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Comparative Evaluation of Modified ZN Staining and Sandwich ELISA Based Detection of *Cryptosporidium* spp. Isolated from Cattle and Buffalo Calves in and around Kolkata

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Abstract

Apicomplexan protozoa *Cryptosporidium* species are responsible for significant gastrointestinal disease in humans and animals around the globe. Accuracy in the early diagnosis of cryptosporidiosis is crucial for managing the disease effectively and preventing its transmission. The study aimed to compare two methods for diagnosing *Cryptosporidium* in clinical samples: the modified Ziehl-Neelsen staining technique and the Sandwich Enzyme Linked Immunosorbent Assay. A total of 268 faecal samples from cattle and buffalo calves aged less than 3 months, showing symptoms of diarrhoea, were collected and examined. The faecal samples were processed initially by the modified Ziehl-Neelsen staining technique followed by Sandwich ELISA (Bio-X Diagnostics, SA) as per the manufacturer's protocol. The modified Ziehl- Neelsen technique and Sandwich ELISA exhibited 13 (4.85%) and 48 (17.91%) numbers of positive samples, respectively, among 268 samples. The outcome of this study displays the enhanced sensitivity of the sandwich ELISA method in detecting cryptosporidiosis compared to the modified Ziehl- Neelsen technique.

Keywords: *Cryptosporidium* spp., Cattle and buffalo calves, Modified ZN staining, Sandwich ELISA

Introduction:

Many species of *Cryptosporidium*, which are notable apicomplexan protozoan parasites, induce severe gastrointestinal disease in vertebrates and humans (Abeywardena et al., 2015). Cryptosporidiosis has emerged as the known cause of waterborne outbreaks of gastroenteritis, even in disinfected water resources (CDC, 2025). This is because the *Cryptosporidium* oocyst can resist chlorination and can survive for a prolonged period in the environment (Mittal et al., 2014). A total of 26 *Cryptosporidium* species, along with more than 70 genotypes, have been identified (Qi et al*.,* 2015). *C. parvum, C. andersoni,* and *C. bovis* have been identified as the most significant species affecting bovines (Mirhashemi et al., 2015). The *C. parvum* species presents a notable obstacle for profitable livestock farming and creates challenges for public health professionals (Kaupke and Rzezutka, 2015; Galuppi et al., 2016). This protozoan parasite was first discovered by Edward Ernest Tyzzer in 1907 in the small intestine of mice (Tyzzer, 1907), and the first human cryptosporidiosis was identified in 1976 (Nime et al., 1976; Meisel et al., 1976). Cryptosporidiosis in the bovine has been reported from different parts of the world with a 100% infection rate in some herds (Olson et al., 2004; Ayinmode and Fagbemi, 2010). The first recorded detection of *Cryptosporidium* oocysts in India occurred in Uttar Pradesh, utilising faecal samples obtained from buffaloes and zebu cattle (Dubey et al., 1992). Instances of cryptosporidiosis have been documented in Puducherry (Kumar et al., 2004), West Bengal (Roy et al., 2006), Karnataka (Rekha et al., 2016), and Punjab (Bhat et al., 2013).

In zoonotic cryptosporidiosis, cattle (neonatal calves) play as an important source of dissemination of infection (Preiser et al., 2003; Smith et al., 2004; Chalmers et al., 2005; Kiang et al., 2006; Xiao and Feng, 2008). The principal mode of transmission for the illness is the consumption of sporulated oocysts through contaminated feed and water (Amer et al., 2013). Infected or carrier animals can discharge significant amounts of oocysts (Romero-Salas et al., 2016), hence serving as a potential source of infection for susceptible populations. Clinical signs, including yellow-coloured, sometimes blood-tinged, profuse watery diarrhoea, are observed prominently (Fayer and Ungar, 1986).

The modified Ziehl-Neelsen staining technique is considered to be the 'gold standard' for the detection of *Cryptosporidium* spp. (OIE, 2008), whereas the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are also useful diagnostic tools (Cho et al., 2012; Mirhashemi et al., 2015).

Molecular methods like PCR can even diagnose as low as 1-2 oocyst(s) per sample (Hawash et al., 2015).

This study aims to assess the modified Ziehl-Neelsen (mZN) staining technique and coproantigen-based sandwich ELISA for the rapid detection of *Cryptosporidium* spp. These two methods were compared to assess the diagnostic sensitivity for their simplicity, reliability, and widespread applicability.

Materials and Methods:

Collection of faecal samples

A total of 268 diarrhoeic faecal samples were collected from 217 cattle calves and 51 buffalo calves, respectively, from November 2022 to May 2023. The faecal samples were collected directly from the rectum. After collection, the samples were divided into two parts and kept in sterile labelled Ziploc bags and stored at 4°C for further processing. One part was used for modified ZN staining, and the remainder for sandwich ELISA.

Modified Ziehl-Neelsen staining (mZN staining)

With the help of a toothpick, faecal smears were prepared on a clean, grease-free glass slide and left to air dry. The air-dried smears were fixed by absolute methanol for 5 minutes, and then these slides were held transiently on a flame and put to cool down. Concentrated carbol fuchsin was poured over the dried smear and allowed to stain for 20-30 minutes. The stained smears were then washed under running tap water. After that, the slides were destained with 1% acid alcohol solution (1% HCl in 70% absolute alcohol) for 15-30 seconds and washed immediately under running tap water. The destained slides were then counterstained with methylene blue for 5 minutes. These slides were washed under running tap water for 5 minutes and kept in a slanting position for air drying. The stained slides were observed under high-power (x40) illumination followed by oil immersion (x100) lens, respectively (Garcia et al., 1983).

The faecal samples were then tested using the sandwich ELISA method with the MonoscreenAg ELISA® kit for detecting *Cryptosporidium* spp., following the instructions provided by the manufacturer.

Results and Discussion:

Currently, multiple techniques exist for the identification of cryptosporidiosis in diverse clinical specimens; however, the method suitable for routine screening of faecal samples from diarrhoeal cases must demonstrate acceptable sensitivity and specificity while delivering clinically pertinent, cost-effective, and prompt results, especially in areas susceptible to waterborne diseases (Mittal et al., 2014). After modified Ziehl–Neelsen staining, the oocysts appeared as bright red stained against a blue background, round to oval structures containing distinct internal structures (Figure 1). A total of 13 (4.85%) samples were found to be positive by microscopy. Previously a very high prevalence rate, i.e., 20.9%, was found positive for oocysts of *Cryptosporidium* species from West Bengal (Bhanja et al., 2023). *Cryptosporidium* has been shown to be prevalent in dairy cattle worldwide, with a prevalence of 7.1% in cattle in Egypt (Mahfouz et al*.,* 2014), 10.2% in dairy cattle in England and Wales (Smith et al*.,* 2014), and between 10.7% and 41.5% in dairy calves in Brazil, India, France, and Ethiopia (Meireles et al*.,* 2011; Venu et al*.,* 2012; Delafosse et al*.,* 2015; Wegayehu et al*.,* 2016). The low prevalence of *Cryptosporidium* in cattle compared with other studies may be attributed to differences in the methodology used for detection of *Cryptosporidium,* which could partially explain the discrepant results (Inpankaew et al*.,* 2017). Additionally, the overall low prevalence found in this study, compared to other studies from different areas, suggests that cattle might get infected by whatever type of *Cryptosporidium* is available in the specific locations where each study took place.

A coproantigen-based sandwich ELISA revealed 48 (17.91%) faecal samples were found positive for *Cryptosporidium* oocysts. The sandwich ELISA revealed a significantly higher number of *Cryptosporidium* oocysts. All positive samples from the modified acid-fast stain were also positive by sandwich ELISA. Among the two methods employed in the present study, sandwich ELISA was found to be the most sensitive, i.e., 17.91% (48/268), in comparison to modified ZN staining, 4.85% (13/268). This observation was in agreement with the findings of Mirhashemi et al. (2015), clearly highlighting the lack of sensitivity of direct smear examination in detecting *Cryptosporidium* oocysts. Vastert et al. (2025) compared different diagnostic methods for the detection of *C. parvum* in faeces in both acute and chronic diarrhoeic calves and found that the sensitivities of microscopic detection, Crypto-Strip, and ELISA were 37%, 78%, and 71%, respectively. Radfar et al. (2013) concluded that capture ELISA was more efficient than the mZN technique for detecting *C. parvum* in faecal samples. Conversely, Mittal et al. (2014) revealed that stool microscopic modified acid-fast staining exhibits more sensitivity than ELISA for detecting *Cryptosporidium* in stool samples; nevertheless, ELISA demonstrated superior specificity compared to microscopy. A commercially available ELISA kit (Rajkhowa et al., 2006) was satisfactory for detecting cryptosporidiosis in mithun. Recently in Kuwait, Abdou et al. (2022) reported that 15.25% of cattle were suffering from cryptosporidiosis as detected by ELISA. In comparison to the mZN technique, dipstick ELISA kits offered the benefits of reduced time consumption and ease of execution, eliminating the need for an ELISA

microplate reader or other specialist apparatus. The superior performance of sandwich ELISA can be linked to its mechanism of detection, which binds both the capture and detection antibodies to *Cryptosporidium* oocyst antigen. This higher detection rate even at lower antigen concentrations can be attributed to this dual binding of antigens. One probable cause for the contrast in detection rates could be the arbitrariness in oocyst shedding patterns in collected samples. It can be that the quantity of *Cryptosporidium* oocysts shed in some samples might be less than the limit of mZN staining but still within the sensitivity range of sandwich ELISA. The inference of this study is significant concerning upgrading the sensitivity of *Cryptosporidium* diagnosis. The superior sensitivity of sandwich ELISA can be notably useful in patients having low-level infections, thus leading to better management of the morbid populations, earlier medical interventions, and eventually reducing or breaking the transmission cycle of the pathogen.

Conclusion:

This study shows that sandwich ELISA is better than modified ZN (mZN) staining at finding *Cryptosporidium* oocysts. Further surveys could be done to explore the commercial and practical application of sandwich ELISA in a larger clinical population. The study's illations are expected to help better the decision-making in clinical settings, resource allocation, and the expansion of a more effective blueprint for *Cryptosporidium* diagnosis and surveillance, which in turn will provide an upgrade to overall disease control measures and improve public health outcomes.

Conflicts of interest:

Authors declare no conflict of interest for this investigational report.

Ethical approval:

Authors maintained all ethical concern during sample collection and do not require IAEC certificate as it's not experimental.

Contributions:

All the authors equally participated in designing, data analysis and interpreting the results, drafting, editing the manuscript and approved the final version of the manuscript.

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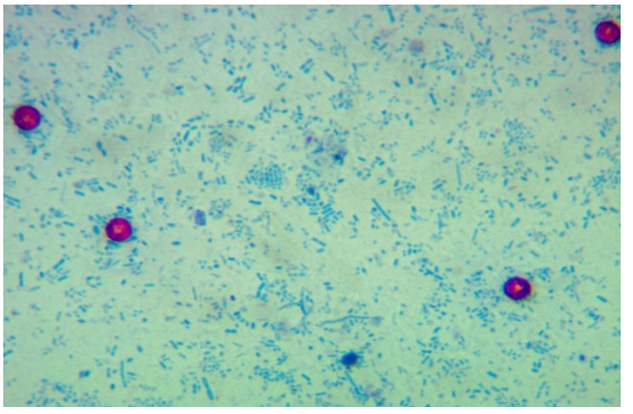

Figure 1: Microscopic view of *Cryptosporidium* oocysts in modified Z-N stain under (x100)

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