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Evaluation of three commercial rapid kits to detect *Cryptosporidium parvum* in diarrhoeic calf stool

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ABSTRACT

The aim of this study was to evaluate three commercially available rapid immunochromatographic tests for detection of *Cryptosporidium parvum* antigens in faeces of naturally infected neonatal diarrhoeic calves. FASTest® CRYPTO strip, FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® were compared for their sensitivity, specificity, positive predictive value and negative predictive value using a cumulative positivity as gold standard. In addition, the agreement between each test and the gold standard was evaluated by Cohen's Kappa (k) value. The highest infection rate was observed by FASTest® CRYPTO-GIARDIA Strip (65.15%), followed by FASTest® CRYPTO strip (63.64%) and TETRASTRIPS® (56.06%,). A very good diagnostic performance of all the three tests was observed. FASTest® CRYPTO strip ($k = 0.935$) and FASTest® CRYPTO-GIARDIA Strip ($k = 0.968$) had the highest sensitivity (100%) while TETRASTRIPS® ($k = 0.875$) had the highest specificity (100%). *Eimeria* spp oocysts were present in six samples but cross-reaction with this protozoan was not observed. These assays were not time-consuming and very easy to perform and to read. Based on our results, we recommend the use of FASTest® CRYPTO strip, FASTest® CRYPTO-GIARDIA Strip or/and TETRASTRIPS® for detection of *C. parvum* antigens in faeces of neonatal diarrhoeic calves.

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Calf diarrhoea; *Cryptosporidium parvum*; rapid immunochromatographic tests

Introduction

Diarrhea is a common disease in calves and can become a serious problem causing high rates of morbidity and mortality (Cho and Yoon 2014). Major enteric pathogens associated with calf diarrhoea include viruses (i.e. Bovine Rotavirus, Bovine Coronavirus, Bovine Viral Diarrhea Virus, Bovine Torovirus, Bovine Norovirus, Nebovirus), bacteria (i.e. *Salmonella enterica*, *Escherichia coli*, *Clostridium perfringens*), and protozoa such as *Cryptosporidium parvum* (Cho and Yoon 2014). Accurate and rapid detection of such a large number of potential aetiological agents during severe diarrhoea outbreaks in calves can quickly aid to implement appropriate interventions, decreasing economic losses to breeders and improving animal welfare (McGuirk 2008; Foster and Smith 2009).

Cryptosporidium parvum is a common cause of diarrhoea in neonatal calves (Björkman et al. 2003, 2015; Trotz-Williams et al. 2005; Singh et al. 2006). This apicomplexan protozoan parasite is recognised as highly infectious enteric pathogen and is transmitted through faecal-oral route by ingestion of oocysts that are excreted in the faeces of infected hosts. *Cryptosporidium parvum* is reported to mainly infect pre-weaned calves from 5 days to 2 months of age (Santìn et al. 2004). Symptoms of cryptosporidiosis in calves include watery diarrhoea and loose stool (Björkman et al. 2015), sometimes accompanied by depression, inappetence, fever, dehydration and/or poor condition (Björkman et al. 2003). Cryptosporidiosis in calves has been reported from different parts of the world. For instance, reported prevalence values of *C. parvum* were 5% and 11% in healthy and diarrhoeic calves in Sweden (Björkman et al. 2003), 40.6% in dairy calves from farms with a history of diarrhoea in Canada (Trotz-Williams et al. 2005), or 25.68% and 50% in non-diarrhoeic and diarrhoeic neonatal dairy calves in India (Singh et al. 2006). In addition to *C. parvum*, *Cryptosporidium andersoni*, *Cryptosporidium bovis*, *Cryptosporidium ryanae* and *Cryptosporidium ubiquitum* can also be identified in calves (Björkman et al. 2015; Wegayehu et al. 2016). *Cryptosporidium parvum*, *C.*

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ubiquitum, and *C. andersoni* are also recognised zoonotic agents, and persons in contact with infected cattle are at risk of contracting these infections (Jiang et al. 2014; Li et al. 2014). Other *Cryptosporidium* species that may be zoonotic include *Cryptosporidium bailey*, *Cryptosporidium canis*, *Cryptosporidium felis*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*, and *Cryptosporidium suis* (Ghazy et al. 2015).

Diagnosis of cryptosporidiosis can be carried out by using faecal smears stained by the modified Ziehl–Neelsen technique (Björkman et al. 2003; Singh et al. 2006; Wegayehu et al. 2016), Sheather's sucrose flotation solution (Trotz-Williams et al. 2005; Singh et al. 2006) or direct immunofluorescence assays (DIA) (Björkman et al. 2015; Mirhashemi et al. 2015). Enzyme-linked immunosorbent assays (ELISA) are also available for detection of specific *Cryptosporidium* coproantigens (Cho et al. 2012; Mirhashemi et al. 2015). Molecular methods involving polymerase chain reaction (PCR) assays are needed to identify different *Cryptosporidium* species in cattle faeces (Jiang et al. 2014; Björkman et al. 2015; Mirhashemi et al. 2015; Wegayehu et al. 2016). All these methods are time-consuming and expensive, requiring well-equipped laboratories and well-trained, skilled personnel.

In recent years, commercial immunochromatographic assays have been marketed for rapid detection of *C. parvum* alone or *C. parvum* and other major enteric pathogens in faeces from diarrhoeic calves (Muccio et al. 2004; Klein et al. 2009; Cho and Yoon 2014). These tests enable detection of *C. parvum* antigens in unconcentrated stool within few minutes and can be used in the field. The present study was conducted to assess the diagnostic performance of three rapid commercial kits for detection of *C. parvum* in cattle.

Materials and methods

Between July 2016 and May 2017, 132 calf stool samples were tested to evaluate the performance of three rapid tests. All of these samples came from naturally infected diarrhoeic calves aged 1 to 84 days (mean age = 12 days, median age = 8 days). They were of Holstein-Friesian (− = 102) or Mucco Pisano ($n = 4$) breed and crossbred ($n = 26$), including 68 females and 64 males. Diarrheic calves were born and bred on four dairy cattle farms where cases of neonatal calf diarrhoea occurred over the sampling time. The farms were located in the province of Pisa (43°43′N 10°24′E), Tuscany, central Italy. Diarrhea was defined as a condition in which faeces were semi-formed/pasty, loose (i.e. faeces stay on top of bedding), or watery (i.e. faeces sift through bedding) (http://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf).

The calves were sampled on the same day of the onset of diarrhoea and before any possible treatment was given. The stool specimens were collected directly from the anus into clean plastic vials immediately after a gloved, lubricated finger was gently passed through the anus to massage the rectal wall and to stimulate rectal evacuation. After collection, samples were labelled by recording identification number, sex, age and breed of calves, kept at 4 °C in a cold bag, and then transported to the laboratory where they were examined as soon as possible or stored at 4 °C in a refrigerator until the three tests were performed (maximum within 1 day). This study was approved by the Institutional Animal Care and Use Committee of the University of Pisa (D.R. prot. n. 33479/2016).

FASTest® CRYPTO Strip (Vetefarma, Cuneo, Italy), FASTest® CRYPTO-GIARDIA Strip (Vetefarma, Cuneo, Italy), and TETRASTRIPS® (Starfish, Milan, Italy) can detect antigens of *C. parvum* in faeces of diarrhoeic neonatal calves. All samples ($n = 132$) were tested in parallel by FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® according to the manufacturers' guidelines. Briefly, for each of the three assays, a spoonful of faecal sample was collected using the stopper plug and added to the diluent buffer contained in the sample tube supplied by each manufacturer. The specimen dilution buffer was gently mixed three times to obtain a homogenous stool suspension. The arrow of each strip was correctly inserted and allowed to stand for 1 minute into the stool suspension. Then the strips were removed and placed on a dry horizontal surface at room temperature. After 5 (FASTest® CRYPTO Strip and FASTest® CRYPTO-GIARDIA Strip) or 10 (TETRASTRIPS®) minutes, reading was carried out by visual inspection. The diagnostic interpretation of the three kits in agreement with manufacturers' instructions is as follows. The presence of the control band indicates a valid test result. Every single appearance of additional bands, each of them with a determined colour, indicates positivity to a different specific pathogen. Results were recorded as *C. parvum* positive or *C. parvum* negative, as these kits provide only qualitative results. In addition to *C. parvum*, the FASTest® CRYPTO-GIARDIA Strip allows to determine also the occurrence of *Giardia duodenalis* coproantigens while the TETRASTRIPS® is designed to detect also Rotavirus, Coronavirus and *Escherichia coli* K99$^+$ in the same calf stool specimen. Therefore, positivity to these pathogens was also recorded.

Upon arrival at the laboratory, an aliquot of each faecal sample was submitted to routine flotation method using a commercial nitrate solution (Coprosol®, Candioli Farmaceutici S.p.A., Torino, Italy) with specific gravity 1200, and examined by light microscopy. Parasitic agents were identified by their morphologic characteristics. The Ziehl-Neelsen method was not applied to the samples because it is time-consuming and tedious and requires experienced microscopists to accurately identify the oocysts (Morgan et al. 1998).

Since there is no available gold standard diagnostic technique for detection of *Cryptosporidium* oocysts (Smith 2008), the performance of the three diagnostic kits was assessed based on the assumption that the pooled results from at least two tests accurately reflected the true infection status. Hence, a cumulative positivity was used as gold standard and we considered as positive samples those that were positive by at least two of the three tests applied. Cumulative positivity has previously been used as diagnostic gold standard in other studies where individual tests were compared and their performances were calculated based on comparison with results obtained by different methods combined (Goodman et al. 2007; Knopp et al. 2008; Steinmann et al. 2008; Utzinger et al. 2008; Habtamu et al. 2011; Paştiu et al. 2015). Infection rates were determined as number of positive samples/number of examined samples ×100 along with the corresponding 95% confidence interval (95% CI). Sensitivity (Se), specificity (Sp), positive predictivevalue (PPV) and negative predictive value (NPV) of each kit were calculated. The Cohen's Kappa (*k*) was also calculated as a measure of the degree of agreement between each kit and the gold standard. Cohen's Kappa values were interpreted as poor ($k < 0.01$), slight ($k = 0.01$–0.20), fair ($k = 0.21$–0.40), moderate ($k = 0.41$–0.60), substantial ($k = 0.61$–0.80) and excellent ($k = 0.81$–1.00).

Results

Overall, 82/132 (62.12%, 95% CI = 53.85–70.40) samples were positive for *C. parvum*, either alone or together with other enteric pathogens, by at least two of the three tests applied at the time of sampling. Infection rates of *C. parvum* according to FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip or TETRASTRIPS® were 84/132 (63.64%, 55.43–71.84%), 86/132 (65.15%, 57.02–73.28%) and 74/132 (56.06%, 47.59%–64.53%), respectively. In addition, coinfections of Rotavirus with Coronavirus, *C. parvum* with *G. duodenalis*, or *C. parvum* with Rotavirus and Coronavirus were detected in 2/132 (1.51%, 0.00–2.24%, 9) samples each. The three assays yielded valid test results with all specimens because control lines were always obtained. *Eimeria* spp oocysts were identifed by microscopy in 6/132 (4.55%, 0.99–8.10%) *C. parvum* negative samples.

Table 1. Measures of the comparative performance of three commercially available rapid immunochromatographic tests (FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip, and TETRASTRIPS®) to detect *Cryptosporidium parvum* in faeces of 132 diarrhoeic calves aged 1 to 84 days in Italy.

	Immunochromatographic tests		
Comparative performance	FASTest® CRYPTO Strip	FASTest® CRYPTO-GIARDIA Strip	TETRASTRIPS®
True positives	82	82	74
False positives	2	4	0
True negatives	48	46	50
False negatives	0	0	8
Sensitivity	100%	100%	90.24%
Specificity	96%	92%	100%
Positive predictive value	97.62%	95.35%	100%
Negative predictive value	100%	100%	86.21%

The diagnostic Se of FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® to detect *C. parvum* on the same 132 faecal samples from diarrhoeic calves was 100%, 100% and 90.24%, respectively, while their diagnostic Sp was 96%, 92% and 100%. The agreement between each kit and the gold standard was excellent ($k = 0.935$, 0.968 and 0.875, respectively). Measures of the comparative performance, including PPV and NPV, are summarised in Table 1.

Discussion and conclusions

FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® are three commercially available rapid immunochromatographic tests that can be used to detect antigens specific for *C. parvum* in faecal samples from neonatal calves with diarrhoea. The present study evaluated their diagnostic performance using cumulative positivity as gold standard. Our results show that Se was >90% and Sp was >91% for all the three commercial kits. This is in agreement with data on Se and Sp reported in the inserts of the kits. Diagnostic Se and Sp of both FASTest® CRYPTO Strip and FASTest® CRYPTO-GIARDIA Strip stated by the manufacturer are 96.7% and 99.9%, respectively, while the stated diagnostic Se and Sp of TETRASTRIPS® for *C. parvum* are 95.5% and 94.1%. No cross-reactions with *Eimeria* spp were observed. Moreover, we pointed out that a rate as high as 62.12% of diarrhoeic calves were spreading *C. parvum,* which is transmissible to humans, with most cases of zoonotic infections resulting from exposure to infected cattle (Jiang et al. 2014;

Björkman et al. 2015). This is consistent with the results of a study performed in Canada where *C. parvum* was commonly found (40.6%) among 7- to 21-day-old dairy calves (Trotz-Williams et al. 2005) and with another study conducted in the United States where up to 66.7% of calves at 2 weeks of age were found be infected (Santìn et al. 2004). Cattle are considered the main reservoir of *C. parvum* and immunosuppressed people are highly susceptible to the infection, particularly patients with acquired immune deficiency syndrome (Hunter and Nichols 2002; Leitch and He 2011). In Italy, *C. parvum* has previously been reported in 20.6% of cattle farms (Duranti et al. 2009) while *Cryptosporidium* oocysts were detected in 11.4% of calves aged 2 to 240 days and three species of *Cryptosporidium* other than *C. parvum* were identified, these were *C. bovis*, *C. ryanae* and *C. ubiquitum* (Di Piazza et al. 2013).

Clinical cryptosporidiosis is difficult to diagnose in neonatal calves because the signs mimic those of many other entero-pathogens. Thus, due to the manifestations of disease, it may be misdiagnosed. Diagnostic tests should be performed to rule out a variety of different enteric pathogens that cause similar signs before a bacterial, viral or protozoan causative agent is associated with the clinical presentation. Stool analysis is the only practical means to identify *C. parvum* infection. As already mentioned, currently there are a number of stool examination procedures for diagnosis of cryptosporidiosis. All of these methods can detect either oocysts, or antigens, or DNA specific for *C. parvum* with high sensitivity and/or specificity. However, they require expensive laboratory equipment and it takes a long time between the examination of affected calves and the diagnosis of cryptosporidiosis. Conversely, our findings show that FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® are sensitive and specific but much less time-consuming and much easier to use than faecal smear, faecal flotation, DIA, ELISA and PCR for diagnosis of cryptosporidiosis in diarrhoeic calves. The use of highly specific monoclonal antibodies directed against single epitopes ensures excellent specificity of the kits tested for the detection of *C. parvum* antigens. The availability of accurate and rapid diagnostic assays for *C. parvum* that can immediately be used at the time of animal examination not only can help facilitate diagnosis but also allows for timely implementation of appropriate intervention strategies and control procedures. *Cryptosporidium* transmission to young calves can come from many sources including other calves, their dams, animal handlers, other animals and the environment. The life cycle of the parasite allows it to multiply rapidly in the host leading to the rapid spread of the disease within a susceptible group of animals. Infected animals can shed millions of infectious oocysts into the environment. The oocysts can survive many commonly used farm disinfectants and water chlorination treatment, making it difficult for farmers, veterinarians and water suppliers to control or inactivate it. Currently, there is no vaccine available and treatment options are limited. Given these issues, *C. parvum* can cause serious disease outbreaks in susceptible calves, leading to significantly reduced farm incomes in severe cases. Accurate diagnosis is crucial. Effective management solutions can significantly reduce the parasite burden on farm and thereby the impact of disease (Wells and Thomson 2014). When cryptosporidiosis is diagnosed within a short time, measures to prevent the spread of infection to other animals and to prevent zoonotic transmission, such as separation of affected animals and disinfection of contaminated facilities, can be implemented quickly. Other advantages of these rapid assays are that a large number of samples can be processed quickly with minimum effort and the need for technical expertise or specialised laboratory equipment is virtually nil, since interpretation of results is non ambiguous and does not require any special skill. Therefore, these commercial rapid assays can be used conveniently and reliably in the field to determine the infection status of diarrhoeic calves with *C. parvum*. On the other hand, although of less clinical importance, other *Cryptosporidium* species can be found in calves (Björkman et al. 2015; Wegayehu et al. 2016). A disadvantage of the rapid assays used in the present study is that they are unable to detect *Cryptosporidium* species other than *C. parvum*. Thus, these tests might be a useful addition to, but not a substitute for molecular methods in the diagnosis of cryptosporidiosis to species level.

FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® have the added advantage of simultaneously being able to detect *G. duodenalis* or Rotavirus, Coronavirus, and *E. coli* K99^{+} other potential causes of diarrhoea in calves, just in one-step. In this study, the use of FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® allowed us to identify mixed infections by Rotavirus and Coronavirus, *C. parvum* and *G. duodenalis* as well as *C. parvum*, Rotavirus and Coronavirus. A potential risk for zoonotic transmission of *G. duodenalis* from cattle has been reported (McDaniel et al. 2014). Of the 132 samples tested in this study, 6/132 (4.55%, 0.99–8.10%) were co-infected. Mixed infections in diarrhoeic calves have previously been reported (Björkman et al. 2003; Cho and Yoon 2014) and can increase the

risk of clinical signs. The observation of coinfections is not surprising since major enteric pathogens share the faecal-oral route of transmission. By combining FASTest® CRYPTO-GIARDIA Strip and TETRASTRIPS® in parallel, it takes only about 15 minutes to obtain diagnosis for five major enteric pathogens, sample collection and preparation excluded. Therefore, combination of these two tests might greatly improve the diagnosis of calf diarrhoea. However, whether the two assays were accurate methods to detect *G. duodenalis*, Rotavirus, Coronavirus and *E. coli* K99^{+} in calf faeces was not evaluated in the present study.

To conclude, most cattle breeders have to face cryptosporidiosis at some time. Results of our study show that FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip, and TETRASTRIPS® are accurate, helpful, fast and effective tools for the bovine practitioners to diagnose cryptosporidiosis by *C. parvum* in neonatal calves with diarrhoea, requiring minimal supplies. A further advantage of these coproantigen detection assays is that they can be used to test large numbers of samples in a cost effective manner. Therefore, the technical advantages of these assays should be taken into consideration when choosing commercially available rapid diagnostic tests to be used in the field.

Disclosure statement

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