Brief Report

Comparative Sensitivity Between Fecal Sedimentation And Fecal Antigen Elisa For Diagnosis Of Fasciola Infection In Cattle

Naim Che-Kamaruddin * and Nur Mahiza Md Isa

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Comparative Sensitivity between Fecal Sedimentation and Fecal Antigen Elisa for Diagnosis of *Fasciola* Infection in Cattle

Naim Che-Kamaruddin [1,2,*] and Nur Mahiza Md Isa [2,*]

[1] Tropical Infectious Diseases Research and Education Centre (TIDREC), Higher Center of Excellence (HICoE), Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[2] Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia
* Correspondence: naimck@um.edu.my (N.C.-K.); nurmahiza@upm.edu.my (N.M.M.I.)

Abstract: The detection of *Fasciola* eggs in ruminant fecal samples typically occurs around 15 weeks post-infection during patent infections. The recent introduction of coproantigen ELISA (cELISA) diagnostics, which capture antigenic enzymes in fecal material, holds promise for monitoring *Fasciola* fecal egg count (FFEC) to implement control programs, particularly targeted treatments. This study compared two study groups, those with positive FFEC (FFEC+ve) and negative FFEC (FFEC-ve), to assess the sensitivity of Flukefinder® sedimentation and a cELISA kit. This study reports that the coproantigen ELISA detection limit was 4.5 eggs per gram (epg) for a 100% positivity rate. Additionally, a moderate, statistically significant positive correlation (r^2=0.716, p-value < 0.01) and an odds ratio of 1.96 (p-value < 0.01) were observed between FFEC and *Fasciola* coproantigen from cELISA. This implies that for every single unit increase in FFEC, there is a 96% likelihood of obtaining a higher optical density (OD) reading from the cELISA test. The results suggest that cELISA can complement fecal sedimentation tests, serving as a valuable tool for detecting cattle with high FFEC. This capability facilitates targeted treatment strategies, thereby aiding in the control and prevention of the spread of fasciolosis within the studied area.

Keywords: coproantigen ELISA; farm; liver fluke; parasite egg count

1. Introduction

Fasciolosis is an emerging neglected tropical disease that has a significant detrimental impact on ruminant livestock production [1,2]. Fasciolosis is typically diagnosed coproscopically such as fecal sedimentation to detect the *Fasciola* eggs. However, the *Fasciola* eggs can only be detected during patent infection period which is from 15 weeks after ingesting *Fasciola* metacercariae [1]. Other diagnostic techniques includes serological assays of host IgG antibody response, which is less accurate to diagnose active infection as the IgG remains detected for months even after successful treatment. Abattoir examinations are conducted post-mortem, which have limitations in early detection as infections are identified only after the animal has been slaughtered. This delay enables the *Fasciola* to persist and spread within the herd before preventive measures can be implemented. However, the abattoir examinations can still provide crucial insights into the extent of the infection, tissue damage, and other pathological changes [2].

Coproantigen enzyme-linked immunosorbent assay (cELISA) has emerged as a diagnostic method for detecting fasciolosis in ruminants over the past decade. This cELISA captures metabolic antigens produced by newly excysted juvenile (NEJ) and adult *Fasciola* [1], which has demonstrated the effectiveness by offering a high sensitivity and specificity for fasciolosis diagnosis in sheep and cattle [3]. The primary antigen released by *Fasciola* is cathepsins-L, which, upon interaction with the host's digestive enzymes and acids, can be circulated and subsequently retained in the biliary system [1,3] Additionally, cathepsin-L can be detected even during the pre-patent period with a low fluke burden, as low as two flukes [1].

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Fasciolosis surveillance utilizing cELISA is widely employed for detecting *Fasciola hepatica* in temperate regions [3–6], while its application for detecting *F. gigantica* is comparatively limited [4]. Generally, the sensitivity of detection period for both *Fasciola* species via cELISA is similar, occurring approximately four to seven weeks before eggs become detectable in sheep (six to nine weeks post-infection (WPI) for *F. hepatica* and seven to eleven WPI for *F. gigantica*) [4]. During the patent infection, the eggs passed to the environment upon defaecation by *Fasciola*-infected animals and sustain the fasciolosis occurrences within the area. Therefore, early and accurate detection of fasciolosis in ruminant livestock is crucial for controlling the spread and reducing the economic loss due to the infection [7]. Hence, the aim of the present study was to compare the sensitivity between fecal sedimentation and fecal antigen ELISA (cELISA) for the diagnosis of fasciolosis in free-grazing cattle within an area endemic to *F. gigantica*.

2. Materials and Methods

2.1. Fecal sample

Detailed description on the sample collection work is described in a previous study **[**8**]**. Briefly, ruminant fecal samples were collected for fasciolosis surveillance in farms in Taiping, Malaysia. The sampling was conducted from February to August 2020. To ensure precision, the inclusion criteria for the present analysis were exclusively tailored to encompass samples from cattle. This selection was guided by adherence to the manufacturer's protocol, as the commercial cELISA kit utilized in this study had been specifically optimized for fecal samples from only sheep and cattle.

A total of 92 fecal samples were randomly selected through an Excel datasheet and included in the present study. The samples were categorized into two study groups based on *Fasciola* positivity, determined through Flukefinder® fecal sedimentation. Group FFEC+ve comprised 46 samples where *Fasciola* eggs were detected, while FFEC-ve group included 46 samples where *Fasciola* eggs were not detected.

2.2. Preparation of fecal supernatant for cELISA

Fecal supernatants were prepared at a ratio of 1:1 in ProClin® 300 (Sigma-Aldrich, CAS#55965-84-9), using 2 g of each cattle fecal sample. To ensure homogeneity and prevent clumping, fecal samples were thoroughly vortexed before incubating overnight at 4°C. Following incubation, the suspension was thawed to room temperature for 30 minutes, vortexed, and subsequently centrifuged for 10 minutes at 1,000 x g (RCF) to yield the supernatant. A volume of 500 µL of the supernatant from all sample were aliquoted and stored at -20°C for future use. The prepared supernatant remains viable for *Fasciola* coproantigen detection for up to six weeks [5].

2.3. Coproantigen ELISA analysis

In the present study, a commercial semi-quantitative coproantigen ELISA kit (MM3-COPRO-BIOK 201, Bio-X Diagnostics, Jemelle, Belgium) was employed. The analysis was performed in accordance with the manufacturer's instructions. This indirect sandwich cELISA kit utilizes two distinct coatings on alternate strips, consisting of monoclonal and polyclonal antibodies, to minimize false-positive results. The kit provided an avidin-peroxidase conjugate, a highly sensitive agent for detecting *Fasciola* coproantigen, higher sensitivity of identifying one NEJ or adult *Fasciola* at minimum [9]. The second conjugate in this cELISA was the MM3 monoclonal antibody (mAb), recognized as the most sensitive and specific among available mAbs for binding with *Fasciola* coproantigen [1]. A volume of 100 µL of the supernatant was loaded into both monoclonal and polyclonal antibody-coated wells, with samples duplicated for result averaging. The provided reference *Fasciola* antigen was used as a positive control, while deionized distilled water served as the blank control to assess the extent of nonspecific binding in the procedure.

Optical densities (OD) were measured at a wavelength of 450 nm, and the cut-off for positivity value was determined using the quality control (QC) datasheet supplied with the kit. An OD exceeding 8 was considered as a positive result.

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2.4. Statistical analysis

The Kolmogorov-Smirnov test was applied to analyze the raw data, indicating a non-normal distribution of values. The obtained OD results for the tested samples underwent one-way ANOVA analysis to determine the total variance and coefficient of variation (CV) for each sample, with the results expressed as a percent CV. The potential association between the OD values obtained with cELISA and the *Fasciola* fecal egg count (FFEC) was assessed through Spearman's rank correlation. The association is considered statistically significant when the p-value < 0.01. All statistical analyses were carried out using R statistical software (version 1.3.1073).

3. Results

The cELISA optical density (OD) for the blank controls and polyclonal antibody coated wells in all microplates in the present study remained below the cut-off value. In contrast, positive controls using the crude *Fasciola* antigen exhibit above the cut-off value indicating that the OD readings analyzed in this study were validated.

The range of *Fasciola* eggs from the FFEC+ve samples in the present study varied from 1 egg per 2 grams (0.5 egg per gram, epg) to 113 eggs per 2 grams (56.5 epg). The median value of 2 epg was observed, indicating a skewed distribution of epg in the samples obtained from free-grazing cattle in the present study.

Figure 1 illustrates the linear correlation between *Fasciola* fecal egg count (FFEC) and the concentration of *Fasciola* coproantigen, as measured by the optical density (OD) readings in the cELISA. A statistically significant moderate positive correlation (r^2=0.716, p-value < 0.01) was observed, indicating that higher egg counts are associated with higher OD values. The FFEC+ve group demonstrated a lower limit of detection (LoD) for the cELISA, set at approximately 4.5 epg. Samples with more than 4.5 epg consistently exhibited a 100% positivity rate. The highest observed OD value corresponded to the highest egg count in this study, reaching 113 eggs per 2 grams (56.5 epg). This finding emphasizes the sensitivity of the cELISA method in detecting *Fasciola* antigens, particularly in samples with higher egg counts.

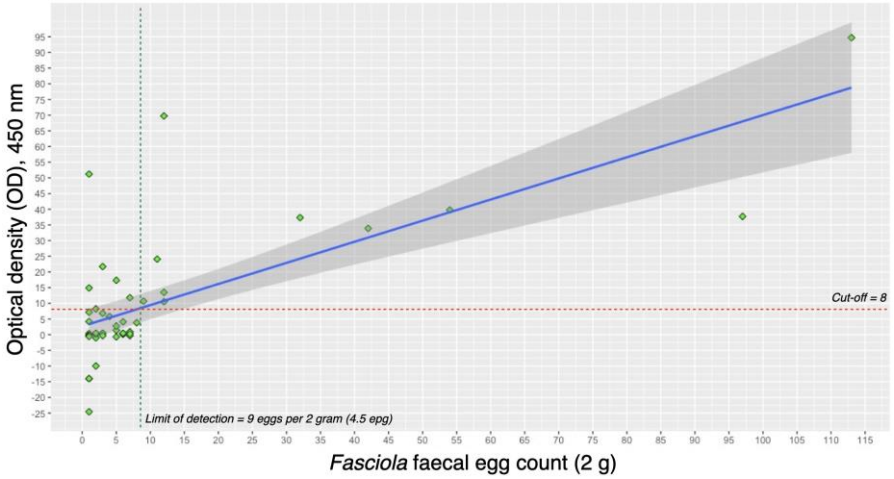

Figure 1. Linear correlation depicting the association between *Fasciola* fecal egg count (FFEC) and coproantigen concentration levels, measured through optical density (OD-cELISA), in the FFEC+ve group. The correlation coefficient was moderately statistically significant (r^2=0.716, p-value < 0.01). The limit of detection for 100% positivity in cELISA from samples in this study was determined at 4.5 eggs per gram (epg). Dashed red line indicates the cut-ff value (8).

Descriptive overview of the Flukefinder® and cELISA results is presented in Table 1 and Figure 2. Out of 46 samples from the FFEC+ve group, 36 exhibited 4.5 epg or fewer, while 10 had more than 4.5 epg. Among the samples with 4.5 epg or fewer, only 6 (16.7%) tested positive through cELISA. In

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contrast, all samples with more than 4.5 epg showed a positive result with cELISA. The range of optical density (OD) values from cELISA-positive samples varied from 11 to 94, with a median OD value of 34.

Within the FFEC-ve group, 5 samples (10.87%) tested positive for *Fasciola* coproantigen. Notably, the range of coproantigen, as indicated by the optical density (OD) readings, in these 5 samples varied from 11 to 39 OD values, with a median OD value of 16.

Table 1. Groups of fecal samples categorized according to Flukefinder® results, for a comparative analysis of *Fasciola* fecal egg count (FFEC) and the concentration of *Fasciola* antigen based on cELISA. The odds ratio (OR) of cELISA positivity to FFEC is presented.

Group, based on Flukefinder®	Frequency of positivity from Flukefinder® (%)	Frequency of positivity from cELISA (%)	Odd ratio of positivity in cELISA
Total fecal samples (N=92)	46 (50.0%)	21 (22.8%)	1.96 (p-value < 0.01)
FFEC+ve (n=46)	46 (100.0%)	16 (34.8%)	-
- *≤ 4.5 epg (Range: 0.5 – 4.5 epg)*	*36 (100.0%)*	*6 (16.7%)*	-
- *> 4.5 epg (Range: 5.5 – 56.5 epg)*	*10 (100.0%)*	*10 (100.0%)*	-
FFEC-ve (n=46)	0 (0.0%)	5 (10.9%)	-

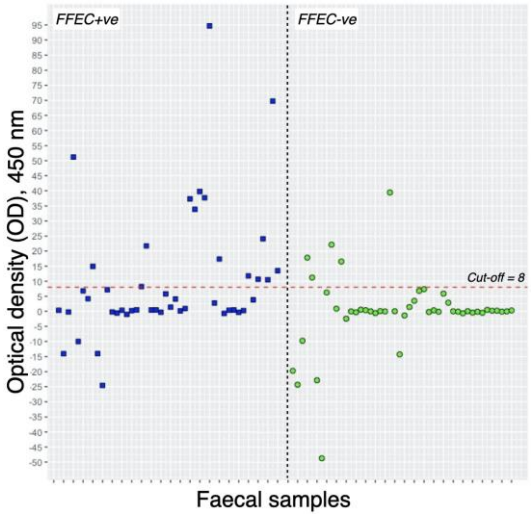

Figure 2. Scatter plot of cELISA results between samples detected with *Fasciola* eggs from Flukefinder® (FFEC+ve) and vice versa (FFEC-ve). Dashed red line indicates the cut-ff value (8).

4. Discussion

Early detection of fasciolosis in ruminant livestock is crucial for effective parasite control and management [9,10]. The present study aimed to compare the sensitivity of two diagnostic methods, fecal sedimentation and fecal antigen ELISA (cELISA) for the diagnosis of fasciolosis in free-grazing cattle within an area endemic to *F. gigantica*. The exploration of this correlation has the potential to enhance the understanding of the applicability of coproantigen ELISA in monitoring parasite egg counts. This information is vital for preventing the spread of fasciolosis, as untreated animals contribute to continuous environmental contamination with *Fasciola* eggs, especially in free-ranging animals with a higher prevalence [8].

Out of the total FFEC+ve group, only 16 samples tested positive in cELISA. The present study delves into the distribution of *Fasciola* eggs per gram (epg), revealing that samples with more than 4.5 epg demonstrated a 100% positivity rate compared to those with fewer epg. The 30 fecal samples

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undetected with *Fasciola* coproantigen, despite the presence of *Fasciola* eggs, could be attributed to the absence of active infection of newly emerged juveniles (NEJ) and adult flukes [1]. Additionally, the retention of eggs after parasite elimination through treatment is common, leading to false positives in coproscopic examinations such as Flukefinder® [10]. Coproantigen ELISA proves to be a more sensitive diagnostic method compared to coproscopic examination [1,4], detecting antigens from *Fasciola* during active infection. With the lowest detection limit observed at 4.5 epg in the present study, it is noteworthy that these 30 samples were only documented with less than 4 epg.

Other possible justification is that due to the skewed distribution of egg counts, a phenomenon commonly observed in naturally infected animals. In naturally infected populations, there is often a wide range of egg counts among individual animals [10–12], with some having very low counts and others having significantly higher counts. This variability can be influenced by several factors, including the stage of infection, host immune response, and individual variations in susceptibility [10,11]. The skewed distribution implies that animals in the sample population may have low egg counts, falling below the detection limit of the coproantigen test. The sensitivity of cELISA is limited in detecting very low-level infections, especially when the concentration of antigen in the feces is below the assay's threshold [1]. Thus, this natural variability in egg distribution highlights the complexity of diagnosing parasitic infections in field settings. It underscores the importance of considering the limitations of diagnostic methods and interpreting results in the context of the specific characteristics of the study population. Future research could explore alternative or complementary diagnostic approaches to improve sensitivity, especially in situations where egg distribution is skewed.

As the moderate positive correlation between the diagnostic tests, the estimation of *Fasciola* coproantigen concentration through cELISA proves to be a reliable predictor of egg counts, aligning with previous findings [1], which is consistent with the correlation observed in the present study. Furthermore, the elevation of *Fasciola* coproantigen correlates with egg counts in naturally infected animals [5]. The examination of the odds ratio between *Fasciola* fecal egg count (FFEC) and the optical density (OD) correlation yielded a value of 1.96 (CI: 1.61 ± 2.38, p-value < 0.01). This indicates that for every single unit increase in FFEC, there is a 96% likelihood of obtaining a higher OD reading from the coproantigen ELISA test. The odds ratio underscores that *Fasciola* coproantigen in the samples can serve as an estimate for the egg count.

5. Conclusions

The present study reveals a moderate positive correlation between *Fasciola* coproantigen concentration and fecal egg count (p-value < 0.01), offering a promising diagnostic tool for cattle fasciolosis. The establishment of a lowest detection limit of at least 4.5 eggs per gram in cELISA resulted in 100% positivity, providing a reliable threshold for identifying active *Fasciola* infection. This finding suggests that the application of cELISA holds potential for monitoring the distribution of parasite eggs and identifying cattle with a high parasite egg burden. Such targeted identification can facilitate precise treatment strategies, thereby contributing to the effective control and limitation of the spread of fasciolosis.

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Institutional Review Board Statement: The work described in this study involved the use of non-experimental animal and non-invasive sampling. The fecal samples collected were non-invasive and non-painful procedures following clearance number UPM/IACUC/AUP-007/2019.

Data Availability Statement: The raw datasets supporting this article's findings are available from NCK upon written request. Please send a request to naimck@um.edu.my to request access to the data.

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Conflicts of Interest: The authors declare no conflicts of interest.

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