TF-Test techniques for the laboratory diagnosis of gastrointestinal parasites of humans and animals

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Summary
Intestinal parasites inhabit the intestinal tract of humans and animals, causing damages whose severity depends on several factors related to the parasite and the host. Immunocompromised individuals are more likely to develop severe forms of parasitic infestation. The diagnosis of the gastrointestinal parasitosis is mainly performed by the examination of the feces, which consists of the direct visualization and identification of the parasites eliminated through the feces. These tests are generally low sensitive and the microscope slides contain a large number of impurities, which can impair the result of the diagnosis. In order to improve the diagnostic accuracy, a new parasitological technique called Three Fecal Test (TF-Test) was developed. To further improve its diagnostic accuracy, few modifications of the original protocols have been made with the years. In this study the performance of these new techniques to detect gastrointestinal parasites in human and animal fecal samples was described and discussed in relation to the performance of other conventional coprological tests. It could be concluded that the TF-Test conventional and modified can be used for the diagnosis of several human and animal parasites, with satisfactory results.

Introduction

Intestinal parasites are highly prevalent in the world population, in both developed and developing countries. Diseases caused by intestinal paraistes affect more than one billion people (Menezes 2008, WHO 2015, CDC 2017). Several species of helminths and protozoa inhabit the human intestinal tract, where they can cause damage, whose severity depends on several factors related to the parasite and the host. Immunocompromised individuals are more likely to develop severe forms of the disease. The associated symptoms are diverse and generally nonspecific. As they are similar to those caused by other pathogens, the clinical diagnosis need to be confirmed by laboratory tests (Garcia *et al.* 2017).

The laboratory diagnosis of intestinal parasitosis is mainly performed by the examination of the feces, which consists of the direct visualization and identification of the parasites eliminated through the feces (Gomes *et al.* 2004, Carvalho *et al.* 2015). The identification is made by parasites reading the microscopy slides prepared after concentrating the parasites and eliminating faecal impurities. When most infections are mild, the sensitivity of the diagnostic techniques and kits currently available is low and inadequate (Carvalho *et al.* 2012).

The most commonly coproparasitological techniques used for diagnosis are centrifugation-flotation by saturated solution of zinc sulfate (Faust *et al.* 1938), saturated solution of sodium chloride (Willis 1921), spontaneous sedimentation (Hoffman *et al.* 1934) and formalin-ether concentration technique (Ritchie 1948). However, none of these techniques can identify all parasites when used separately (Oliveira-Sequeira *et al.* 2002). Also, the main limitation of the sedimentation technique is the large amount of debris which makes it difficult

to visualize the parasite (Garcia 2001). Therefore, there was a need for a more sensitive technique to obtain reliable results (Gomes *et al.* 2004). In order to improve the diagnostic quality, a new parasitological technique called Three-Faecal Test (TF-Test) was developed (Hoshino-Shimizu *et al.* 2002, Gomes *et al.* 2004).

The TF-Test technique is recommended for the laboratory diagnosis of enteroparasitosis by the Brazilian Ministry of Health, through the Plan for Surveillance and Control of Enteroparasitosis (Brasil 2005). With the purpose of promoting a higher concentration of parasitic structures with a reduction in the amount of faecal debris, improvements were made to the operational protocol, resulting in the TF-Test Modified technique (Carvalho *et al.* 2015). The modified technique uses formalin-ethyl acetate centrifugal sedimentation, spontaneous sedimentation, and flotation (Gomes *et al.* 2004).

The principles used in both the TF-Test and TF-Test Modified techniques of were adapted and applied in the diagnosis of intestinal parasites of animals. New techniques have then been developed for the parasitological examination of faecal samples in veterinary medicine as a TF-Test Conventional technique for sheep (Lumina *et al.* 2006), TF-Test Modified/Dog and TFGII/Dog for dogs (Coelho *et al.* 2013, 2015) and TF-Test Coccidia for cattle (Inácio *et al.* 2016). The evolution of the TF-Test technique, as well as its new applications in the laboratory diagnosis of intestinal parasites in humans and animals, are described in this review.

Diagnosis of intestinal parasites in the human medicine area

TF-Test Conventional

The developed Three Faecal Test technique is based on the collection of three faecal samples on alternate days. This increases the sensitivity with higher concentration of parasites (Gomes *et al.* 2004).

The TF-Test technique consists of a commercial Kit TF-Test (Figure 1) (Biobrasil Ciência e Tecnologia®) which includes: (I) three collector tubes containing preservative liquid based on neutral buffered formalin solution to collect faecal samples on three alternating days; (II) a laboratory set consisting of two filter meshes (400 and 200 µm) coupled with a centrifuge tube that allows the simultaneous processing of the three samples resulting in a single faecal sediment; and (III) operational protocol based on the centrifugal sedimentation principle formalin-ethyl acetate for the concentration of parasites (Gomes *et al.* 2004).

Figure 1. *TF-Test kit and its components.*

By means of this technique, it is possible to identify the most prevalent parasitic species in humans, with detection of helminths like *Ascaris lumbricoides, Schistosoma mansoni, Taenia* spp., *Enterobius vermicularis, Trichuris trichiura, Hymenolepis nana, Hymenolepis diminuta,* hookworms, protozoa such as *Entamoeba histolytica, Giardia duodenalis, Lodamoeba butschlii, Blastocystis* spp., *Endolimax nana* and *Entamoeba coli* (Borges *et al.* 2011).

The collection of three samples on alternate days with single processing improves the diagnostic efficacy, especially in patients with mild infections. This technique has the advantage of presenting less faecal impurities in the microscopy slide, it is easy to execute and it is inexpensive (Gomes *et al.* 2004).

The TF-Test Conventional technique was evaluated and compared to conventional techniques (Kato-Katz, Lutz/Hoffman, Faust and Rugai) in a study conducted on 1,102 individuals living in four different municipalities in the state of São Paulo (Gomes *et al.* 2004). The diagnostic sensitivity using the TF-Test technique varied from 86.2% to 97.8%, being significantly higher ($p > 0.01$) than that observed when using the Coprotest kit and the combination of conventional techniques (Gomes *et al.* 2004).

It is also important to highlight a very positive point for the TF-Test Conventional technique, in relation to sample preservation, which can be maintained at room temperature for up to 30 days (Garcia *et al.* 2007). This is an important advantage, since for the realization of the FLOTAC technique (Cringoli 2010), samples must be kept under vacuum and at most 4 °C (Rinaldi *et al.* 2011).

TF-Test Modified

Despite the advantages offered by the TF-Test Conventional (Gomes *et al.* 2004), the microscopy slide still had a concentration of debris. In 2010, in view of the constant improvement in diagnostic efficacy, a modification was made to the original protocol of the TF-Test Conventional technique, which was renamed TF-Test Modified. In this technique, the TF-Test kit is also used to collect faecal

samples, but the operational protocol includes three methods for parasite concentration: formalin-ethyl acetate centrifugal sedimentation, spontaneous sedimentation, and spontaneous fluctuation (Carvalho *et al.* 2015).

TF-Test Modified showed higher diagnostic sensitivity (98.38%) when compared to the TF-Test Conventional fecal processing (83.12%). Additionally, this method was superior (100.0%) to TF-Test Conventional (81.03%) and the Helm-Test/Kato-Katz technique (39.66%) when only the species detected by the latter were considered (Carvalho *et al.* 2015).

Table I. *Results of comparisons of different coproparasitological techniques (Rugai* et al. *1954, Kato-Katz 1972, Gomes* et al. *2004, Carvalho* et al. *2015).*

Positivity of fecal samples in four different parasitological techniques (%)			
TF-Test Modified	TF- Test Conventional	Helm Test	Rugai, Mattos e Brisola
42.23	36.76	5.03	4.16
Sensitivity test considering the values of the Helm Test technique (%)			
TF-Test Modified	100.00		
TF-Test Conventional	81.03		
Helm Test	36.66		
Sensitivity test considering the values of the TF-Test technique (%)			
TF-Test Modified	98.38		
TF-Test Conventional	83.12		

Diagnosis of intestinal parasites in the veterinary medicine area

TF-Test Conventional in the diagnosis of intestinal parasitoses of sheep

The greatest losses in world sheep farming are caused by gastrointestinal disorders of parasitic origin (Suarez 2009). Species detected and reported as most damaging in production are *Haemonchus contortus, Eimeria* spp., *Thichostrongylus* spp. e *Strongyloides* spp. (Cavalcante *et al.* 2009, Emery *et al.* 2016).

In intensive production systems, coccidia infections can cause damage, especially in newborn lambs parasitized with *Eimeria* spp. (Cavalcante *et al.* 2009). Several species can attack herds and cause mortality and poor performance (Reeg *et al.* 2005). Several factors, such as the low sensitivity of parasite detection techniques, allow us to conclude that the parasite involvement in the herds may be underestimated. Nevertheless, to guide the management strategies in the problematic of the herd it is indispensable to determine the occurrence of parasites by coproparasitological techniques (Bansal *et al.* 2015).

Within this context, considering its high sensitivity showed in the diagnosis of intestinal parasitoses of humans, in 2006, it was proposed to modify the TF-Test Conventional technique for use in the diagnosis of intestinal parasitoses of sheep (Lumina *et al.* 2006). The proposed protocol was compared with the modified Gordon & Whitlock (G&W) method (Gordon and Whitlock 1939) and the results obtained with these two techniques were not statistically different ($p > 0.005$) in relation to the detection of *H. contortus* (Lumina *et al.* 2006).

However, the TF-Test technique was more ($p < 0.001$) efficacious in detecting of oocysts of *Eimeria* spp. compared to the G&W method (Gordon and Whitlock 1939 - modified), and also ($p < 0.05$) in detecting *Strongyloides* spp. (Lumina *et al.* 2006).

Thus, the adaptation of TF-Test as a diagnostic technique for intestinal parasitoses in sheep was beyond all expectations, which led to its evaluation in other animal species.

TF-Test Modified / Dog

Dogs can be considerable sources of transmission of gastrointestinal parasites to humans (Xiao *et al.* 2007, Rodie *et al.* 2008, Bridger and Whitney 2009). *Ancylostoma* spp., *Toxocara canis, Trichuris vulpis* e *Dipylidium caninum*, *Giardia duodenalis* and *Cryptosporidium* spp. (Katagiri *et al.* 2008, Martins *et al.* 2012), are helminths and protozoa with zoonotic potential high prevalent in dogs.

The control of gastrointestinal parasites depends on diagnostic, investigation and follow up methods. In order to propose a more accurate diagnosis of parasitoses in dogs, the TF-Test Modified/Dog technique was developed, based on the principles of TF-Test Conventional and Modified (Coelho *et al.* 2013).

In TF-Test Conventional technique, two microscopy slides, one for eggs with density above 1.19 g/mL and another for eggs below this density have to be prepared and read. In order to facilitate the procedure, a new protocol was developed by adding the basic principles that are centrifugal sedimentation, spontaneous flotation and sedimentation, improve and make the TF-Test more practical.

The performance of the TF-Test Modified/Dog technique was compared with other techniques such as centrifugation flotation by saturated solution of zinc sulfate, simple flotation by saturated solution of sodium chloride and direct microscopy

in examining 106 faecal samples from dogs. The TF-Test Modified/Dog technique showed higher sensitivity (98.41%), than Zinc Sulfate Solution (79.36%), TF-Test Conventional (73.02%), Sodium Chloride Solution (55.22%) and direct examination (30.16%) (Coelho *et al.* 2013).

These results may be related to several factors: collection of faecal samples on three alternate days, the use of preservative solution, double filtering of samples, and stages of parasitic concentration (Garcia 2007).

TFGII/Dog

In the continuity of this research line, with the objective of providing a high sensitive diagnosis for the gastrointestinal parasites of dogs, protocol improvements were proposed, thus giving rise to the TFGII/Dog technique. This technique based on the same principles of the TF-Test Conventional and TF-Test Modified (formalin-ethyl acetate centrifugal sedimentation, spontaneous sedimentation and flotation), demonstrated to be more sensitive compared with the main conventional coproparasitological techniques [spontaneous fluctuation using saturated sodium chloride solution d = 1.20 g/mL (Willis 1921), centrifugation-flotation in zinc sulphate solution d = 1.18 g/mL (Faust *et al.* 1938), centrifugation-flotation in saturated sugar solution d = 1.18 g/mL (Sheather 1923) and spontaneous sedimentation (Hoffman *et al.* 1934, Lutz 1919)] in detecting gastrointestinal helminths and protozoa in a study carried out with 250 samples of 50 naturally infected dogs, with Kappa value agreement = 0.739 (Coelho *et al.* 2015).

Both S-Sedimentation and TFGII/Dog demonstrated the higher sensitivity values (80.0%) against 39.0% of CF-Zinc Sulfate and CF-Sugar, and 37.0% of SF-Sodium Chloride. However, TFGII/Dog stood out for its capability of detecting eight genera of parasites while the others remained between six and seven genera. This detection of multiple parasitic infections is also due to the samples collection on alternate days, processed in a single time (Coelho *et al.* 2015). The difficulty in the diagnosis of gastrointestinal parasites in dogs likely depends on the fact that the elimination of their evolutionary forms is varying and occurs intermittently. Additionally, the hyper saturated solutions of sodium chloride or sucrose destroy the cellular structures (Coelho *et al.* 2015).

Although the spontaneous sedimentation technique has been shown to be the most suitable for the detection of *Ancylostoma* spp. (most prevalent parasites in the analysed samples), it was not able to detect with the same accuracy as the TFGII/Dog, helminths like *Taenia* spp., *Dypilidium* spp. and *Trichuris* spp., as well as coccidians and gastrointestinal flagellates (Coelho *et al.* 2015). For these species, the centrifugal-flotation technique in zinc sulphate performed better (48% positive). However, none of the comparative techniques identified as many different genera of parasites as TFGII/Dog in this study (Coelho *et al.* 2015). The use of this new technique can be easily included in the laboratory routine, since the kit is totally disposable, practical, cost similar to conventional techniques and/or other commercial kits and presents sensitivity and accuracy superior to any other common technique in the laboratory routine (Coelho *et al.* 2015). However, there was a need for a new protocol for the protozoan *Cryptosporidium* spp., to better visualize it, because of its small size, and the presence of debris in the microscopy slide which makes diagnosis difficult.

TF-Test Coccidia

Protozoa of the *Cryptosporidium* genus are important zoonotic pathogens that infect mammals, birds, reptiles, amphibians and fish (Fayer 2010, Xiao 2010). These parasites cause diarrhoea in calves (Vargas *et al.* 2014), with delayed growth, mortality and economic losses (Olson *et al.* 2004, Santín *et al.* 2008, Couto and Bonfim 2012).

The TF-Test Coccidia technique was validated in the bovine herd to optimize the diagnosis of *Cryptosporidium* spp. Currently, for the diagnosis of *Cryptosporidium* spp. specific concentration and permanent colouring techniques are recommended, but they are very costly in routine vision (Gomes *et al.* 2004, Garcia 2007, Carvalho *et al.* 2012, Carvalho *et al.* 2015).

The Malachite Green negative staining is a temporary microscopy slide technique (Hussein 2011) that can present a large amount of impurities in the microscopy slide, impairing the identification of the protozoa. Permanent microscopy slide techniques are not universally recommended for the detection of *Cryptosporidium* sp. oocysts because they have low specificity and sensitivity (Kaushika *et al.* 2008, Elsaf 2013). The permanent staining techniques most frequently used for detecting *Cryptosporidium* spp. are Ziehl-Neelsen (Tahvildar-Biderouni and Salehi 2014) and Kinyoun (Elsaf 2013).

The TF-Test Coccidia includes the collection of fecal samples on three alternate days, following by the application of the laboratory protocol for concentrating and staining the oocysts (Inácio *et al.* 2016). To validate this technique, faecal samples were taken from newborn calves, from herds with high frequency of cryptosporidial infection (Fayer and Santín 2009, Silverlås *et al.* 2010).

TF-Test Coccidia, the Malachite Green negative staining and the nested PCR showed statistic

agreement (Kappa = 1.000) in resulting 34 negative samples of a total of 68. As for the positive samples, the TF-Test Coccidia and the Malachite Green agreed that 34 were positive while the nested PCR was able to confirm 24 positive samples (Inácio *et al.* 2016). The TF-Test Coccidia technique presented excellent results, showing a clearer microscopy slide with low amount of impurities, good morphology and oocysts concentration. In the microscopy slides, it was possible to visualize more than seven oocysts of *Cryptosporidium* spp. per field with the objective lens of 60x without immersion. This favoured the detection of this parasite in the laboratory routine and reduced the error of diagnostic interpretation (Inácio *et al.* 2016). The TF-Test Coccidia made it possible to perform a temporary microscopy slide with a microscope reading with dry objective lens in a laboratory routine, since the techniques of permanent slides are rarely used routinely because they are very time-consuming and laborious (Inácio *et al.* 2016).

Overview

The TF-Test was compared with the Kato-Katz technique in detecting geohelminths (Siqueira *et al.* 2011). While Kato-Katz technique detected more geohelminths than TF-Test, it did not detect protozoa, which makes it a limitation. Conversely, TF-Test was able to detect protozoa and geohelmintes. However, in another comparison study, some authors found that Kato-Katz detected more parasites than TF-Test (Nacife *et al.* 2018), but fecal samples were from one day collection only, this might have decreased the effectiveness of the technique.

The TF-Test also obtained good results when compared to other coproparasitological techniques (Kato-Katz, Hoffman-Pons-Janer, Willis and Baermann-Moraes) in the detection of human parasites, providing greater or equal sensitivity (Carvalho *et al.* 2015).

TF-Test Conventional and TF-Test Modified techniques proved to be effective in the diagnosis. The modifications added to the protocol of the TF-Test Modified technique were significant for the improvement of the diagnostic performance, which can result in an estimable scientific and social contribution.

To date, for the veterinary medicine area, according to the authors' knowledge, no alternative method of parasite concentration has been presented that combines the advantages of sample preservation, easy execution, low operational cost and sensitivity, such as TF-Test in sheep (Lumina *et al.* 2006) and dogs (Coelho *et al.* 2013, Coelho *et al.* 2015). The TF-Test Modified/Dog, compared to the other techniques, demonstrated better performance and high sensitivity, which makes it suitable for the diagnosis of gastrointestinal parasites in dogs. And based on the good results of study of the TF-Test Coccidia technique, it can be applied in the veterinary medicine field for faecal diagnosis of *Cryptosporidium* spp. oocysts.

General consideration

In the described studies, new approaches are proposed using the TF-Test technique. From these operational changes it has been possible to carry out the diagnosis of several human and animal parasites, with satisfactory results.

Although these results are satisfactory, the technique still needs adjustments mainly for an evolution and an automated perspective because there are still some limitations such as excess of steps in the protocol and this entails in losses of parasites in the process that consequently decreases the sensitivity.

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