	Periscreen™ strip
Dry off			
Delaval SCC	1.00	0.58 (435, P<0.0001)	0.75 (249, P<0.0001)
Multistix® strip		1.00	0.60 (242, P<0.0001)
Periscreen™ strip			1.00
Freshening			
Delaval SCC	1.00	0.45 (364, P<0.0001)	0.52 (259, P<0.0001)
Multistix® strip		1.00	0.75 (259, P<0.0001)
Periscreen™ strip			1.00

Table 2: Summary of the results of logistic regression analysis of the ability of milk esterase activity measured by the Multistix® and Periscreen™ stripsto predict subclinical mastitis (SCM) and intramammary infection (IMI) in quarter milk samples obtained from dairy cows at dry off and freshening.

	n	Optimal strip cut-point for esterase activity	AUC	Sensitivity	Specificity	+LR	κ
Subclinical mastitis							
Dry off							
Multistix® strip	435	≥ trace	0.71	0.50	0.88	4.2	0.31
Periscreen™ strip	249	≥ trace	0.75	0.51	0.98	25.5	0.32
Freshening							
Multistix® strip	364	≥ trace	0.63	0.28	0.98	14	0.18
Periscreen™ strip	364	≥ trace	0.66	0.32	1.00	∞	0.22
Intramammary infection							
Dry off							
Multistix® strip	435	≥ trace	0.60	0.52	0.64	2.1	0.10
Periscreen™ strip	249	≥ trace	0.66	0.66	0.66	1.9	0.19
Freshening							
Multistix® strip	364	≥ trace	0.65	0.36	0.94	6.0	0.34
Periscreen™ strip	364	≥ trace	0.75	0.54	0.94	9.0	0.48

n = number of quarters used in the analysis; AUC – area under the response characteristic curve; κ = kappa coefficient, which is a measure of agreement between the prediction of SCM or IMI using an increased esterase activity of trace or higher measured by the two methods and a diagnosis by the reference method; +LR = positive likelihood ratio.

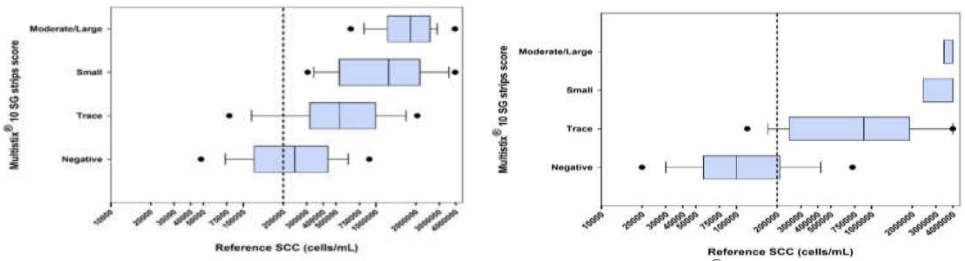

Figure 1: Top panel – Box and whiskers plot of the association between the Multistix® strip scores and somatic cell count (SCC) measured by the reference method in 435 quarters at dry off. The shaded box represents the first and third quartile, the vertical line in the shaded box represents the median value, the whiskers represent the 10th and 90th percentiles, and filled circles represent data points outside this percentile range. The vertical dashed black line indicates the SCC cut-point for subclinical mastitis (200,000 cells/mL).
Bottom panel - Box and whiskers plot of the association between the Multistix® strip scores and SCC measured by the reference method in 364 quarters at freshening.

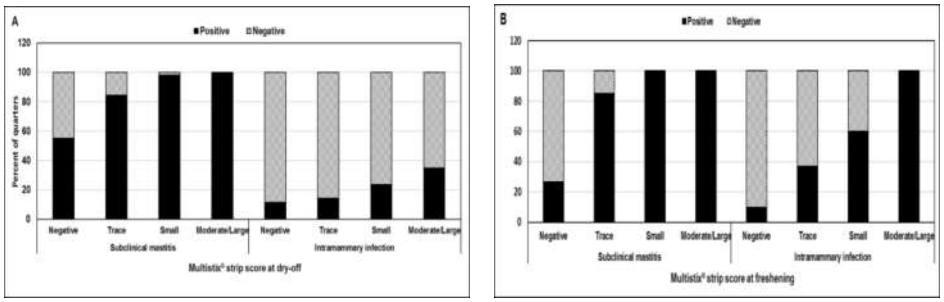

Figure 2: Panel A – Associations between Multistix® strip scores and the presence of subclinical mastitis (SCM) or an intramammary infection (IMI) for 435 quarters at dry-off.
Panel B – Associations between Multistix® strip scores and the presence of SCM or IMI for 364 quarters at freshening.

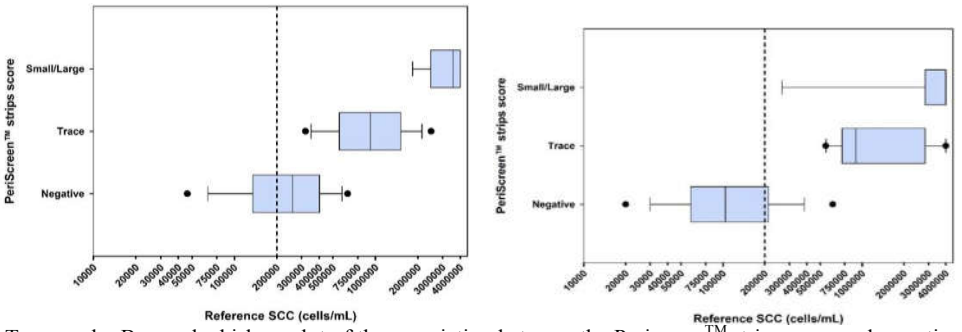

Figure 3: Top panel – Box and whiskers plot of the association between the Periscreen™ strip scores and somatic cell count (SCC) measured by the reference method in 249 quarters at dry off. The Periscreen™ strip scores were categorized as negative, trace, or small/large. See Fig. 1 legend for more details.
Bottom panel - Box and whiskers plot of the association between the Periscreen™ strip scores and SCC measured by the reference method in 259 quarters at freshening.

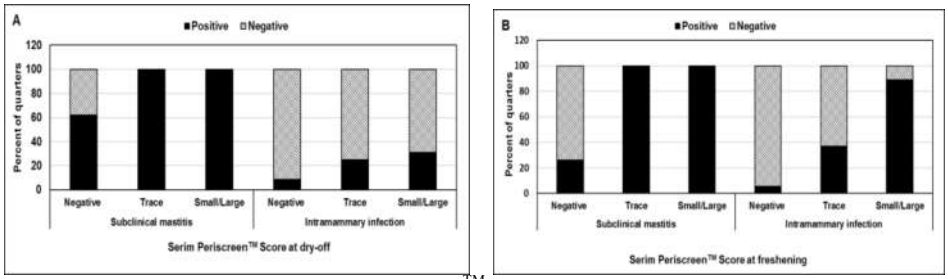

Figure 4: Top panel – Associations between the Periscreen™ score and the presence of subclinical mastitis (SCM) or an intramammary infection (IMI) for 249 quarters at dry-.
Bottom panel – Associations between the Periscreen™ score and the presence of SCM or an IMI for 259 quarters at freshening

DISCUSSION

This appears to be the first study evaluating the clinical performance of Multistix® and Peri Screen™ strips for identifying SCM in dairy cattle at dry-off and early lactation based on the semi-quantitative measurement of leukocyte esterase activity in milk. The major findings of our study were that the clinical utility of the Multistix® and PeriScreen™ strips for diagnosing SCM was only fair as clinically useful tests typically have an AUC >0.80 and κ>0.6. We therefore conclude that the Multistix® and PeriScreen™ stripsdo not provide clinically useful cow-side tests for diagnosing SCM in lactating dairy cattle.

Milk esterase activity was extensively studied in the 1960's as a possible diagnostic method for SCM. Several studies reported a rapid increase in milk esterase activity either parallel with or immediately after an increase in milk SCC (Forster *et al.,* 1961; Booth *et al.,* 1965; Marquardt and Forster, 1965; Marquardt *et al.,* 1966). In the initial study, Forster and colleagues recommended the development of simple colorimetric procedure to assay esterase, since phenol is a hydrolytic product of the substrate, phenyl acetate (Foster *et al.,* 1961); however, the procedure used to measure esterase activity at that time was a manometric system involving the Warburg apparatus (Warburg manometric techniques) that was time-consuming and required trained personnel and expensive equipment (Luedecke *et al.,* 1967). Consequently, measuring esterase as a mastitis diagnostic tool lost favor and by 1968, milk esterase activity was believed to offer little practical diagnostic benefit over the milk leukocyte count (Prasad, and Newbould, 1968).

Our results are similar to that previously reported (Forster *et al.,* 1961; Booth *et al.,* 1965; Marquardt, and Forster, 1965; Marquardt *et al.,* 1966) in that a higher milk esterase activity is associated with an increased probability of SCM, as determined by SCC >200,000 cells/mL. This finding was expected on the assumption that milk esterase activity is correlated with milk leukocyte count, as increased milk SCC reflects a marked increase in milk leukocyte count (Kitchen, 1981; Pyörälä, 2003; Constable *et al.,* 2016). However, the exact source of esterase in cow's milk has not been confirmed (Marquardt *et al.,* 1966; Prasad and Newbould, 1968), and approximately 70% of the esterase activity in cow's milk is due to non-enzyme proteins, with only 20% of the activity being enzymatic in origin (Downey and Andrews, 1965). Because esterase activity in plasma is typically 1,000 to 2,000 times the activity in milk (Marquardt and Forster, 1965), it is possible that some of the milk esterase activity is due to increased blood-milk permeability and movement of plasma constituents into the mammary gland and secretions (Marquardt *et al.,* 1966), or the intrinsic properties of non-enzymatic proteins for hydrolysis (Downey and Andrews, 1965).

The lack of agreement between milk esterase activity and the presence of IMI based on culture results may be attributed to the failure to isolate mastitis pathogen from the majority of quarter milk samples with elevated SCC. The latter situation may result from mastitis episodes where bacteriais not the cause of udder inflammation, intermittent shedding or low concentration of the mastitis pathogen from the infected gland, intracellular location of the pathogens and the presence of bacterial growth inhibitors in milk, or the spontaneous elimination of the infection from the udder (Constable *et al.,* 2016). Furthermore, culture negative quarter samples with increased SCC may reflect delayed healing of infection where the pathogens are eliminated from the udder while the infiltration of leukocytes continues until complete healing has occurred.

The performance of the esterase reagent strips in milk is likely to have been impacted by matrix effects, where the high protein and fat content of milk relative to urine may have contributed to their suboptimal performance. The strips and color scales were developed for the diagnosis of infection in the urine, where the protein concentration is usually lower than 1 g/L, and in ascites, where the protein concentrations is less than 30 g/L but fat is not present. For comparison, milk is a complex physicochemical suspension; with the bulk tank milk for this dairy herd averaging 32 g/L of protein and 36 g/L of fat. Protein could decrease leukocyte esterase activity, either by the presence of an inhibitor of this enzyme, or by physical interference with the reagent pad detergent or enzyme substrate (Gülberg *et al.,* 2007; Nousbaum *et al.,* 2007). The protein concentration may also provide a possible explanation for the slightly better performance of PeriScreen™ strip sover the Multistix® strips, where the PeriScreen™ strips were developed to be used in patients with ascites where the protein content is ≈30 g/L and closer to that of milk (≈36 g/L) than urine (1 g/L). Milk fat may also have interfered with the reaction of the strip through physical interference with the detergent in the reagent pad.

The costs of the three tests used in this study were US $0.36, $4.00, and $2.33 for the Multistix® strips, PeriScreen™ strips, and DCC test respectively, although the DCC test cost does not include the purchase cost of the analyzer. For comparison, the California Mastitis Test (CMT) costs $0.04/test. Because of its cow-side application, much lower cost, and acceptable sensitivity and specificity values in this study population, there does not appear to be a persuasive reason for preferring the Multistix® or PeriScreen™ strips over the CMT.

Aprimary goal of our laboratory is to find a rapid, accurate on-farmor cow-side test for identifying

quarters with SCM or IMI. We considered that sensitivity and specificity of the tests under evaluation were equally important. On this basis, AUC, κ, and +LRare useful indices for the overall performance of a clinical test. Ideally, such a test should have an AUC>0.90, a sensitivity close to 100%, specificity as high as possible, and +LR >10. Using the optimal cut-points identified during logistic regression, the AUC for both Multistix® and PeriScreen™ strips indicated low to moderate accuracy at freshening and dry-off, respectively. Similarly, the κ coefficient indicated fair agreement between both the Multistix® and Peri Screen™ strips result and the reference method at both freshening and dry-off in classifying quarters by infection status. Although the +LR was >10 for diagnosing SCM using the Multistix® strip at freshening and the Peri Screen™ strip at dry off and freshening, the sensitivity of the two strips was too low to make them useful. Our findings therefore indicate an overall suboptimal performance of both esterase reagent strips in milk. Consequently, neither strip appears to provide a clinically useful test for identifying quarters with SCM at dry off or freshening.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

DISCLOSURE

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الفائدة الاكلينيكية لاثنين من الاشرطة الكاشفة عن انزيم الاستيريز المتحرر من الكريات البيضاء لتشخيص مرض التهاب الضرع تحت السريري في الماشية الحلابه

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يعد مرض التهاب الضرع في الماشية من المسببات الضاره للعائد الاقتصادي لمزارع الالبان عالمياً ليس فقط نتيجة انخفاض انتاج اللبن وانخفاض جودة الحليب المنتج والتخلص من الحيوان المنتج للبن مبكراً أو تكاليف العلاج ولكن ايضا نتيجة فقد ونفوق الحيوانات المصابة نفسها. إجريت هذه الدراسه لالقاء الضوءعلي الصورة تحت السريرية للحيوانات المصابة بمرض التهاب الضرع وافضل الطرق للتشخيص المبكر للمرض وباقل تكلفه اثناء الأسبوع الاخير من الحلابه قبل التجفيف والاسبوع الاول بعد الولادة. حيث تم تقييم الكشف عن انزيم الاستيريز بأستخدام اختبارات سريعة متداولة في الأسواق للأستخدام الآدمي للكشف عن هذا الانزيم في البول والسائل البريتوني. تم تجميع عينات حليب من الابقار الحلابه الموجود في المزرعة التعليمية بجامعة إلينوي بالولايات المتحدة الأمريكية حيث قد تم زيارة المزرعة مرة علي الأقل أسبوعياً وتم تجميع العينات بواقع عينة من كل غدة (ربع)علي حده من ١١٥ بقرة حلابه في اخر موسم الحليب وقبل تجفيفها ومن ٩٢ بقرة خلال اليوم الرابع الي السابع بعد الولادة. تم تقييم استخدام اشرطة الماتيستيكس والبريسكرين في الحليب والتي تستخدم للكشف عن انزيم الاستيريز في البول والسائل البريتوني في الانسان علي التوالي حيث تم اختبار قدره هذه الاشرطة المختلفه للكشف عن انزيم الاستيريز في اللبن لتشخيص مرض التهاب الضرع تحت السريري ومقارنتها بعدد الخلايا الجسمية للبن المقاسة اليكترونياً باستخدام جهاز الديلفال وكذلك نتائج الزرع للكشف عن وجود عدوي بأحد الميكروبات المسببة لالتهاب الضرع كطرق مرجعيه للكشف عن المرض. وقد تبين عدم قدرة هذه الاشرطة للكشف عن انزيم الاستيريز في اللبن للتشخيص المبكر للأصابة بالتهاب الضرع تحت السريري والذي قد يرجع الي احتواء الحليب علي نسبه عالية من البروتين والدهون والتي قد تتعارض مع او تعوق التفاعل الانزيمي لهذه الاشرطة للكشف عن وجود انزيم الاستيريز.

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