Evaluation of two faecal occult blood diagnostic tests for detection of non-perforating abomasal lesions in cattle

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Research article

Keywords: Abomasal lesion, abomasal ulcer, bovine, diagnostic test, faecal occult blood test, guaiac, tetramethylbenzidine

Posted Date: September 6th, 2019

DOI: <https://doi.org/10.21203/rs.2.13955/v1>

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Abstract

Non-perforating abomasal lesions have been observed with a high prevalence in slaughtered dairy cattle. Of all lesions found 62-66% were reported to be bleeding and 41-44% were reported to be chronic. The diagnostic properties of two faecal occult blood tests for detection abomasal lesions were evaluated. First, an in vitro titration test was used to determine the detection limit for blood in faeces for each of the tests. A tetramethylbenzidine-based test detected 2 ml blood and a guaiac-based test detected 4.5 ml of blood per 1,000 g faeces. Then, to determine if blood is degraded by rumen microbes, an in vitro study of blood fermentation in rumen fluid was conducted. Blood or haemoglobin in the quantities used did not break down when fermented for 48 hours. Lastly, faeces from 1,553 cattle was collected in a slaughterhouse and tested for the presence of blood the same day and 2 days after. The faecal results were compared with the presence of abomasal lesions in the cattle investigated. Cattle with ≥ 4 lesion subtypes considered to be bleeding had more positive test results. Cattle with ≥ 4 subtype Ib lesions had 1.7 times higher odds of testing positive in the tetramethylbenzidine test on Day 2 (95% confidence interval: 1.4 – 1.9) and cattle with ≥ 4 subtype Ic lesions had 1.9 times higher odds of testing positive (95% confidence interval: 1.6 – 2.3). However, 20% false-positive results were also observed, and a positive test can not necessarily be interpreted as the cow having abomasal lesions.

Introduction

Abomasal lesions at the time of slaughter have been reported with a prevalence of up to 84% in dairy cattle [1], 66% in fattening bulls [2], and up to 77% in veal calves [3]. All but a few exceptions are non-perforating lesions of different types, e.g. mucosal erosions, acute or chronic deeper lesions or scars, whereas perforating lesions are very few [1, 4–6]. Of all the lesions, bleeding lesions have been reported with a prevalence between 62–66% and chronic lesions have a reported prevalence between 41–44% [1, 6]. The impact on production and reproduction appears to be limited, but the lesions may still be painful to the affected animal [5, 7]. However, ante-mortem diagnosis of abomasal lesions is challenging, although bleeding lesions should be detectable by detection of occult blood in the faeces. However, blood in faeces can originate from other sources than abomasal lesions, i.e. recent rectal examinations, epistaxis or traumatic lesions in the gastrointestinal tract.

Few veterinary tests are available for detection of occult blood in faeces within minutes. The only veterinary test for occult blood we can acquire is the SUCCEED Equine Fecal Blood Test (Freedom Health LLC, Ohio, USA). This tests is an equine immunochemical test for detection of albumin from undigested blood in the hindgut or haemoglobin in the foregut. The cost of this test is approximately 40 € per test, which is too costly for most dairy farmers to use routinely.

Traditional faecal occult blood tests in humans have included the use of products with the one of the active compounds orthotolidine, benzidine or guaiac to detect haemoglobin [8, 9]. The orthotolidine and benzidine tests have been reported as able to detect volumes of blood as low as 0.04 ml per 100 g human faeces [9], while the detection limit of the guaiac test initially was reported between 3.4 and 8 ml of blood per 100 g human faeces [8]. Even though orthotolidine and benzidine were the favoured test compounds in the 1970s their use were diminished substantially due to their carcinogenic effects [10, 11]. After the partial phasing out

of the orthotolidine test, the guaiac tests were the most sensitive and cost effective tests in human medicine. An example of the most sensitive guaiac faecal occult blood test currently known is the Hemocult II SENSА (Beckman Coulter, Brea, California, USA). This test is reported with a detection limit as low as the equivalent of 1.6 ml of blood per 100 g human faeces [12].

The guaiac tests do not have the same sensitivity for detection of blood as the orthotolidine or benzidine tests. Therefore a compound like orthotolidine and benzidine, but with less carcinogenic potential was investigated for its blood detecting abilities, namely tetramethylbenzidine (TMB) [11, 13]. TMB has been reported as having almost the same sensitivity for detection of blood as the benzidine tests [11]. A TMB test has been reported to have a detection limit between 3 and 5 ml blood per 100 g human faeces with a sensitivity of 69% in 13 samples with a measured blood volume between 3.0–4.9 ml (mean 3.8 ml) per 100 g human faeces. However, samples with lower amounts of blood were also found positive using the TMB test. The TMB test was positive in 5/30 of samples containing between 1.0 and 1.9 ml (mean 1.5 ml) blood per 100 g human faeces [14].

Even though the use of orthotolidine tablet tests have been described a few times in veterinary medicine throughout the last fifty years [15–17], a more recent slaughterhouse study used a guaiac test specific for human faecal samples [2]. However, the production of this guaiac test has been discontinued. A potentially more sensitive human TMB test than the guaiac test is the Hemo-fec (Med-Kjemi, Asker, Norway). The Hemo-fec test is currently produced with a listing price of roughly 0.11 € per test, while the human faecal occult blood guaiac test Hemocult II SENSА is produced with a listing price of approximately 1.30 €.

The main objective of this study was to estimate the diagnostic sensitivity and specificity of the Hemo-fec and the Hemocult tests used on bovine faecal samples for detection of different types of non-perforating abomasal lesions in dairy cattle. First, we should determine if haemoglobin is degraded by the rumen microbes in rumen fluid and then determine the detection limit.

Methods

To address the objective, we carried out two experiments and conducted one observational study.

Experiment A:

To determine the detection limit for detection of bovine blood in bovine faeces with two faecal occult blood tests Hemo-fec and Hemocult II SENSА, a titration experiment was performed.

Faeces from three clinical healthy cows used for teaching at the Large Animal Hospital at University of Copenhagen was collected before the morning sweeping. None of the cows had any rectal examinations performed within weeks prior to the experimental study. Only manure with no visible contamination (e.g. bedding, straw etc.) was collected. All faecal samples were tested for the presence of occult blood with the SUCCEED and the Hemo-fec test. The SUCCEED Equine Fecal Blood Test (Freedom Health LLC, Aurora, Ohio, USA) was used to verify that the negative control samples were haemoglobin negative. Only faecal samples

which had tested negative with the two faecal tests were used for the experimental setup testing used to determine the detection limit of the Hemo-fec and the Hemocult II SENSAs tests.

The experiment was made in 4 batches each with 4 samples of 1,000 g faeces. Batch 1 was composed of faeces from two animals. Batch 2 was made with faeces from one cow and Batch 3 had two cows contributing with faeces. Batch 4 was made with faeces from one cow.

For each batch, a total of 4,000 g of faeces were weighed in 4 plastic bags containing 1,000 g of faeces each. Fresh bovine blood was added to 3 of the 4 bags in different volumes as shown in Table 1. Bovine blood in volumes of 5, 25 and 50 ml was added in Batch 1; in Batch 2 and 3, blood in volumes of 0.5, 2 and 4.5 ml were added to each of the three different bags; while Batch 4 had volumes of blood at 0.25, 0.75 and 1 ml added to each bag (Table 1). Faeces and blood were mixed thoroughly with the hands through the plastic bag for 10 minutes to distribute the blood evenly in the faeces. The mixture was observed several times through the clear plastic during the mixing to ensure an even distribution of the blood. Three samples from each bag were placed in separate pre-labelled containers with a total volume of 100 ml. The sample containers were filled almost to the top with the faecal and blood mixtures. Three samples of the bag with no added blood were also made for control purposes. The reason for the slightly skewed series of blood volumes was that larger volumes were tested first to ensure a positive reaction; and then the detection limit was determined by diminishing the volume of blood until it was undetectable by the Hemo-fec test as shown in Table 1.

The first test was done immediately following the mixture of blood into faeces were placed in the smaller sample containers, the second test was made 8–10 hours after the first test and samples were tested in intervals of 10–12 hours after the second test with a total of 6 tests of each sample container and 2 tests of each sample bag.

Before testing the samples with the Hemo-fec test a table setup was prepared. Hand towel paper was laid out to protect the surface and to act as a writable surface. A piece of filter paper of approximately 4 cm x 1 cm was placed for each sample on top of the hand towel paper, a cotton swap was placed beside each strip of filter paper and a ball pen was used to make identification for each sample easy by writing the samples name above the piece of filter paper. By using the cotton swap, a small amount of faecal matter was applied to the filter paper (approximately a piece of faeces corresponding to the size of a small pea). When the sample was applied to the filter paper, the cotton swap was used to thin the sample in one end to better let the reagents pass through the faecal sample and down to the filter paper.

The Hemo-fec test was used according to the instructions of the manufacturer: One drop from the Reagent A vial immediately followed by one drop from the Reagent B vial were added on top of each sample applied to the filter paper. The results were interpreted within 10 seconds after the drop of Reagent B was made. A bluish-greenish-yellowish colour of the Hemo-fec test appearing on the filter paper was considered a positive result. If none of the colours mentioned above was observed within 10 seconds after the drop of Reagent B had been added, the test was considered as negative. All test samples were stored at 5° C between tests to ensure the highest accuracy [17].

All the samples from Batches 1–4 were also tested using the Hemocult II SENSE test. The Hemocult II SENSE test cards were prepared 20–24 hours after the batches were mixed. The manufacturer's instructions were followed and a test card was made for each of the faecal-blood mix samples: a test card contains two test windows at the front under a stamped out lid and a stamped out lid at the back. Faecal matter (approximately pea sized) was obtained from the faecal containers with a cardboard spatula. The small amount of faecal matter was smeared to the two test fields one at the time and the card board lid was placed on top of the sample. The test card was dried for at least 48 hours. To assess the test, two drops of the reagent were applied on each sample from the back and one drop was applied to the control patch for comparison of a positive or negative test. The results were evaluated and registered within 60 seconds after application of the solvent.

Experiment B

This experiment was carried out to determine if rumen microbes can degrade haemoglobin. Rumen fluid from two clinically healthy Danish Jersey heifers (serving as teaching animals at the University of Copenhagen) was harvested through a rumen fistula before morning feeding. The rumen fluid was filtered through two layers of household cheesecloth to remove the large feed particles. Filtered rumen fluid were mixed with a standard solution buffer in the ratio 1:2. From this mixture 90 ml of sample was transferred into three 100 ml glass containers, which served as fermentation chambers. Bovine blood in the volumes of 0, 1, 5 and 10 ml was added to each of four fermentation chambers and fermented for 48 hours at 39.5° C. The fermentation was conducted using a standardized protocol [18]. The fourth fermentation chamber with 0 ml blood, but still containing rumen fluid and standard buffered solution, served as negative control.

After fermentation, the 4 mixtures were poured into 100 ml plastic containers and tested for the presence of haemoglobin with the Hemo-fec test. The filtrate was transferred to filter paper using a cotton swap. Both the filter paper and the cotton swap were tested with Hemo-fec according to the manufactures guidelines as described above. After 10 seconds the tests were evaluated and the results were recorded.

Observational study

Faeces from 1,553 cattle were collected during an abattoir survey studying abomasal lesions described in a previous publication [1]. After evisceration of the cattle, the intestines were further divided. The rectal ampulla was opened in the longitudinal direction. An incision of 10–30 cm was made with a scalpel between the sigmoid colon and the rectum. When the intestine curled back from the incision, a faecal sample was collected from an area away from the incision to avoid blood contamination from the environment and cut mucosa.

The faecal samples were analysed using the Hemo-fec test as described above. The analysis of each sample was done on the day of faeces collection (Day 0) and 2 days after collection (Day 2). This was done to potentially increase the probability of a positive test, as the erythrocytes will decay over time and make the haemoglobin more accessible to the Hemo-fec reagents [17]. The faecal samples were stored at 5° C between test days to ensure the highest accuracy [17].

Abomasal lesions were also recorded as previously described, and approximately the same sample of cattle was used [1]. Briefly, lesions were scored as subtype Ia: superficial erosions of the mucosa, sometimes only identifiable due to lack of rugae; subtype Ib: lesions with local haemorrhage and a punched-out appearance, and where there were no obvious alterations of the mucosa near the subtype Ib lesions; subtype Ic: lesions with a crater-like appearance, where the edges of the lesions were bulging and often detritus was found at the surface of the lesion; and subtype Id: lesions where the mucosa makes retention towards the lesion in a star-shape, or holes in the spiral mucosal folds [6].

No analytical statistics were carried out for Experiment A and B, because the results grouped specifically with no need for statistical tests. The detection limit was determined based on the lower concentration detected for each test.

In the observational study, cow was used as the study unit, and the diagnostic sensitivity was estimated for each combination of lesion types as the proportion of cows being Hemo-fec positive at Day 0 or Day 2, respectively, among those with a given lesion type combination. The diagnostic specificity was estimated as the proportion of Hemo-fec negative cows among those without any lesions.

Many cows had multiple lesions and multiple lesion types, and to assess if this had an impact on the diagnostic sensitivity, we first carried out a univariable logistic regression for each of the lesion subtypes Ia, Ib, Ic and Id, and then a multivariable logistic regression.

The outcome was the Hemo-fec score (positive or negative) and the predictors were lesion subtypes. Lesion subtypes Ia, Ib and Ic were categorised in 0, 1, 2, 3, and ≥ 4 lesions, while lesion subtype Id was only grouped in 0, 1 and ≥ 2 lesions based on the distribution of observations. The multivariable logistic model was reduced using the likelihood ratio test, and $P < 0.05$ was considered statistically significant. The analyses were carried out in R v. 3.5.1 [19] with the logistic regression done using the `glm()`-function.

Results

The Hemo-fec results for determination of the detection limit in Experiment A are shown in Table 2. All samples containing blood in concentrations below 1 ml per 1,000 g faeces were negative and all samples with concentrations above 1 ml per 1,000 g faeces were positive in the Hemo-fec test during the different points in time the samples were tested. At the concentration 1 ml blood per 1,000 g faeces, some results were positive and some were negative. Hence, the detection limit was 2 ml per 1,000 g faeces for the Hemo-fec test. The results from the Hemocult II SENSAs are shown in Table 3. All samples with concentrations higher than 2 ml per 1,000 g faeces were positive and the rest were negative. Thus, the detection limit was 4.5 ml per 1,000 g faeces for the Hemocult II SENSAs tests.

The filtrated rumen fluid had characteristic colour changes correlating with the amount of added blood. The negative control was light brownish of colour, whereas the three samples with 1, 5 and 10 ml of added blood had an increasing dark reddish-brownish-blackish discolouration when compared with the negative control. The pH of the rumen fluid ranged between pH 6.83 and 6.99 after the fermentation.

The filtrate of the control with no added blood was negative for haemoglobin when tested using the Hemo-fec test. The filtrates with added blood were all positive when tested with the Hemo-fec reagents. The four types of test results are illustrated in Figure 1.

Samples from 1,553 cattle from 311 herds were included in the study. These included 1,298 Holsteins, 147 crossbreeds, 84 Red Danish, 8 Hereford, 6 Danish Red Holstein, 5 Jersey, 2 Limousine, 2 Highland cattle and one cow of unknown breed. Of these 298 were first parity cows, 410 were second parity cows, and 745 cows were of higher parities; 2 were bulls and the remaining 98 animals were heifers. Ten faecal samples were excluded due to not being tested after 48 hours (Day 2). Overall, 19% tested positive in the Hemo-fec at Day 0 and 34% tested positive at Day 2.

A general finding for all lesions and abomasal alterations was that the proportion of positive Hemo-fec samples was higher on Day 2, also for cattle with no lesions (Table 4). The diagnostic sensitivity was generally within the range of 16.6–21.3% on Day 0 and 27.1–38.6% at Day 2, except for cows with oedema of which there was only 15 (Table 5).

The results of the univariable logistic regression are shown in Table 5. Lesion subtypes Ib and Ic were significantly associated with the Hemo-fec result on Day 0 ($P = 0.01$ and $P = 0.001$, respectively), and on Day 2 ($P = 0.0006$ and $P = 0.0001$, respectively). No multivariable associations existed for Day 0, as the model was reduced to results identical to the univariable model for lesion subtype Ic. For Day 2, the multivariable results are shown in Table 6. Overall, only cattle with ≥ 4 lesions were identified with results deviating from the baseline for both lesion subtypes Ib and Ic. Cattle with ≥ 4 subtype Ib lesions had 1.7 times higher odds of testing positive in the Hemo-fec test on Day 2 (95% confidence interval (CI): 1.4–1.9)) and cattle with ≥ 4 subtype Ic lesions had 1.9 times higher odds of testing positive (95% CI: 1.6–2.3).

Discussion

The Hemo-fec test had a detection limit of 2 ml blood per 1,000 g of faeces, which was better than for the Hemoccult II SENSA (4.5 ml blood per 1,000 g of faeces). Rumen microbes did not appear to break down substantial amounts of haemoglobin when incubated for 48 hours. Furthermore, in the observational study of potentially bleeding abomasal ulcers the diagnostic sensitivity of the Hemo-fec test was low for faecal samples tested on the same day as collected, but higher (in the range of 27.1–38.6 %) for faecal samples tested 2 days after collection. For lesion subtypes Ia and Id, where blood is generally not expected to occur, the Hemo-fec test appeared with no diagnostic value. Animals with ≥ 4 lesions of subtypes Ib or Ic had significant more positive Hemo-fec results 2 days after faecal collection than cattle with no lesions.

As we expected the results of Experiment A showed that the Hemo-fec test was more sensitive than the guaiac-based Hemoccult II SENSA test for detecting blood in faeces in an experimental setting. Therefore we used the Hemo-fec test for the observational study. However, we also observed a relatively high proportion of false-positives (20.8% and 27.1% at the same day of collected faeces and to days post collection, respectively) in the animals with no observed abomasal lesions. We do not know if these animals had been subject to e.g. a rectal examination before they were sent for slaughter to ensure they were not too far into gestation (according to the legislation in the European Union, it is illegal to transport animals in the last 10th

of the gestational period [20]) and the prevalence of pregnant cattle at slaughter tend to be high in Denmark [21]. Generally, the proportion of positive Hemo-fec samples compared to the lesion types found in the abomasum of the animals were at 16.6–21.3% at Day 0 and noticeably higher at Day 2 with 32.5–43.5% (when animals with oedema were excluded).

Cows with ≥ 4 lesions of subtype Ib or Ic were more likely to test positive ($P < 0.01$ and $P < 0.001$, respectively). Since both lesion subtypes are expected to be bleeding, these results support our expectations. We did not take into consideration the size nor the site of the lesions. One could speculate that a large lesion would bleed more than several smaller ones, and with a bleeding lesion located closer to the aboral part of the abomasum, the blood would be less affected by digestive juices than a lesion located in the most oral part of the abomasum. If the haemoglobin is not broken down by the digestive juices of the abomasum, the location of a lesion would not have an impact on the Hemo-fec test result of the faeces. Nonetheless, our results showed a clear association between having many potential bleeding lesions (subtypes Ib and Ic) and the detection of blood in the faeces. Furthermore, our results showed a higher sensitivity when samples are stored for 2 days. This could be an indication of the blood degrades in the faecal matter when stored cold for 2 days, releasing the haemoglobin from the erythrocytes and thereby give a quicker positive reaction with smaller quantities of blood in the sample. We did not experience this in Experiment A. All samples except one were either positive or negative at all 6 tests within a test period of up to 58 hours. The exception was the mix of 1 ml per 1,000 grams of faeces. The samples from this mixture were all positive when the mixture were first tested. At the second test all samples were negative. After this the samples tested both positive and negative at the different tests intervals, however, they tested mostly negative. At the last test, the plastic bag in which the blood was mixed with the faeces, tested positive and the three samples taken at the first test all tested negative. The most likely explanation for this could be that the blood was not equally distributed in the faeces and this resulted in these sporadic positive or negative samples. Another explanation for the variable results could be that the reading of the test was questionable. However, a picture of each test result was taken to re-assess the results afterwards. An overall difference between Experiment A and the observational study was observed. In Experiment A we mixed faeces with different quantities of fresh blood, whereas the blood in the faeces in the observational study must have went through some digestive processes, if the blood originated from the abomasum or from the upper gastrointestinal tract. Even though we showed that fermentation in rumen juice did not break down larger quantities of blood, it is not unlikely that the digestive processes of the gastrointestinal tract makes the haemoglobin more accessible for the Hemo-fec test. Another explanation could be that the Hemo-fec test gave a false-positive reaction due to other substances in the bovine faecal matter. The cows donating faeces in Experiment A were situated at a Large Animal Hospital at University of Copenhagen, which provides different types of feed than normally for both dairy breeds and beef breeds. The hospital fed the cows ad libitum wrap and restricted concentrate, whereas most Danish dairy cows are feed a mixed ratio with large quantities of e.g. corn silage. Cox [22] showed that TMB has a positive reaction with e.g. yellow corn. However, if the Hemo-fec indeed reacted with the corn residues in the bovine faeces, the proportion of positive results would be expected to be a lot higher since most included cows from the slaughter house were of dairy cattle, which are most often fed with corn silage. However, the majority of animals did not provide positive results as shown in Table 5. It would not have been possible to determine the specific ration given to the individual cows in the study.

Haemoglobin has been demonstrated to have a relatively low absorption through the intestinal mucosa in rats and dogs (2.5% and 30%, respectively) [23, 24] and a low degree of both absorption and degrading through the gastrointestinal tract in humans [8, 25]. These three species are all omnivorous and it must be expected that some of their iron uptake originate from ingested haemoglobin. We were not able to identify any literature supporting this in ruminants. However, we demonstrated that the haemoglobin did not break considerably down when incubated for 48 hours in rumen fluid. Smaller amounts of blood might be relevant, but the observational study suggested that a significant number of lesions would still be required, and we were primarily interested in determining if blood could be degraded or not. If lower concentrations should be detected in vitro, a titration test setup for blood in rumen fluid should be set up to determine the lowest possible detection range according to blood in rumen fluid before incubation, but this was not pivotal to our study. However, should small quantities of blood be able to pass the ruminal fluid without being degraded, then small quantities of blood can enter the gastro-intestinal system due to e.g. epistaxis, bleedings from the lungs, teething, licking a bleeding wound or other smaller lesions in the mouth, oesophagus and upper or lower airways and thus result in false positive results.

Likewise, any rectal examinations without sufficient amounts of rectal gel or gentleness could also give false positive results due to minor lesions or tares in the rectal mucosa. Additionally, our investigation was carried out in a slaughterhouse, where blood is present all over, and there might have been a risk of contaminating of the faecal samples with blood.

We established the detection limit of the Hemo-fec test to be 2 ml blood in 1,000 g faeces. In a small sample of e.g. 100 g faeces, this corresponds to a blood volume of 0.2 ml blood. Whereas this is a small amount of blood, it may occur in a single concentrated drop due to blood contamination from the surroundings from the abattoir or due to a prior rectal examination, and subsequently result in a false-positive reaction. The reason for why we say there were found false-positive samples, were due to the macroscopic findings in the abomasa regarding lesions and other alterations. Although, with the high number of false-positive results we must assume that some of them are due to haemoglobin originating from the gastrointestinal-tract, whether it may be to ulcerations or rectal examinations. Some of the false-positive could also be caused by a reaction between the Hemo-fec reagents and some plant oxidases in the faeces. TMB are known to react with a number of different oxidases e.g.: broccoli, yellow corn, cucumber, mushroom, onion, etc. [22]. As mentioned above, Danish dairy cows consume a large quantity of corn silage, which potentially could be an explanation for the false-positive tests, however, we would have expected a higher overall risk of testing positive if this should be the key contributor. The manufactures guidelines warned that oxygen from the air can act as an oxidizer for the TMB reagent and give a positive result. However, this should be compensated for by using the short period of time, where the test is recorded as positive or negative, nonetheless, no false-positive reactions were recorded in Experiment A.

A positive sample collected in a farm situation would have possible higher reliability than those collected at the abattoir, because blood would normally not contaminate the environment, and the veterinary practitioner usually know if they have performed a rectal examination prior to testing a cow.

Several veterinary sources describe the use of a diagnostic faecal occult blood test in the pursuit of a diagnostic test for bleeding in the intestinal tract [2, 15–17]. Three papers out of the four uses or mentions

the use of the orthotolidine tablet called Hematest (Ames Co, Elkhart, Indiana, USA). E.g. the Payton and Glickman paper [17] evaluated two commercial tests for occult blood, the Hematest, the commercial guaiac test Hemoccult and a non-commercial guaiac tincture. They concluded that the non-commercial guaiac tincture was the most sensitive test for occult blood *in vitro* with a detection limit of 0.33 ml per 100 g bovine faeces; the Hematest was reported with a detection limit of 0.59 ml blood per 100 g bovine faeces, and the Hemoccult test was reported with a detection limit of 0.91 ml per 100 g bovine faeces. This study also showed that most faecal samples were positive 7–9 hours past infusion with the heparinized blood to the abomasum with both tests. The results are not similar to what Payton and Glickman [17] found, but the relation between the two tests are to a certain extent similar with the orthotolidine test being more sensitive towards blood than the Hemoccult test.

Smith and colleagues [16] also used the Hematest as a diagnostic test for abomasal lesions and found the diagnostic sensitivity to be 0.77 (20 test-positive among 26 cattle with abomasal lesions and a total of 296 head of cattle) and a diagnostic specificity of 0.97 (with 8 false-positive Hematests of 270 ulcer-negative cattle in a total population of 296 head of cattle). However, this study was based on hospitalized cows and was not a slaughter population like our study.

Hund and colleagues [2] used the TMB test Combur Test (Roche Diagnostics GmbH, Vienna, Austria) as a reference for blood and the guaiac test Hemo FEC (Roche Diagnostics GmbH, Vienna, Austria) as their diagnostic test. They reported an overall test sensitivity of Hemo FEC of 16% and an overall Combur Test sensitivity of 73% in relation to abomasal lesions. Their findings showed the TMB test to be more sensitive than the guaiac test, which supports our findings in Experiment A and B.

In conclusion, the Hemo-fec test had a very high analytical sensitivity *in vitro*. *In vivo* it can detect multiple lesions of the bleeding lesion subtypes Ib and Ic. However, there is a high risk of a false-positive results, and caution should be made when proceeding to decision-making following the diagnostic process.

Abbreviations

Faecal occult blood: FOB

Faecal occult blood test: FOBT

Tetramethylbenzidine: TMB

Declarations

Acknowledgements

The authors acknowledge Kurt Bach and the technicians of the Large Animal Teaching Hospital at University of Copenhagen for caring for the cows donating faecal matter Experiment A; and the technicians of Rørrendegård for providing the rumen fluid for experiment B. The authors also acknowledge Hanne Helene Hansen for incubating the blood with rumen fluid for 48 hours.

Funding

This work was supported by The Milk Levy Foundation, Aarhus, Denmark with funding grants in 2016 and 2017. TrioLab, Brøndby, Denmark donated a box of Hemocult II SENSEA tests.

Availability of data and materials

The datasets used and analysed for this study are available from the corresponding author on request.

Authors' contributions

SLM and NC planned the studies and SLM executed the studies and collected the data. SSN and SLM performed the statistical analyses and SSN and SLM interpreted the findings. SLM drafted the manuscript and all authors commented on and approved the final version.

Ethical approval

Experiments A and B were covered by a general ethical approval for the experimental animals at University of Copenhagen. The faecal matter was collected after it had left the cow and did not require an ethical approval. The blood mixed with the faecal matter were drawn either through a catheter or as a part of the teaching of veterinary students to collect blood and make venepunctures in this ethical approval by The Animal Experimentation Inspectorate (2015-15-0201-00683). The ethical approval of the harvest of rumen fluid via a rumen fistula were given by The Animal Experimentation Inspectorate (2012-15-2934-00648).

The observational study did not require an ethical approval since the samples were collected after the cattle were slaughtered.

Consent for publication

Not applicable.

Competing interests

The authors have no conflict of interests to declare.

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Tables

Table 1

The different volumes of blood added to 1,000 grams of bovine faecal matter in the different batch numbers in an in vitro experiment of finding the diagnostic sensitivity of bovine blood in bovine faecal matter using a human test. Batches 2 and 3 were similar in volumes of blood, and were made to investigate if pooling the faecal matter from different cows had an impact on the results

Batch number	Amount of blood (ml) added to 1,000 g faeces			
	Bag 1	Bag 2	Bag 3	Control
1	5	25	50	0
2	0.5	2	4.5	0
3	0.5	2	4.5	0
4	0.25	0.75	1	0

Table 2

The experimental in vitro setup testing the analytical SE of bovine blood in bovine faecal matter in different titrations tested with Hemo-fec. The letters A, B and C are the replicas made for each sample, and the letters “N” and “P” represents the negative and positive samples, respectively. “Plastic bag” is tests from the plastic bag where the faecal matter and blood were mixed together. The plastic bag is tested when the mixture has taken place and at the last test. Batches 1, 3 and 4 were made with several donors whereas Batch 2 was made with one donor.

Batch 1	ml blood per 1,000 grams faeces	Replicate	Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
	5	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	25	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	50	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	0/control	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
Batch 2	ml blood per kg faeces	Replicate/Time (h)	Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
	0.5	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
	2	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	4.5	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	0/control	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
Batch 3	ml blood per kg faeces	Replicate/Time (h)	Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
	0.5	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
	2	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	4.5	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	0/control	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N

Batch 4	ml blood per kg faeces	Plastic bag Replicate/Time (h)	N					
			Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
0.25		A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
0.75		A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
1		A	P	N	P	N	N	N
		B	P	N	P	N	N	N
		C	P	N	N	P	N	N
		Plastic bag	P					P
0/control		A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N

Table 3

In vitro test of the analytical SE of the Hemocult II SENSAs test using bovine faeces and blood. The letters A, B and C are the replicas made for each sample, and the letters “N” and “P” represented the negative and positive samples, respectively. “Plastic bag” included tests from the plastic bag where the faecal matter and blood were mixed together. The plastic bag was tested when the mixture been made and at the last test. Batches 1, 3 and 4 were made with several donors whereas Batch 2 was made with one donor.

Batch 1	ml blood per 1,000 grams faeces	Replicate	Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
	5	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	25	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	50	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	0/control	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
Batch 2	ml blood per kg faeces	Replicate/Time (h)	Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
	0.5	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
	2	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
	4.5	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	0/control	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
Batch 3	ml blood per kg faeces	Replicate/Time (h)	Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
	0.5	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
	2	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
	4.5	A	P	P	P	P	P	P
		B	P	P	P	P	P	P
		C	P	P	P	P	P	P
		Plastic bag	P					P
	0/control	A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N

Batch 4	ml blood per kg faeces	Plastic bag Replicate/Time (h)	N Time after mixing the faeces and blood					
			0	8- 10	20- 24	32- 34	44- 46	56- 58
0.25		A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
0.75		A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N
1		A	P	N	P	N	N	N
		B	P	N	P	N	N	N
		C	P	N	N	P	N	N
		Plastic bag	P					P
0/control		A	N	N	N	N	N	N
		B	N	N	N	N	N	N
		C	N	N	N	N	N	N
		Plastic bag	N					N

Table 4

Results of Hemo-fec on slaughtered cattle the same day the faecal sample was obtained and two days after faecal sampling where cow was the study unit. The diagnostic sensitivity with the corresponding confidence interval for both days are shown

Lesion subtype or alteration	N ¹	Hemo-fec test results Day 0				Hemo-fec test results Day 2			
		HF+ ²	HF- ³	P(T+ LT) ⁴	CI ⁵	HF+ ²	HF- ³	P(T+ LT) ⁴	CI ⁵
No lesions	96	20	76	0.208	[0.127-0.290]	26	70	0.271	[0.182-0.360]
Ia	661	110	551	0.166	[0.138-0.195]	215	446	0.325	[0.290-0.361]
Ib	503	102	401	0.203	[0.172-0.233]	192	311	0.382	[0.339-0.424]
Ic	760	155	605	0.204	[0.173-0.234]	283	477	0.372	[0.338-0.407]
Id	108	23	85	0.213	[0.182-0.244]	47	61	0.435	[0.342-0.529]
Ia+Ib	955	178	777	0.186	[0.157-0.216]	335	620	0.351	[0.321-0.381]
Ia+Ic	1138	213	925	0.187	[0.157-0.217]	399	739	0.351	[0.323-0.378]
Ia+Id	721	124	597	0.172	[0.143-0.201]	243	478	0.337	[0.303-0.372]
Ib+Ic	997	197	800	0.198	[0.167-0.228]	365	632	0.366	[0.336-0.396]
Ib+Id	568	117	451	0.206	[0.175-0.237]	219	349	0.386	[0.346-0.426]
Ic+Id	797	160	637	0.206	[0.175-0.237]	297	500	0.373	[0.339-0.406]
Ia+Ib+Ic	1267	239	1028	0.189	[0.159-0.218]	447	820	0.353	[0.326-0.379]
Ia+Ib+Id	991	186	805	0.188	[0.158-0.217]	351	640	0.354	[0.324-0.384]
Ia+Ic+Id	1553	288	1265	0.187	[0.163-0.211]	523	1030	0.352	[0.323-0.381]
Ib+Ic+Id	1019	200	819	0.196	[0.166-0.227]	371	648	0.364	[0.335-0.394]
Ia+Ib+Ic+Id	1278	241	1037	0.189	[0.159-0.218]	451	827	0.353	[0.327-0.379]
Oedema	15	8	7	0.533	[0.281-0.786]	9	6	0.600	[0.352-0.848]
Hyperplasia	855	152	703	0.178	[0.152-0.203]	282	573	0.330	[0.298-0.361]
Nodules	499	105	394	0.210	[0.175-0.246]	198	301	0.397	[0.354-0.440]
Scar	58	12	46	0.207	[0.103-0.311]	22	36	0.379	[0.254-0.504]
SP _{HF0} ⁶	0.792								
SP _{HF2} ⁷	0.729								

¹N: the number of animals in the category; ²HF+: the number of positive Hemo-fec samples; ³HF-: the number of negative Hemo-fec samples; ⁴ P(T+|LT): the SE regarding the category, except for No lesions, where it corresponds to 1 - Sp; ⁵CI: confidence interval of the SE; ⁶SP_{HF0}: the specificity of the Hemo-fec test in regard of abomasal lesions and alterations at day 0; ⁷SP_{HF2}: the specificity of the Hemo-fec test in regard of abomasal lesions and alterations at day 2

Table 5

Univariable fixed effects of abomasal lesion subtype and amount of abomasal lesion subtypes on the Hemo-fec test at Day 0 and Day 2.

Variable	Level	N ¹	Hemo-fec test results Day 0			Hemo-fec test results Day 2		
			Estimate ²	SE ³	P-value	Estimate ²	SE ³	P-value
Lesion subtype Ia	Intercept		-1.389	0.084	< 0.0001	-0.640	0.070	< 0.0001
	1	322	-0.305	0.175	0.082	-0.172	0.140	0.22
	2	142	-0.060	0.230	0.79	0.240	0.185	0.19
	3	103	-0.460	0.299	0.12	-0.395	0.235	0.09
	≥ 4	94	0.016	0.270	0.95	-0.022	0.229	0.93
Lesion subtype Ib	Intercept		-1.536	0.081	< 0.0001	-0.776	0.066	< 0.0001
	1	168	-0.117	0.225	0.60	0.136	0.175	0.44
	2	101	0.074	0.267	0.78	0.185	0.218	0.40
	3	45	0.004	0.398	0.99	-0.019	0.329	0.95
	≥ 4	189	0.046	0.186	0.014	0.553	0.161	0.0006
Lesion subtype Ic	Intercept		-1.602	0.095	< 0.0001	-0.835	0.077	0.0001
	1	315	-0.042	0.180	0.82	0.069	0.144	0.63
	2	182	0.236	0.207	0.26	0.318	0.172	0.06
	3	110	0.326	0.250	0.19	0.429	0.209	0.04
	≥ 4	153	0.663	0.203	0.001	0.691	0.180	0.0001
Lesion subtype Id	Intercept		-1.494	0.068	< 0.0001	-0.711	0.060	0.0001
	1	66	0.270	0.301	0.37	0.405	0.255	0.11
	≥ 2	42	0.047	0.399	0.91	0.520	0.315	0.10

¹N: amount of cows with the different amounts of lesion subtypes, also please notice one cow can have several lesion subtypes present at the same time and therefore one cow can be registered at more than one level; ²Estimate of the probability of testing Hemo-fec positive with the given amount of lesion subtypes; ³SE: standard error of the estimate

Table 6

Multivariable logistic regression of Hemo-fec Day 2 results in relation to lesion subtype Ib and Ic.

	Hemo-fec Day 2	Level	Estimate ¹	SE ²	P-value
Lesion subtype Ib		Intercept	-0.911	0.086	< 0.0001
		1	0.125	0.176	0.48
		2	0.191	0.220	0.39
		3	-0.071	0.332	0.83
		≥ 4	0.504	0.162	0.002
Lesion subtype Ic		1	0.043	0.145	0.77
		2	0.305	0.173	0.077
		3	0.395	0.211	0.061
		≥ 4	0.654	0.181	0.0003

¹Estimate of the probability of testing Hemo-fec positive with the given amount of lesion subtypes; ²SE: standard error of the estimate

Figures

Figure 1

Result of Hemo-fec test of fermentation of blood in rumen fluid. Picture A shows the negative control, picture B shows the 1% blood/rumen fluid mix. Picture C shows the 5% blood/rumen fluid mix and picture D shows the 10% blood/rumen fluid mix