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# Evaluation of immunochromatographic point-of-care tests for the detection of calf diarrhoea pathogens in faecal samples

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## Summary

We have evaluated the diagnostic performance of immunochromatographic point-of-care tests (POCT) for the detection of rotavirus, coronavirus, *Escherichia (E.) coli* F5, *Cryptosporidium (C.) parvum*, *Clostridium (Cl.) perfringens* and *Giardia (G.) intestinalis* in fresh and thawed faecal samples from calves aged up to six months with diarrhoea. We performed POCTs to detect rotavirus, coronavirus, *E. coli* F5, *C. parvum*, *Cl. perfringens* and *G. intestinalis* on fresh samples in a field study and re-evaluated the performance for *C. parvum*, *Cl. perfringens* and *G. intestinalis* using thawed samples. We calculated the performance based on the results of the reference methods, which were RT-qPCR for the detection of rota- and coronavirus and bacteriological culturing and PCR to detect *E. coli* F5 and *Cl. perfringens* α and $\beta_2$ toxins. *C. parvum* was detected by phase-contrast microscopy and *G. intestinalis* by immunofluorescence microscopy. We collected 177 faecal samples from diarrhoeic calves. We found good performance for the POCT targeting rotavirus (sensitivity (SE)=92.9 %; specificity (SP)=95.6 %) and *C. parvum* (SE=63.3 %; SP=96.2 %). For *E. coli* F5, the number of true positive samples (n=1) was too low to evaluate the performance. The POCT to detect coronavirus gave a poor performance (SE=3.3 %; SP=96.6 %) and the POCT to detect *Cl. perfringens* a moderate

## Zusammenfassung

**Evaluierung von immunochromatographischen Point-of-Care Tests für den Nachweis von Durchfallerregern in Kälberkot**

Ziel dieser Studie war es, in einer Feldstudie die Validität von immunchromatographischen Point-of-Care Tests (POCT) zum Nachweis von Rotavirus, Coronavirus, *Escherichia (E.) coli* F5, *Cryptosporidium (C.) parvum, Clostridium (Cl.) perfringens* und *Giardia (G.) intestinalis* an frischen und aufgetauten Kotproben von Kälbern bis zu einem Alter von sechs Monaten mit Durchfall zu beurteilen. In der Feldstudie wurden POCTs zum Nachweis von Rotavirus, Coronavirus, *E. coli* F5, *C. parvum, Cl. perfringens* und *G. intestinalis* an 177 frischen Kotproben eingesetzt. In der Reevaluierung wurde die Validität der POCTs zum Nachweis von *C. parvum, Cl. perfringens* und *G. intestinalis* an aufgetauten Kotproben erneut beurteilt. Basierend auf den Ergebnissen der Referenzmethoden wurde die Validität der Tests berechnet. Als Referenzmethode wurde die RT-qPCR zum Nachweis von Rota- und Coronavirus eingesetzt, eine bakteriologische Kultivierung mit anschließender PCR für den Nachweis von *E. coli* F5 und *Cl. perfringens* α- und $\beta_2$-Toxin. *C. parvum* wurde in der Phasenkontrastmikroskopie und *G. intestinalis* in der Immunofluoreszenzmikroskopie nachgewiesen. In der

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performance (SE=52.8 %; SP=78.2 %). *G. intestinalis* POCT showed a higher sensitivity to immunofluorescence microscopy in thawed than in fresh faecal samples (SE=43.9 % versus SE=29.2 %). There are substantial differences in diagnostic performance between the commercially available immunochromatographic POCTs. Still, POCT can make a valuable contribution to the diagnosis and prevention of calf diarrhoea.

**Abbreviations:** βCOV = beta Coronavirus; CPG = Cysts per gram of faeces; GE = Genome equivalents; κ = Cohen's Kappa; NPV = Negative predictive value; OPG = Oocysts per gram of faeces; PA = Percent agreement; POCT = Point-of-care test; PPV = Positive predictive value; RVA = Rotavirus A; SE = Sensitivity; SP = Specificity

Feldstudie zeigten die POCTs eine moderate bis gute Validität für den Nachweis von Rotavirus (Sensitivität (SE)=92,9 %; Spezifität (SP)=95,6 %) und *C. parvum* (SE=63,3 %; SP=96,2 %). Für *E. coli* F5 war die Anzahl der positiven Proben (n=1) zu gering, um die Qualität des Tests zu bewerten. Der POCT für den Nachweis von Coronavirus erzielte eine Sensitivität von 3,3 % und eine Spezifität von 96,6 %, der POCT für den Nachweis von *Cl. perfringens* erreichte eine Sensitivität von 52,8 % und eine Spezifität von 78,2 %. Der POCT für den Nachweis von *G. intestinalis* zeigte eine höhere Empfindlichkeit bei aufgetauten als bei frischen Kotproben (SE=43,9 % vs. SE=29,2 %). Die Ergebnisse bestätigen deutliche Unterschiede in der Validität zwischen den kommerziell erhältlichen immunchromatographischen POCTs. Trotzdem können POCT-Ergebnisse einen wertvollen Beitrag zur Diagnose und Prävention von Kälberdurchfall leisten.

## Introduction

Neonatal calf diarrhoea is a common cause of death in the pre-weaning period and half the fatalities among unweaned calves have been attributed to diarrhoea (Torsein et al. 2011; Cho et al. 2014; Windeyer et al. 2014). Diarrhoea can be caused by infectious factors, such as viruses (e.g. rotavirus, coronavirus, bovine virus diarrhoea virus, norovirus, torovirus, nebovirus), bacteria (e.g. *Escherichia (E.) coli, Clostridium (Cl.) perfringens, Salmonella (S.) enterica*) and protozoa (e.g. *Cryptosporidium (C.) parvum, Giardia (G.) intestinalis, Eimeria* spp.) (Foster & Smith 2009; Cho et al. 2014; Gillhuber et al. 2014). Concurrent infections with two or more pathogens are common and the presence of more than one pathogen frequently leads to increased mortality and morbidity (Blanchard 2012; Al Mawly et al. 2015). Non-infectious risk factors, in particular management practices, calf housing and feeding, are also important in the pathogenesis (Klein-Jöbstl et al. 2014; Al Mawly et al. 2015).

Diagnostic tests require high accuracy (ability to give a true measure), high precision (consistency of results, low variability) and a high ability to give a correct positive or negative result. These factors are quantified in terms of test sensitivity and specificity. An understanding of test performance and the prevalence of the pathogen of interest enables the determination of the positive and negative predictive value and helps in the interpretation of a test result (Dohoo et al. 2010). In human and veterinary medicine, point-of-care tests (POCTs) for the detection of various pathogens have become commercially available during the previous decade. They need to fulfil the 'ASSURED' (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, deliverable to end-users) criteria and should lead to quick implementation of appropriate interventions at or near the point of patient care (Kosack et al. 2017).

Many POCTs for the detection of calf diarrhoea pathogens are commercially available but their evaluation has yielded contradictory results, with evaluations of POCTs for the rapid detection of bovine rotavirus showing sensitivities of 32.7 %, 71.9 % and 42.3 % and specificities of 46.7 %, 95.3 % and 100.0 % (Klein et al. 2009; Cho et al. 2012; Izzo et al. 2012). Similar variations were observed for bovine coronavirus, where test sensitivities were 28.2 % and 60.0 % and specificities 79.6 % and 96.4 % (Klein et al. 2009; Izzo et al. 2012). Sensitivity and specificity were calculated based on the results of RT-PCR (Klein et al. 2009), qRT-PCR (Izzo et al. 2012) and a multiplex real-time PCR (Cho et al. 2012). The evaluation of POCTs for the detection of *C. parvum* showed good diagnostic performances, with a sensitivity of 75.0 %, 100.0 % and 81.5 % and a specificity of 100.0 %, 94.6 % and 98.6 % based on the results of a modified Ziehl-Neelsen staining, a sedimentation-flotation technique and, in the final study, on the cumulative positivity of the tests used for comparison (Luginbühl et al. 2005; Klein et al. 2009; Papini et al. 2018). The studies indicate that the assessment of diagnostic test performance is substantially affected by the choice of reference method. Our previous evaluation of POCTs has shown low to medium sensitivities (29.2 % and 77.6 %) and high specificities (98.4 % and 91.1 %) for the detection of *G. intestinalis* (detected by immunofluorescence microscopy) and *C. parvum* detected by phase-contrast-microscopy) in diarrhoeic calves (Lichtmannsperger et al. 2019). Nevertheless, there is a lack of independent validation for the majority of commercially available calf diarrhoea POCTs. We have conducted a follow-up investigation to our original work to test whether the point-of-care tests are highly sensitive and specific for the detection of bovine rotavirus, bovine coronavirus, *E. coli* F5, *C. parvum* and *Cl. perfringens* in faeces from diarrhoeic calves in comparison to standard laboratory methods performed with fresh (field study) and thawed (re-evaluation) samples.

## Materials and methods

### Animals and sample processing

This trial was approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine, Vienna. Local veterinarians and farmers were contacted personally, via email or during professional meetings and asked to participate. If the local veterinarian and the farmer were willing to participate, the farms were included (convenient selection) and visited by their local veterinarian or the principal author (KL). If the local veterinarian was willing to take an active part in the study, he or she was instructed on sample collection, POCT implementation, result documentation, sample storage and transport. Between November 2017 and July 2018, the local veterinarian or KL collected diarrhoeic faecal (soft, liquid, watery) samples from the rectum (at least 10 g or 10 ml faeces; according to the standard operating procedure for sample collection and homogenization from Megacor Diagnostik GmbH) of male and female calves less than 180 days of age during the farm visits. Samples were collected in 100 ml collection cups and no calf was sampled more than once.

### Point-of-care test in the field study

During the farm visits, four commercial immunochromatographic POCTs were performed by the local veterinarian or KL. One immunochromatographic POCT (Speed V-Diar 4™, Virbac, Carros, France) was a combined test for the simultaneous detection of four pathogens (bovine rotavirus, bovine coronavirus, *E. coli* F5, *C. parvum*) (Figure 1A); the other three tests were single POCTs for the detection of *G. intestinalis* (FASTest® Giardia Strip, Megacor, Hörbranz, Austria), *C. parvum* (FASTest® Crypto Strip, Megacor) and *Cl. perfringens* (FASTest® C. perfringens, Megacor) (Figure 1B). All POCTs were carried out according to the manufacturer's specifications; the test kits did not provide a positive or negative control. Before testing, all samples were homogenized with a wooden spatula in the 100 ml collection cup.

### Sample processing

Following on-farm testing, fresh faecal samples were transported to the University Clinic for Ruminants at the University of Veterinary Medicine, Vienna by special medical logistics (organized by the local veterinarians) or directly on ice in a polystyrene box within one day. The same or the following day, aliquots were sent for analysis to the appropriate institutes of the University of Veterinary Medicine, Vienna (Parasitology, Virology, Microbiology); the results were used as standards of reference (= reference methods). Parasitological and bacteriological examinations were performed immediately. Aliquots (dry swabs) were taken from each sample and frozen at -80 °C for virological examination. In addition, aliquots of all faecal samples were stored in 20 ml collection cups at -80 °C for further investigation (see re-evaluation of POCT under laboratory conditions).

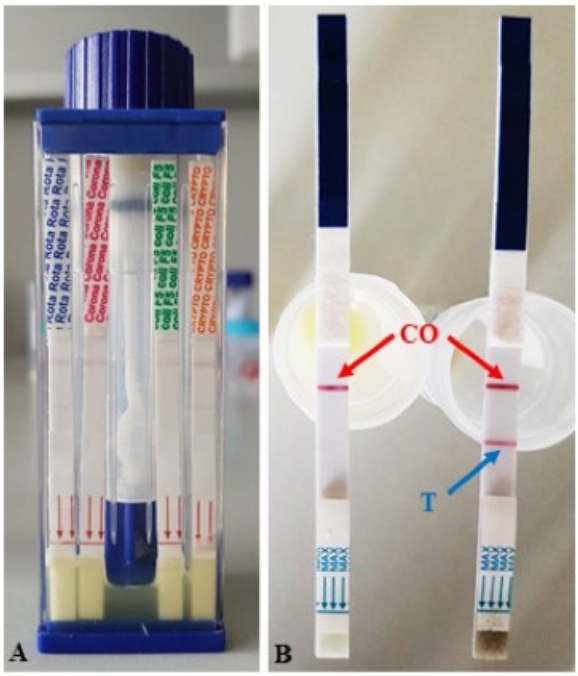


**Fig. 1:** The combined POCT (Speed V-Diar 4™) is illustrated in the left photograph (A). The POCT detects bovine rotavirus, bovine coronavirus, *E. coli* F5 and *C. parvum*. An example of single POCTs (FASTest® Giardia Strip) is given in the right photograph (B), in which the red arrows show the control line (CO) and the blue arrow (T) the test line. Photograph B shows a negative FASTest® Giardia Strip result (one red control line visible) and a positive FASTest® Giardia Strip (red control and red test line visible) result. / Der kombinierte POCT (Speed V-Diar 4™) ist auf dem linken Foto (A) abgebildet. Der POCT weist bovines Rotavirus, bovines Coronavirus, *E. coli* F5 und *C. parvum* nach. Ein Beispiel für einzelne POCTs (FASTest® Giardia Strip) im rechten Foto (B). Die roten Pfeile zeigen die Kontrolllinie (C) und der blaue Pfeil (T) die Testlinie. Es werden ein negatives Testergebnis, eine rote Kontrolllinie sichtbar, und ein positives Testergebnis, eine rote Kontrolllinie und eine rote Testlinie sichtbar, dargestellt.

### Reference methods

Virological examination

At the Institute of Virology, faecal swabs were analysed for betacoronavirus (βCoV) and rotavirus A (RVA). Swabs were placed in 1 ml of sterile phosphate-buffered-saline, vortexed for ten seconds and centrifuged for 1 minute at 15,000 × *g*. Two hundred microliters of supernatant were used for nucleic acid extraction, which was performed on a QIAcube HT instrument using the Cador Pathogen 96 QIAcube HT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls consisting of sample-free extracts (blanks) were produced si-

**Tab. 1:** Primers and probes for the detection of betacoronavirus (ßCoV) and rotavirus A (RVA) by RT-qPCR and primers for the molecular detection of genes encoding for *E. coli* fimbriae F5 and *Cl. perfringens* toxins α and $\beta_2$ using endpoint PCRs. / Eingesetzte Primer und Sonden für die Detektion von Betacoronavirus (ßCoV) und Rotavirus A. Auflistung der Primer für den molekularen Nachweis von *E. coli* F5 und den Nachweis von *Cl. perfringens* α Toxin and $\beta_2$ Toxin.

| Target | Primer/probe sequences | Annealing (°C) | Reference |
|---|---|---|---|
| ßCoV[a] | ßCoV-F 5'-ACGTGGTGTTCCTGTTGTTATAGG-3'<br>ßCoV-R 5'-AACATCTTTAATAAGGCGACGTAACAT-3'<br>ßCoV-Probe:<br>FAM-5'-CCACTAAAGTTTATGGCGGCTGGGATG-3'-TAMRA | 60 | Spiss et al. 2012 |
| RVA[b] | RVA7-1F: 5'-RCATRACCCYCTATGAGC-3'<br>NVP3-R: 5'-GGTCACATAACGCCCC-3'<br>RVA7pr.1:<br>FAM-5'-ATAGTTAAAGCTAACACTGTCAAAAACCTAAA-3'-TAMRA | 60 | Otto et al. 2015<br>Pang et al. 2004 |
| F5[c] | for: 5'-TATTATCTTAGGTGGTATGG-3'<br>rev: 5'-GGTATCCTTTAGCAGCAGTATTTC-3' | 50 | Franck et al. 1998 |
| α toxin[d] | for: 5'-GTTGATAGCGCAGGACATGTTAAG-3'<br>rev: 5'-CATGTAGTCATCTGTTCCAGCATC-3' | 55 | Yoo et al. 1997 |
| $\beta_2$ toxin[d] | for: 5'-AGATTTTAAATATGATCCTAACC-3'<br>rev: 5'-CAATACCCTTCACCAAATACTC-3' | 50 | Jost et al. 2005 |

[a]βCoV = betacoronavirus, [b]RVA = rotavirus A; Described for detection of canine respiratory coronavirus but also validated for bovine coronavirus nucleic acid detection (pan betacoronavirus 1 RT-qPCR), [c]F5 = *E. coli* fimbriae F5, [d]α toxin and $\beta_2$ toxin of *Cl. perfringens*

multaneously in each extraction process. RT-qPCRs were carried out using qScript XLT 1-Step RT-qPCR ToughMix (Quantabio, Beverly, USA) on a Rotor-Gene Q5plex (Qiagen). Table 1 gives the sequences of primers and probes and references. Positive and negative controls were run side-by-side with each RT-qPCR approach. Dilutions of synthetic RNA transcripts derived from cloned cDNA including the target sequences were used for absolute quantification. Sensitivity for both RT-qPCR assays was determined by running doubles of serial dilution rows of βCoV and RVA RNA transcripts. Results are given in genome equivalents per millilitre (GE/ml) swab lysate. The limit of detection was assessed with 10 GE/µl in the reaction, which is equivalent to $4.3x10^3$ GE/ml swab lysate. Dilutions of the RNA transcripts were used to create a standard curve; GE values for 1 µl of RNA extracts were calculated with the Rotor-Gene software according to the given concentrations of the transcripts.

Bacteriological examination

All samples were examined at the Institute of Microbiology for the presence of *E. coli* and *Cl. perfringens*. Samples were plated onto Columbia agar with 5 % sheep blood and MacConkey agar using the three-phase streaking method and plates were incubated aerobically (Columbia agar, MacConkey agar) and anaerobically (Columbia agar) at 37 °C for 48 h. Bacterial growth was semi-quantitatively graded as light, moderate or heavy depending on the occurrence and number of isolated colonies in streaking sections. Characteristic *E. coli* and *Cl. perfringens* colonies were selected and DNA extracted using GenElute™ Mammalian Genomic DNA Miniprep Kit (Merck, Darmstadt, Germany). Endpoint PCRs were performed to detect virulence-associated factors, including genes for *E. coli* fimbriae F5 and *Cl. perfringens* α and $\beta_2$ toxins, using a Mastercycler® Nexus Gradient Thermal Cycler (Eppendorf Austria GmbH, Vienna, Austria) and amplification protocols as described (Yoo et al. 1997; Franck et al. 1998; Jost et al. 2005). Primers and annealing temperatures are listed in Table 1. DNA from an *E. coli* strain harbouring F5 and a *Cl. perfringens* strain carrying both α and $\beta_2$ toxin genes were used as positive controls. DNA was replaced by PCR-grade water for negative controls. Isolates were considered positive for the virulence factor if amplicons with the same length as the positive control were produced. Randomly selected amplicons were sequenced at LGC Genomics, Berlin, Germany to confirm amplification of the target gene.

Parasitological examination

All faecal samples were examined for *G. intestinalis* and *Cryptosporidium* at the Institute of Parasitology. The quantitative detection of cysts/oocysts in faecal samples was performed as described (Lichtmannsperger et al. 2019). Cysts/oocysts were purified by the sodium acetate-acetic acid-formalin solution (SAF) method. The pellet was re-suspended in phosphate-buffered-saline and used for further analysis. The immunofluorescence assay (IFA) the Merifluor® (Merifluor® *Cryptosporidium*/Giardia Meridian Bioscience Inc., Cincinnati, USA) test was performed according to the manufacturer's instructions to detect *G. intestinalis* and the number of cysts

**Tab. 2:** Co-infections of the pathogens in faecal samples of diarrhoeic calves. Occurrence of bovine rotavirus (RVA), bovine coronavirus (βCoV) detected by RT-qPCR; *E. coli* and *Cl. perfringens* detected by bacteriological culturing and subsequent endpoint PCR (*E. coli* fimbriae F5; α and $\beta_2$ toxin for *Cl. perfringens*), *Cryptosporidium* spp. *(Crypto)* and *G. intestinalis (Giardia)* detected by phase-contrast-microscopy and immunofluorescence microscopy, respectively. One sample was positive for *E. coli* F5 (not included in the table). / Ko-Infektionen der untersuchten Pathogene im Kot von Kälbern mit Durchfall. Auftreten von bovinem Rotavirus (RVA) und bovinem Coronavirus (βCoV) nachgewiesen mittels RT-qPCR; *E. coli* und *Cl. perfringens* nachgewiesen mittels bakteriologischer Untersuchung und anschließender PCR zum Nachweis von Virulenz assoziiertem Faktor F5 und α und $\beta_2$ Toxin. *Cryptosporidium* spp. *(Crypto)* wurde in der Phasen-Kontrast-Mikroskopie und *G. intestinalis (Giardia)* in der Immunfluoreszenzmikroskopie nachgewiesen. Eine Probe zeigte ein positives Ergebnis für *E. coli* F5 (nicht in der Tabelle inkludiert).

| | Pathogen | | | | | | |
|---|---|---|---|---|---|---|---|
| **Pathogen co-infection** | **RVA** | **βCoV** | ***E. coli*** | ***Cl. perfringens* (α)** | ***Cl. perfringens* ($\beta_2$)** | ***Crypto*** | ***Giardia*** |
| **RVA** | 42 | 9 | 42 | 12 | 5 | 31 | 3 |
| **βCoV** | 9 | 60 | 60 | 14 | 5 | 30 | 16 |
| ***E. coli*** | 42 | 60 | 174 | 41 | 17 | 98 | 48 |
| ***Cl. perfringens* (α)** | 12 | 14 | 41 | 41 | 17 | 25 | 6 |
| ***Cl. perfringens* ($\beta_2$)** | 5 | 5 | 17 | 17 | 17 | 9 | 1 |
| ***Crypto*** | 31 | 30 | 98 | 25 | 9 | 98 | 21 |
| ***Giardia*** | 3 | 16 | 48 | 6 | 1 | 21 | 48 |

is given as cysts per gram of faeces (cpg). To detect *Cryptosporidium*, a disposable haemocytometer (C-Chip, NanoEnTek Inc., Pleasanton, USA) was used. The slide was screened under a phase-contrast microscope (PCM) (Nikon Labophot-2, Nikon Instruments Inc., Tokyo, Japan) with 200 × magnification for oocysts. The number of *Cryptosporidium* oocysts is given in oocysts per gram of faeces (opg).

### Reevaluation of POCTs under laboratory conditions on thawed samples

In June 2019, faecal samples were selected by simple randomization and thawed in the 20 ml collection cups overnight under room temperature in the laboratory. The blinded second author (KF) then carried out single POCTs (FASTest® Giardia Strip, n=137; FASTest® Crypto Strip, n=130; FASTest® C. perfringens, n=170; all manufactured by Megacor) following the manufacturer's specifications. Before testing, all samples were homogenized with a wooden spatula. The re-evaluation was carried out in collaboration with the manufacturing company to evaluate test performance following freezing.

### Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics Version 24 (IBM®, New York, USA) and Microsoft Excel 2010. Test performance, i.e. true positive, true negative, false positive, false negative, specificity (SE), sensitivity (SP), positive predictive value (PPV), negative predictive value (NPV), percent agreement (PA) and Cohen's Kappa (κ), was calculated based on comparison with the reference methods for each of the POCTs. The percent agreement was calculated as the sum of true positive and true negative results divided by the number of samples. Cohen's Kappa was calculated to describe the agreement between the POCTs and the reference methods. Kappa values can range from 0 to 1 and were interpreted as follows: ≥0.81 very good agreement; 0.61 to 0.80 good agreement; 0.41 to 0.60 moderate agreement; 0.21 to 0.40 fair agreement; and ≤0.2 poor agreement (Thrusfield & Christley 2018).

Test performance was calculated for the results of the combined POCT (Speed V-Diar 4™; targeting bovine rotavirus, bovine coronavirus, *E. coli* F5, *C. parvum*) and the three single POCTs (FASTest® Giardia Strip, FASTest® Crypto Strip, FASTest® C. perfringens Strip) carried out on 177 fresh faecal samples in the field study. To calculate test performance under laboratory conditions on thawed samples, 137, 130 and 170 faecal samples were randomly selected to test for *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) and *Cl. perfringens* (FASTest® C. perfringens Strip), respectively. Not all faecal samples were subjected to re-evaluation as this part of the work was undertaken in collaboration with the POCT manufacturer and no more POCT kits were available.

## Results

### Farm visits, animals and sample collection

Four veterinary practices (6 veterinarians) actively participated and undertook 47 farm visits; one author performed 27 farm visits. Seventy farms were visited, of which four were visited twice (74 farm visits). The farms were located in the federal districts of Salzburg (n=26), Lower Austria (n=24), Styria (n=7), Upper Austria (n=3), Burgenland (n=6) and Tyrol (n=4). The number of calves sampled per farm ranged from 1 to 10 (mean=2.5; median=2); the average farm size

**Tab. 3:** Contingency tables for combined point-of-care test (POCT) (Speed V-Diar 4™), targeting bovine rotavirus (RVA), bovine coronavirus (βCoV), *E. coli* fimbriae F5 and *C. parvum* and three single POCT for the detection of *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) and *Cl. perfringens* (FASTest® C. perfringens Strip) used in the field study. Test performance was calculated based on the results of the reference laboratory methods. / Kreuztabellen für den kombinierten Schnelltest (Speed V-Diar 4™) zum Nachweis von bovinem Rotavirus (RVA), bovinem Coronavirus (βCoV), *E. coli* F5 und *C. parvum*. Weiters Kreuztabellen für drei Einzel-Schnelltestreifen für den Nachweis von *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) und *Cl. perfringens* (FASTest® C. perfringens Strip). Die Tests wurden im Feldversuch durchgeführt. Die Ergebnisse der jeweiligen Referenzmethode wurden zur Berechnung der Testqualität herangezogen.

| | POCT | Pathogen | POCT result | Reference positive | Reference negative | Total |
|---|---|---|---|---|---|---|
| Field study | Speed V-Diar 4™ | RVA | Positive | 39 | 6 | 45 |
| | | | Negative | 3 | 129 | 132 |
| | | | Total | 42 | 135 | 177 |
| | | βCoV | Positive | 2 | 4 | 6 |
| | | | Negative | 58 | 113 | 171 |
| | | | Total | 60 | 117 | 177 |
| | | *E. coli* F5 | Positive | 1 | 2 | 3 |
| | | | Negative | 0 | 174 | 174 |
| | | | Total | 1 | 176 | 177 |
| | | *C. parvum* | Positive | 62 | 3 | 65 |
| | | | Negative | 36 | 76 | 112 |
| | | | Total | 98 | 79 | 177 |
| | FASTest® Giardia Strip | *G. intestinalis* | Positive | 14 | 2 | 16 |
| | | | Negative | 34 | 127 | 161 |
| | | | Total | 48 | 129 | 177 |
| | FASTest® Crypto Strip | *C. parvum* | Positive | 76 | 7 | 83 |
| | | | Negative | 22 | 72 | 94 |
| | | | Total | 98 | 79 | 177 |
| | FASTest® C. perfringens Strip | *Cl. perfringens* | Positive | 28 | 27 | 55 |
| | | | Negative | 25 | 97 | 122 |
| | | | Total | 53 | 124 | 177 |

was approximately 56 livestock units. Overall, 177 calves were included and their ages ranged from 1 to 164 days (mean=27; median=12). In total 108 female and 69 male Simmental, Brown Swiss, Holstein and cross-breed calves were sampled. All 177 faecal samples were tested with the combined (Speed V-Diar 4™; targeting bovine rotavirus, bovine coronavirus, *E. coli* F5, *C. parvum*) and the three single POCTs (FASTest® Giardia Strip; FASTest® Crypto Strip; FASTest® C. perfringens Strip) by the same researcher (n=87 samples) or by the local veterinarian (n=90 samples) on-farm during the farm visits.

## Occurrence of calf diarrhoea pathogens in the reference laboratory method

*Bovine rotavirus A* and *bovine coronavirus*

Of the 177 faecal samples, 42 (23.7 %) and 60 (33.9 %) yielded positive results in the reference methods for bovine rotavirus and bovine coronavirus, respectively. Table 2 summarizes the occurrence of enteropathogens and co-infections in faecal samples from the field study. Mean viral excretion (GE/ml) was $1.2x10^7$ (median=$1.3x10^6$; SD=$4.1x10^7$) for bovine rotavirus (n=42) and $3.3x10^8$ (median=$8.1x10^6$; SD=$7.1x10^8$) for bovine coronavirus (n=60).

*E. coli* and *Cl. perfringens*

*E. coli* was isolated from 174 (98.3 %) of the 177 faecal samples. Bacterial growth on MacConkey agar was light (n=2), moderate (n=8) or heavy (n=164). Only three of the 177 (1.7 %) faecal samples gave negative results for *E. coli* in bacteriological culturing. The gene encoding fimbriae F5 was detected by PCR in 1 of the 174 (0.6 %) *E. coli* isolates. *Cl. perfringens* was isolated from 53 (29.2 %) of the 177 faecal samples. Growth of *Cl. perfringens* appeared to be light (n=13), moderate (n=32) or heavy (n=8) on Columbia agar plates. In 41 and 17 *Cl. perfringens* isolates, the α and the $\beta_2$ toxin was detected by PCR, respectively. All *Cl. perfringens* $\beta_2$ toxin-positive isolates (n=17) were also positive for the α toxin gene.

*G. intestinalis* and *Cryptosporidium*

In total, 48 (27.1 %) and 98 (55.4 %) of the 177 faecal samples were positive for *G. intestinalis* and *Cryptosporidium*, respectively. The mean cyst and oocyst excretion rates were 10,108 (median=1,308; SD=19,244) and $1x10^5$ (median=$1x10^6$; SD=$3x10^6$) for *G. intestinalis* and *Cryptosporidium* spp. (Lichtmannsperger et al. 2019).

## Test performance compared to the reference laboratory methods

For each test implemented in the field study and in the re-evaluation, we constructed two by two contin-

**Tab. 4:** Contingency tables for three single point-of-care tests (POCT) for the detection of *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) and *Cl. perfringens* (FASTest® C. perfringens Strip) used in the re-evaluation under laboratory conditions carried out on thawed samples by the blinded second author. Test performance was calculated based on the results of the reference laboratory methods. / Kreuztabellen für drei Einzel-Schnelltestreifen für den Nachweis von *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) and *Cl. perfringens* (FASTest® C. perfringens Strip). Die Einzel-Schnelltests wurden unter Laborbedingungen an aufgetauten Kotproben durch die geblindete Zweitautorin in der Reevaluierung durchgeführt. Die Ergebnisse der jeweiligen Referenzmethode wurden zur Berechnung der Testqualität herangezogen.

| | POCT | Pathogen | POCT result | Reference positive | Reference negative | Total |
|---|---|---|---|---|---|---|
| Re-evaluation | FASTest® Giardia Strip | *G. intestinalis* | Positive | 18 | 1 | 19 |
| | | | Negative | 23 | 95 | 118 |
| | | | Total | 41 | 96 | 137 |
| | FASTest® Crypto Strip | *C. parvum* | Positive | 63 | 6 | 69 |
| | | | Negative | 13 | 48 | 61 |
| | | | Total | 76 | 54 | 130 |
| | FASTest® C. perfringens Strip | *Cl. perfringens* | Positive | 34 | 36 | 70 |
| | | | Negative | 18 | 82 | 100 |
| | | | Total | 52 | 118 | 170 |

gency tables to compare the results of the reference laboratory tests (standard of comparison) with those of the POCTs. The tables enabled us to deduce the number of true positive, false positive, true negative and false negative results and Table 3 shows the data for the field study carried out on fresh samples, with Table 4 giving the results of the re-evaluation under laboratory conditions on thawed samples. Based on this information, we calculated test sensitivity, specificity, positive and negative predictive values, percent agreement and Cohen's Kappa for all of the POCT in the field (Table 5) and in the re-evaluation (Table 6).

### Test performance of POCT in the field study carried out on fresh samples

The combined POCT was positive for bovine rotavirus in 45 (25.4 %),

**Tab. 5:** Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), percent agreement (PA) and Cohen's Kappa for the combined POCT (Speed V-Diar 4™) targeting bovine rotavirus (RVA), bovine coronavirus (βCoV), *E. coli* carrying virulence-associated factor F5 and *C. parvum* and three single point-of-care tests for the detection of *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) and *Cl. perfringens* (FASTest® C. perfringens Strip). The combined and three single point-of-care tests were carried out on-farm on fresh samples (n=177) during the farm visits. Test performance was calculated based on the results of the reference laboratory methods. / Sensitivität, Spezifität, positiver prädiktiver Wert (PPV) und negativer prädiktiver Wert (NPV), Prozent Übereinstimmung (PA) und Cohen's Kappa für den kombinierten Schnelltest (Speed V-Diar 4™) zum Nachweis von bovinem Rotavirus (RVA), bovinem Coronavirus (βCoV), *E. coli* F5 und *C. parvum* und drei Einzel-Schnelltestreifen zum Nachweis von *G. intestinalis* (FASTest® Giardia Strip), *C. parvum* (FASTest® Crypto Strip) und *Cl. perfringens* (FASTest® C. perfringens Strip). Der kombinierte Schnelltest und die drei Einzel-Schnelltestreifen wurden an frischen Durchfallkotproben (n=177) in den Betrieben durchgeführt. Die Ergebnisse der jeweiligen Referenzmethode wurden zur Berechnung der Testvalidität herangezogen.

| Field study<br>Pathogen | Sensitivity (95 % CI[a]) | Specificity (95 % CI) | PPV[b] (95 % CI) | NPV[c] (95 % CI) | PA[d] | Cohen's Kappa (95 % CI) |
|---|---|---|---|---|---|---|
| RVA[e] | 92.9 (85.1, 100.6) | 95.6 (92.1, 99.0) | 86.7 (76.7, 96.6) | 97.7 (95.2, 100.3) | 94.9 | 0.86 (0.72, 1.01) |
| βCoV[f] | 3.3 (-1.2, 7.9) | 96.6 (93.3, 99.9) | 33.3 (-4.4, 71.1) | 66.1 (59.0, 73.2) | 65.0 | 0.00 (-0.07, 0.07) |
| *E. coli* F5 | 100.0 (100.0, 100.0) | 98.9 (97.3, 100.4) | 33.3 (-20.0, 86.7) | 100.0 (100.0, 100.0) | 98.9 | 0.5 (0.37, 0.62) |
| *C. parvum* | 63.3 (53.7, 72.8) | 96.2 (92.0, 100.4) | 95.4 (90.3, 100.5) | 67.9 (59.2, 76.5%) | 78.0 | 0.57 (0.43, 0.71) |
| *G. intestinalis* | 29.2 (16.3, 42.0) | 98.4 (96.3, 100.6) | 87.5 (71.3, 103.7) | 78.9 (72.6, 85.2) | 79.7 | 0.35 (0.23, 0.47) |
| *C. parvum*[g] | 77.6 (69.3, 85.8) | 91.1 (84.9, 97.4) | 91.6 (85.6, 97.5) | 76.6 (68.0, 85.2) | 83.6 | 0.67 (0.53, 0.82) |
| *Cl. perfringens* | 52.8 (39.4, 66.3) | 78.2 (71.0, 85.5) | 50.9 (37.7, 64.1) | 79.5 (72.3, 86.7) | 70.6 | 0.31 (0.16, 0.46) |

[a]95 % CI = 95 % confidence interval, [b]PPV = positive predictive value ,[c]NPV = negative predictive value, [d]PA = percent agreement (sum of all true positive and all true negative results divided by the total number of samples), [e]RVA = bovine rotavirus A, [f]βCoV = bovine betacoronavirus, [g]Point-of-care test performance targeting *G. intestinalis* and *C. parvum* has been published (Lichtmannsperger et al. 2019)

**Tab. 6:** Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), percent agreement (PA) and Cohen's Kappa three single point-of-care tests for the detection of *G. intestinalis* (FASTest® Giardia Strip) (n=137 samples), *C. parvum* (FASTest® Crypto Strip) (n=130 samples) and *Cl. perfringens* (FASTest® C. perfringens Strip) (n=170 samples). The re-evaluation was carried out under laboratory conditions on thawed samples by the blinded second author. Test performance was calculated based on the results of the reference laboratory methods. / Sensitivität, Spezifität, positiver prädiktiver Wert (PPV) und negativer prädiktiver Wert (NPV), Prozent Übereinstimmung (PA) und Cohen's Kappa für drei Einzel-Schnelltestreifen zum Nachweis von *G. intestinalis* (FASTest® Giardia Strip) (n=137 Proben), *C. parvum* (FASTest® Crypto Strip) (n=130 Proben) und *Cl. perfringens* (FASTest® C. perfringens Strip) (n=170 Proben). Die Reevaluierung unter Laborbedingungen wurde durch die geblindete Zweitautorin durchgeführt. Die Ergebnisse der jeweiligen Referenzmethode wurden zur Berechnung der Testqualität herangezogen.

| Re-evaluation under laboratory conditions | | | | | | |
|---|---|---|---|---|---|---|
| **Pathogen** | **Sensitivity (95 % CI)** | **Specificity (95 % CI)** | **PPV[a] (95 % CI)** | **NPV[b] (95 % CI)** | **PA[c]** | **Cohen's Kappa (95 % CI)** |
| *G. intestinalis* | 43.9 (28.7, 59.1) | 99.0 (96.9, 101.0) | 94.7 (84.7, 104.8) | 80.5 (73.4, 87.7) | 82.5 | 0.51 (0.36, 0.66) |
| *C. parvum* | 82.9 (74.4, 91.4) | 88.9 (80.5, 97.3) | 91.3 (84.7, 98.0) | 78.7 (68.4, 89.0) | 85.4 | 0.71 (0.53, 0.88) |
| *Cl. perfringens* | 65.4 (52.5, 78.3) | 69.5 (61.2, 77.8) | 48.6 (36.9, 60.3) | 82.0 (74.5, 89.5) | 68.2 | 0.32 (0.17, 0.46) |

[a]PPV = positive predictive value, [b]NPV = negative predictive value, [c]PA = percent agreement (sum of all true positive and all true negative divided by the total number of samples).

for bovine coronavirus in 6 (3.4 %), for *E. coli* F5 in 3 (1.7 %) and for *C. parvum* in 65 (36.7 %) of the samples (n=177). Single *G. intestinalis* and *C. parvum* POCTs showed 16 (9.0 %) and 83 (46.9 %) positive test results from the 177 examined samples (Lichtmannsperger et al. 2019) (Table 3). The POCT targeting *Cl. perfringens* showed positive test results in 55 (31.1 %) of the 177 samples. Of these 55 POCT positive results, 22 *Cl. perfringens* isolates carried α toxin genes and 17 isolates carried the $\beta_2$ toxin gene.

### Test performance of the POCTs implemented in the re-evaluation under laboratory conditions on thawed samples

In the re-evaluation under laboratory conditions, the POCT targeting *G. intestinalis* yielded positive results in 19 (13.8 %) of the 137 thawed analysed samples, the POCT targeting *C. parvum* in 69 (53.3 %) of the 130 thawed samples and the POCT targeting *Cl. perfringens* in 70 (41.2 %) of the 170 thawed samples (Table 4).

## Discussion

We have evaluated the diagnostic performance of immunochromatographic POCTs carried out on fresh (field study) and thawed (re-evaluation) samples for the detection of enteropathogens commonly found in diarrhoeic calves less than half a year of age. We used POCTs to detect bovine rotavirus, bovine coronavirus, *E. coli* carrying F5, *C. parvum*, *Cl. perfringens* and *G. intestinalis* and calculated the test performance based on the results of the reference methods carried out at institutes of the University of Veterinary Medicine, Vienna. Reverse transcriptase-qPCR was used for the detection of bovine rotavirus and bovine coronavirus; bacteriological culturing on Columbia and MacConkey agar and subsequent endpoint PCR for *E. coli* (fimbriae F5) and *Cl. perfringens* (α and $\beta_2$ toxin); phase-contrast-microscopy for *Cryptosporidium* spp.; and immunofluorescence microscopy for *G. intestinalis*.

### Reference methods

No test method (reference method and POCT) can detect a pathogen with 100 % accuracy (Blanchard 2012). However, we assume that the reference methods represented the true infection status (positive or negative) of the calves. The reference methods for detecting calf pathogens are not harmonized, either within Austria or in the European Union. It is likely that sending the samples to different laboratories would lead to contradictory results, although ring-trials are carried out. We expressed the agreement between the POCT carried out on fresh and thawed samples and the reference methods as Cohen's Kappa. Some POCTs have a wide 95 % confidence interval for sensitivity and specificity, reflecting considerable uncertainty about the estimate. Nevertheless, the POCTs were interpreted to be at least as good as the lower limit of the 95 % confidence interval.

### Farm visits, animals and sample collection

The study was conducted with conveniently selected veterinary practices, farms and samples. The participating veterinarians were instructed on sample collection, POCT implementation, result documentation, sample storage and transport. Participating farms had a history of calf diarrhoea, so we expected a high incidence of pathogens in the population. The number of livestock units per farm was approximately 56,

which does not represent the Austrian farm structure. In 2021, the average cattle farm in Austria kept approximately 35 cattle (Rinderzucht Austria 2022). Calf diarrhoea is related to farm size, with diarrhoea cases found on larger farms (large: median 40 cows; small: 28 cows) (Klein et al. 2014). The higher prevalence of calf diarrhoea on larger farms is believed to explain for the high amount of larger farms (on average 56 livestock units) that participated in our study. Most farms were visited once and samples taken from affected animals. Diagnostic results therefore only represent a snapshot of the calves' current infection status.

The high prevalence of the pathogens in our study animals affected the positive and negative predictive value. As the number of true positive faecal samples was high, the positive predictive values increased while the negative predictive values decreased.

### Test performance of POCTs used in the field study on fresh samples

Bovine rotavirus and bovine coronavirus

The combined POCT targeting bovine rotavirus resulted in a high sensitivity (92.9 %) and specificity (95.6 %) calculated based on the results of RT-qPCR (reference method). Nine of the 42 RT-qPCR positive samples were classified incorrectly by the POCT: three samples were classified as false negative and six samples as false positive (see Table 3). The combined POCT (Speed V-Diar 4™) showed a good performance with a high sensitivity (SE=92.9 %) and specificity (SP=95.6 %) and the agreement between the POCT and the reference method (RT-qPCR) was at least good (lower 95 % CI limit of κ=0.72).

Sixty faecal samples gave positive RT-qPCR results for bovine coronavirus. The combined POCT targeting bovine coronavirus showed a low sensitivity (SE=3.3 %) and a high specificity (SP=96.6 %). The agreement (true positive + true negative divided by the total number of samples) was 65 %, as the number of true negative samples was high. Nevertheless, the agreement expressed as Cohen's Kappa between the POCT and the RT-qPCR was poor (κ=0.00). Cohen's Kappa includes false positive and false negative results as it describes the agreement between two methods beyond chance. Similar results have been previously reported (κ=0.095) (Cho et al. 2012), although the authors of the earlier study were surprised by the poor performance and suggested the re-evaluation of the antibody specificity. In our study, only two of the 60 RT-qPCR-positive faecal samples were correctly identified as positive by the POCT (see Table 3). We therefore agree that it would be useful to re-evaluate the specificity of the antibodies.

*E. coli* and *Cl. perfringens*

In total, 174 of the 177 faecal samples showed bacterial growth of *E. coli* on MacConkey agar (reference method). The number of *E. coli* isolates carrying virulence-associated factor F5 determined by PCR was low, with only 1 of 174 *E. coli* isolates yielding a positive result. The test sensitivity was 100 % as all positive *E. coli* F5 isolates (n=1) were correctly identified; the specificity was 98.9 %. As the occurrence of *E. coli* F5 was very low, the negative predictive value was 100 %. Interpretation of the diagnostic performance of the POCT targeting *E. coli* F5 was not feasible. A previous study found a high agreement (κ=0.823) between an immunochromatographic test and multiplex real-time PCR targeting *E. coli* F5, although here too the sample size was small and the prevalence of *E. coli* F5 low (5/100) (Cho et al. 2012). In the previous decade, the occurrence of *E. coli* F5 in the faeces of diarrhoeic cattle was very low, e.g. 3.2 % of calves (14 out of 429 diarrheic calves younger than 6 days of age) in New Zealand were positive (Al Mawly et al. 2015). A recent systematic review and meta-analysis of the epidemiology of pathogenic *E. coli* of diarrhoeic calves found *E. coli* F5 in 12.9 % of the isolates (Kolenda et al. 2015). The combined POCT (Speed V-Diar 4™) only targets *E. coli* virulence-associated factor F5. However, *E. coli* virulence-associated factors F17, F41 and heat-stable enterotoxin isolated from calves are significantly associated with diarrhoea (Kolenda et al. 2015). All of the tests only target the pathogens of interest and do not test for the occurrence of other genera, species or virulence-associated factors.

The single POCT targeting *Cl. perfringens* showed a moderate sensitivity (SE=52.8 %) and specificity (SP=78.2 %). The agreement between bacteriological culturing on Columbia agar with 5 % sheep blood and the POCT was κ=0.31, with a lower limit of the 95 % CI for Cohen's Kappa of κ=0.16 and an upper 95 % CI limit of κ=0.46. The agreement was at least poor (κ=0.16) and the test cannot be recommended. Reading the test line of the POCT targeting *Cl. perfringens* was especially challenging. The absorption/migration rates and running times varied and the test lines appeared weak or doubtful, although the control lines were clearly visible. This problem might have led to the poor diagnostic performance. The diagnosis of *Cl. perfringens*-associated enteric disease remains a substantial challenge. Previous work has focused on the evaluation of intestinal *Cl. perfringens* counts as a diagnostic tool for enterotoxaemia in calves. *Cl. perfringens* counts assessed in intestinal contents *post mortem* show that intestinal counts have no value for the diagnosis of enterotoxaemia in calves (Valgaeren et al. 2013). Alpha toxin is the major toxin produced by type A strains but its role in intestinal diseases is controversial (Goossens et al. 2017). The isolation of *Cl. perfringens* type A or detection of its major toxin, alpha toxin, from faeces or gastrointestinal content has little if any diagnostic value as the toxin is also found in the gastrointestinal tract of healthy animals (Goossens et al. 2017).

*G. intestinalis* and *Cryptosporidium*

The sensitivity of single POCT (FASTest® Giardia Strip) for *G. intestinalis* was 29.2 % and the specificity 98.4 %; the agreement between the single POCT and immunofluorescence microscopy was fair to moderate (lower 95 % CI limit κ=0.23; upper 95 % CI limit κ=0.47). A low sensitivity (28 % and 26 %) and a high specificity (92 % and 93 %) for two POCTs in comparison with the immunofluorescence microscopy is in accordance with the previous results (Geurden et al. 2010). As the majority of the calves (77.1 %) showed low or moderate cyst excretion, we suggest that the number of cysts was below the detection limit as the test was developed for calves shedding higher numbers of cysts (Lichtmannsperger et al. 2019). The majority of immunochromatographic lateral-flow ELISAs target a surface antigen of *Cryptosporidium* spp. and/or *C. parvum*. *C. parvum* is the primary species in diarrhoeic calves worldwide and in the population we studied in Austria (Feng et al. 2018; Lichtmannsperger et al. 2020). The combined POCT (Speed V-Diar 4™) targeting *C. parvum* showed a moderate sensitivity (SE=63.3 %) and high specificity (SP=96.2 %) and there was at least a moderate agreement between the POCT and the phase-contrast-microscopy (reference method) (lower 95 % CI limit κ=0.43; upper 95 % CI limit κ=0.71). The second POCT (single POCT; FASTest® Crypto Strip) to detect *C. parvum* gave in a higher sensitivity (SE= 77.6 %) and a slightly lower specificity (SP=91.1 %) in comparison to the combined POCT. The agreement expressed as Cohen's Kappa between the single POCT and the phase-contrast-microscopy was at least good (lower 95 % CI limit κ=0.53; upper 95 % CI limit κ=0.82). The positive predictive value for both the combined and the single POCT was high (>90 %), so there is a high likelihood that a positive POCT result correctly reflects a positive case.

**Test performance of POCT in the re-evaluation under laboratory conditions on thawed samples**

We found a noticeable difference for *G. intestinalis* POCT, where Cohen's Kappa (0.51 vs. 0.35) and test sensitivities (43.9 % vs. 29.7 %) were considerably higher in the re-evaluation under laboratory conditions than in the field study (fresh samples). Shear forces generated during freeze–thaw cycles disintegrate parasite stages in faeces and *Cryptosporidium* oocysts are apparently more robust than *Giardia* cysts (Robertson & Gjerde 2004). Distorted *Giardia* cyst walls could be seen after multiple freezing-thawing cycles with low voltage scanning electron microscopy and immunofluorescence microscopy (Erlandsen et al. 1990). This effect might explain the apparently increasing sensitivity, as the manufacturer's specifications note that the immunochromatographic *G. intestinalis* test also detects antigen fragments, increasing the probability of a positive test result. The samples contained low numbers of *G. intestinalis* (17–76,333 cpg) but high numbers of *Cryptosporidium* ($3x10^3$ to $3x10^7$ opg), so the effect might be relevant for *G. intestinalis* (Lichtmannsperger et al. 2019). The test sensitivity for *Cryptosporidium* increased only slightly from 77.6 % to 82.9 %. The number of true positive POCT results was high before freezing due to the high opg values, so we could not determine any effect of freeze-thawing on the destruction of *Cryptosporidium* oocysts. There is a need for further studies on the impact of freezing-thawing cycles on the results of immunochromatographic POCT.

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**Fazit für die Praxis**

Der Einsatz von Schnelltests in der tierärztlichen Praxis/am landwirtschaftlichen Betrieb soll helfen, gezielt Maßnahmen zu implementieren (z.B. Mutterschutzimpfung, spezielle Desinfektion, medikamentöse Prophylaxe), welche die Prävalenz von Kälberdurchfall verringern. Die Ergebnisse der Schnelltests sind Momentaufnahmen und müssen immer im Zusammenhang mit der Anamnese und der klinischen Untersuchung interpretiert werden. Die Validität der Ergebnisse der kommerziell erhältlichen Schnelltests ist sehr unterschiedlich und von der eingesetzten Referenzmethode abhängig.

## References


Al Mawly J, Grinberg A, Prattley D, Moffat J, Marshall J, French N. Risk factors for neonatal calf diarrhoea and enteropathogen shedding in New Zealand dairy farms. Vet J. 2015;203(2):155–160. DOI:10.1016/j.tvjl.2015.01.010

Blanchard PC. Diagnostics of dairy and beef cattle diarrhea. Vet Clin North Am Food Anim Pract. 2012;28(3):443–464. DOI:10.1016/j.cvfa.2012.07.002

Cho YI, Sun D, Cooper V, Dewell G, Schwartz K, Yoon KJ. Evaluation of a commercial rapid test kit for detecting bovine enteric pathogens in feces. J Vet Diagn Invest. 2012;24(3):559–562. DOI:10.1177/1040638712440997

Cho YI, Yoon KJ. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. J Vet Sci. 2014;15(1):1–17. DOI:10.4142/jvs.2014.15.1.1

Dohoo I, Martin W, Stryhn H. Veterinary Epidemiologic Research. 2nd ed. Price Edward Island, Canada: Ver Inc.; 2010.

Erlandsen SL, Sherlock LA, Bemrick WJ. The detection of *Giardia muris* and *Giardia lamblia* cysts by immunofluorescence in animal tissues and faecal samples subjected to cycles of freezing and thawing. J Parasitol. 1990;76(2):267–271.

Feng Y, Ryan UM, Xiao L. Genetic diversity and population structure of *Cryptosporidium*. Trends Parasitol. 2018;34(11):997–1011. DOI:10.1016/j.pt.2018.07.009

Foster DM, Smith GW. Pathophysiology of diarrhea in calves. Vet Clin N Am Food Anim Pract. 2009;25(1):13–36. DOI:10.1016/j.cvfa.2008.10.013

Franck SM, Bosworth BT, Moon HW. Multiplex PCR for enterotoxigenic, attaching and effacing, and shiga toxin-producing *Escherichia coli* strains from calves. J Clin Microbiol. 1998;36(6):1795–1797. DOI:10.1128/JCM.36.6.1795-1797.1998

Geurden T, Levecke B, Pohle H, De Wilde N, Vercruysse J, Claerebout E. A Bayesian evaluation of two dip-stick assays for the on-site diagnosis of infection in calves suspected of clinical *Giardia*sis. Vet Parasitol. 2010;172(3–4):337–340. DOI:10.1016/j.vetpar.2010.05.015

Gillhuber J, Rügamer D, Pfister K, Scheuerle MC. Giardiosis and other enteropathogenic infections: a study on diarrhoeic calves in southern Germany. BMC Res Notes. 2014;7:112. DOI:10.1186/1756-0500-7-112

Goossens E, Valgaeren BR, Pardon B, Haesebrouck F, Ducatelle R, Deprez PR, et al. Rethinking the role of alpha toxin in *Clostridium perfringens*-associated enteric diseases: a review on bovine necro-haemorrhagic enteritis. Vet Res. 2017;48(1):9. DOI:10.1186/s13567-017-0413-x

Izzo MM, Kirkland PD, Gu X, Lele Y, Gunn AA, House JK. Comparison of three diagnostic techniques for the detection of rotavirus and coronavirus in calf faeces in Australia. Aust Vet J. 2012;90(4):122–129. DOI:10.1111/j.1751-0813.2011.00891.x

Jost BH, Billington SJ, Trinh HT, Bueschel DM, Songer JG. Atypical cpb2 genes, encoding beta2-toxin in *Clostridium perfringens* isolates of nonporcine origin. Infect Immun. 2005;73(1):652–656. DOI:10.1128/IAI.73.1.652-656.2005

Klein D, Kern A, Lapan G, Benetka V, Möstl K, Hassl A, et al. Evaluation of rapid assays for the detection of bovine coronavirus, rotavirus A and *Cryptosporidium parvum* in faecal samples of calves. Vet J. 2009;182(3):484–486. DOI:10.1016/j.tvjl.2008.07.016

Klein-Jöbstl D, Iwersen M, Drillich M. Farm characteristics and calf management practices on dairy farms with and without diarrhea: a case-control study to investigate risk factors for calf diarrhea. J Dairy Sci. 2014;97(8):5110–5119. DOI:10.3168/jds.2013-7695

Kolenda R, Burdukiewicz M, Schierack P. A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. coli*. Front Cell Infect Microbiol. 2015;5:23. DOI:10.3389/fcimb.2015.00023

Kosack CS, Page AL, Klatser PR. A guide to aid the selection of diagnostic tests. Bull World Health Organ. 2017;95(9):639–645. DOI:10.2471/BLT.16.187468

Lichtmannsperger K, Hinney B, Joachim A, Wittek T. Molecular Characterization of *Giardia intestinalis* and *Cryptosporidium parvum* from calves with diarrhoea in Austria and evaluation of point-of-care tests. Comp Immunol Microb Infect Dis. 2019;66:101333. DOI:10.1016/j.cimid.2019.101333

Lichtmannsperger K, Harl J, Freudenthaler K, Hinney B, Wittek T, Joachim A. *Cryptosporidium parvum*, *Cryptosporidium ryanae*, and *Cryptosporidium bovis* in samples from calves in Austria. Parasitol Res. 2020;119(12):4291-4295. DOI:10.1007/s00436-020-06928-5

Luginbühl A, Reitt K, Metzler A, Kollbrunner M, Corboz L, Deplazes P. Feldstudie zu Prävalenz und Diagnostik von Durchfallerregern beim neonaten Kalb im Einzugsgebiet einer schweizerischen Nutztierpraxis. Schweiz Arch Tierheilkd. 2005;147(6):245–252. DOI:10.1024/0036-7281.147.6.245

Otto PH, Rosenhain S, Elschner MC, Hotzel H, Machnowska P, Trojnar E, et al. Detection of rotavirus species A, B and C in domestic mammalian animals with diarrhoea and genotyping of bovine species A rotavirus strains. Vet Microbiol. 2015;179(3-4):168–176. DOI:10.1016/j.vetmic.2015.07.021

Pang XL, Lee B, Boroumand N, Leblanc B, Preiksaitis JK, Yu Ip CC. Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. J Med Virology. 2004;72(3)496-501. DOI:10.1002/jmv.20009

Papini R, Bonelli F, Montagnani M, Sgorbini M. Evaluation of three commercial rapid kits to detect *Cryptosporidium parvum* in diarrhoeic calf stool. Ital J Anim Sci. 2018;17(4):1059–1064. DOI:10.1080/1828051X.2018.1452055

Rinderzucht Austria. Zahl der Rinder haltenden Betriebe 2021 in Österreich gesunken [cited 2022 Jun 28]. Available from: https://www.rinderzucht.at/nachricht/20220307-zahl-der-rinder-haltenden-betriebe-2021-in-oesterreich-gesunken.html

Robertson LJ, Gjerde BK. Effects of the Norwegian winter environment on *Giardia* cysts and *Cryptosporidium* oocysts. Microb Ecol. 2004;47(4):359–365. DOI:10.1007/s00248-003-0003-5

Spiss S, Benetka V, Künzel F, Sommerfeld-Stur I, Walk K, Latif M, et al. Enteric and respiratory coronavirus infections in Austrian dogs: serological and virological investigations of prevalence and clinical importance in respiratory and enteric disease. Wien Tierarztl Monat - Vet Med Austria. 2012;99(3–4):67–81.

Thrusfield M, Christley R. Veterinary Epidemiology. 4th ed. New Jersey NY, USA: John Wiley and Sons; 2018.

Torsein M, Lindberg A, Sandgren CH, Persson Waller K, Törnquist M, Svensson C. Risk factors for calf mortality in large Swedish dairy herds. Prev Vet Med. 2011;99(2-4):136–147. DOI:10.1016/j.prevetmed.2010.12.001

Valgaeren BR, Pardon B, Verherstraeten S, Goossens E, Timbermont L, Haesebrouck F, et al. Intestinal clostridial counts have no diagnostic value in the diagnosis of enterotoxaemia in veal calves. Vet Rec. 2013;172(9):237. DOI:10.1136/vr.101236

Windeyer MC, Leslie KE, Godden SM, Hodgins DC, Lissemore KD, Leblanc SJ. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Prev Vet Med. 2014;113(2):231–240. DOI:10.1016/j.prevetmed.2013.10.019

Yoo HS, Lee SU, Park KY, Park YH. Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. J Clinical Microbiol. 1997;35(1):228–232. DOI:10.1128/jcm.35.1.228-232.1997

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