Journal of Zoonotic Diseases
2023, 7 (3): 367-370
doi: 10.22034/jzd.2023.16799
https://jzd.tabrizu.ac.ir/article_16799.html

Short Communication

What is the preferred method for diagnosing cryptosporidiosis in sheep - Modified Ziehl–Neelsen or auramine O staining?

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(Received July 28, 2022, Accepted August 28, 2023)

Abstract
Cryptosporidium is an intestinal apicomplexan protozoon that causes cryptosporidiosis, a diarrheal disease affecting gastrointestinal tract of a wide range of vertebrates, including humans, livestock, wild animals, and birds. In the present study, *Cryptosporidium* spp. infection was diagnosed in sheep using two staining methods: Modified Ziehl–Neelsen (MZN) and auramine O (AO) staining. A total of 260 fecal samples were collected from sheep. All the samples were subjected to the formalin-ether concentration technique. The fecal smears were stained using the MZN (acid-fast stain) and AO (fluorescent stain) staining methods and then observed under a light and a fluorescent microscope, respectively. Cohen's kappa was used to check the significant agreement between the two diagnostic methods. According to the results, there was substantial agreement between the two methods. However, since there are many fluorescent artifacts in sheep feces, the detection rapidity of *Cryptosporidium* spp. in sheep feces by MZN staining is higher than that of AO staining. Therefore, it is suggested to use MZN staining for diagnosing cryptosporidiosis in sheep flocks.
Keywords: *Cryptosporidium*, sheep, auramine, Modified Ziehl–Neelsen

Introduction

Cryptosporidiosis is a zoonotic disease caused by *Cryptosporidium* species as a subgroup of apicomplexans (Carey et al., 2004; Huang and White, 2006). Humans and animals are infected by ingesting *Cryptosporidium* spp. oocysts. These parasites are transmitted through a fecal-oral route due to contact with an infected animal or via food or water. According to previous studies conducted in the United States, Sweden, and the United Kingdom, as well as other developed and developing countries, *Cryptosporidium* spp. can cause persistent diarrheas in humans and animals (Fayer et al., 2000; Insulander et al., 2005).

Members of the genus *Cryptosporidium* are recognized as eukaryotic organisms and obligate

intracellular parasites. These protozoans have a complex life cycle, which involves sexual and asexual reproduction by a single host (Caccio, 2004). Today, different diagnostic tests are used to identify *Cryptosporidium* spp., the most important of which are microscopic and molecular methods. The present study evaluated the efficacy of two microscopic methods, including the Modified Ziehl–Neelsen (MZN) and auramine O (AO) staining, in detecting *Cryptosporidium* spp. oocytes in the feces of sheep.

Materials and methods

In this study, 260 fecal samples were collected directly from the rectum of sheep using sterile latex gloves. Each sample was collected in a clean and leak-proof plastic container, which contained 10% formalin to fix the fecal samples for preservation until further examination (Bukhari and Smith, 1995). All the samples were subjected to the formalin-ether concentration technique. The smears were prepared from fecal concentrates and fixed with methanol for five minutes (Bukhari and Smith, 1995). Subsequently, the fecal smears were stained using the MZN (acid-fast stain) and AO (fluorescent stain) staining methods and then observed under a light microscope at ×100 to ×400 magnification. In the MZN method, the oocysts of *Cryptosporidium* spp. were visualized as bright red round organisms on a pale green background (Fayer et al., 2000), while in the AO staining method, the smears were observed via fluorescence microscopy.

Considering their fluorescent properties, the oocysts of *Cryptosporidium* spp. were observed as light particles on a black background (Ranjbar-Bahadori et al., 2011). Cohen's kappa (Sim and Wright, 2005) was used to investigate the significant agreement between the two diagnostic methods.

Results and discussion

In the present study, the detection percentage of *Cryptosporidium* spp. in the sheep fecal samples was slightly higher using AO staining (9.6%) compared to the MZN method (6.5%) (Table 1). Cohen's kappa (κ) was 0.793. This is the agreement proportion over and above chance agreement. Cohen's kappa (κ) can range from -1 to +1. A kappa (κ) of 0.793 represents a substantial agreement strength. Furthermore, since $p < .001$ (i.e., p is less than .001), our kappa (κ) coefficient is statistically different from zero, significantly. In other words, Cohen's κ was run to find out if there was an agreement between two methods for diagnosis on 260 sheep. There was substantial agreement between the two methods results, $\kappa = 0.793$ (95% CI, 0.634 to 0.951), $p < .001$ (Table 2).

Table 1. Frequency of presence of *Cryptosporidium* spp. in sheep feces using the Modified Ziehl–Neelsen and auramine O staining methods.

positive samples	MZN staining	AO staining
Number of positive samples	17	25
Percentage of positive samples	6.5	9.6

Table 2. The symmetric measures demonstrate Cohen's kappa (κ), which is a statistic designed to consider the chance agreement.

	Value	Asymptotic Standard Error [a]	Approximate T[b]	Approximate Significance
Measure of Agreement	0.793	0.070	13.076	0.000
No. of Valid cases	260			

a. Not assuming the null hypothesis

b. Using the asymptotic standard error assuming the null hypothesis.

In a survey conducted by Ranjbar-Bahadori et al. (2011) on the samples of children and calves in Babol, Iran, the detection percentage of *Cryptosporidium* spp. was higher using AO staining compared to the Ziehl–Neelsen method; therefore, fluorescent staining was introduced as a more accurate method in both humans and calves (Ranjbar-Bahadori et al., 2011).

In this regard, Casemore et al. (1985) examined laboratory methods used to identify of *Cryptosporidium* spp. Generally, various methods are used for the detection of oocysts in the stool. To screen the oocysts of *Cryptosporidium* spp., they used auramine phenol staining and MZN techniques. Their results showed that auramine is significantly superior to the MZN technique (Casemore et al., 1985). Moreover, human studies have shown that fluorescent staining, with 100% sensitivity and 99% specificity, is one of the best available methods for this purpose. It is also a cost-effective and rapid method if the goal is not to identify the species, but to determine the parasite genotype (Khurana et al., 2012).

Additionally, in a previous survey, Khurana et al. (2012) evaluated the experimental methods used for identifying *Cryptosporidium* spp. in HIV/AIDS patients. The Ziehl–Neelsen (ZN) staining, auramine phenol staining, enzyme-linked immunosorbent assay (ELISA) for antigen detection, and polymerase chain reaction (PCR) methods were used to diagnose intestinal cryptosporidiosis. The results showed that auramine phenol is the best diagnostic tool, as it is a simple, highly sensitive, specific, and cost-effective fluorescent staining method, which is also less time-consuming than other techniques (Khurana et al., 2012).

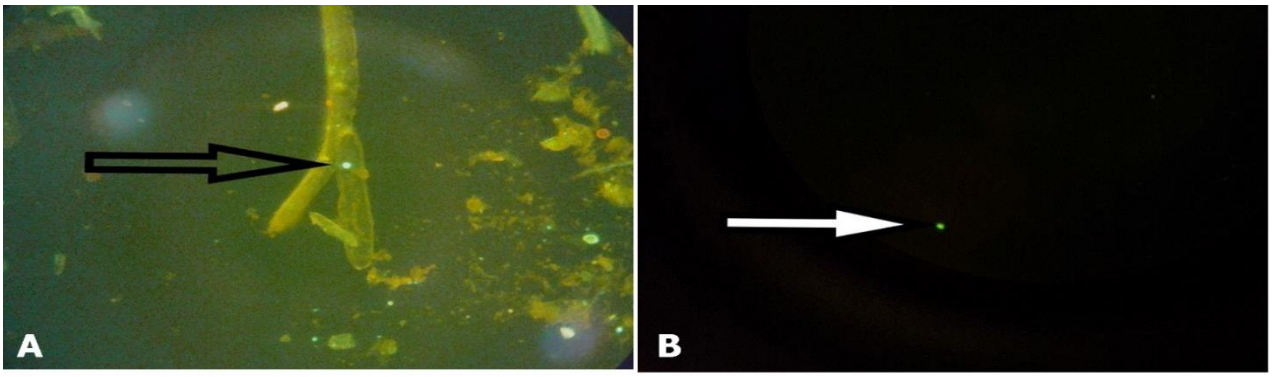

Fig. 1. A: The oocyst of *Cryptosporidium* spp. in sheep feces is observed by the auramine staining method (x400) at the arrow's tip (Original). **B:** The oocyst of *Cryptosporidium* spp. in human feces is observed by the auramine staining method (x400) at the arrow's tip (Original).

Oocysts are completely stained by ZN staining over five minutes, while they are resistant to discoloration by AO staining. Nevertheless, the AO staining method has some advantages over the ZN method, including faster performance and reading. In a study by Al-alousi et al. (2012) in rural areas around Mosul, north of Iraq, no significant differences were found between the AO and ZN staining techniques, and both were found to be specific for the diagnosis of *Cryptosporidium* spp. in fecal samples (Dubey et al., 1990; Taboada et al., 1993). Moreover, to examine the prevalence of *Cryptosporidium* spp., Quadros et al. (2006) evaluated the fecal samples of domestic animals using two staining methods, that is, AO and ZN staining. Based on their results, AO staining could detect more parasites than ZN staining (Taboada et al., 1993).

In the present study, there was substantial agreement between the two methods, while some experiments showed that AO staining is superior to identifying *Cryptosporidium* spp. However, in the

current study, the results obtained from the experiment on sheep feces samples yielded contradictory results. In other words, due to the presence of many fluorescent artifacts in the sheep feces (Figure 1A), unlike human feces (Figure 1B), *Cryptosporidium* detection rapidity decreases.

Conclusion

MZN staining is preferred to AO staining for investigating the prevalence of *Cryptosporidium* spp. in sheep flocks.

Acknowledgments

The idea of this research emerged from the data of thesis No. 96.40, registered at Lorestan University. The authors declare that there are no conflicts of interest.

Conflict of interest statements

The authors declare that there is no conflict of interests.

Ethical approval

Not applicable.

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