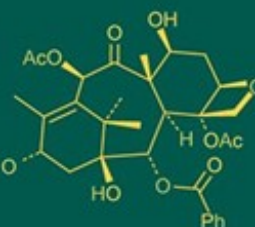
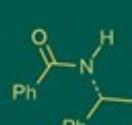


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## Identification and characterization of a *Fasciola*-specific excretory-secretory antigen for development of an antigen-based ELISA for detection of ovine fasciolosis

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### ABSTRACT

Fasciolosis is an important helminthic disease of sheep and other domestic ruminants and is associated with hepatic damage, reduced productivity and considerable economic losses. Conventional diagnosis based on faecal examination has certain limitations, particularly during the prepatent stage of infection, and may consequently underestimate the actual prevalence of infection. The present study was undertaken to develop and evaluate a sandwich enzyme-linked immunosorbent assay (ELISA) for detection of circulating *Fasciola* antigen in sheep serum. Excretory-secretory (ES) products obtained from adult *Fasciola* were used for preparation of specific antibodies employed in the assay. The sandwich ELISA was standardized by optimizing the concentrations of capture antibody, detection antibody and serum dilution. A total of 147 serum samples were collected from sheep presented at selected livestock farms and slaughter facilities during the study period. Based on the reference diagnostic examination, 38 samples were classified as positive for *Fasciola* infection, whereas 109 were classified as negative. The developed ELISA detected circulating *Fasciola* antigen in 35 of the 38 positive samples, while 7 of the 109 negative samples showed positive reactions. The assay demonstrated a sensitivity of 92.1%, specificity of 93.6% and overall diagnostic accuracy of 93.2%. The findings indicate that the developed sandwich ELISA has potential as a useful immunodiagnostic assay for detection of ovine fasciolosis and may complement conventional parasitological methods.

**Keywords:** *Fasciola*, Ovine fasciolosis, Circulating antigen, Sandwich ELISA, Sheep, Immunodiagnosis, Excretory-secretory antigen

### 1. Introduction

Fasciolosis is an economically important trematode infection affecting sheep and other domestic ruminants. The disease is caused by liver flukes belonging to the genus *Fasciola*, with *Fasciola gigantica* being an important species in tropical and subtropical regions, including India. Infection with *Fasciola* may result in hepatic tissue damage, impaired animal health, reduced productivity, poor body condition and condemnation of affected livers at slaughter. The importance of fasciolosis in livestock production and the associated economic losses have been well documented (Mas-Coma *et al.*, 2005; Mas-Coma *et al.*, 2009) [1, 2].

Diagnosis of fasciolosis is commonly performed by demonstration of *Fasciola* eggs in faecal samples. Although faecal examination remains an important diagnostic approach, it has several limitations. Eggs are generally detected only after maturation of the parasite and the onset of egg production. Consequently, animals harbouring immature infections may remain undiagnosed. Intermittent egg shedding and variation in egg output can further influence the sensitivity of conventional coprological examination. Immunological methods have consequently been explored as complementary approaches for the diagnosis of fasciolosis in domestic ruminants, including sheep (Farrell *et al.*, 1981; el-Aziz *et al.*, 2001) [4, 3].

Detection of parasite-derived circulating antigens represents an alternative approach because the presence of parasite antigen in the host may provide an indication of an active infection. Circulating *Fasciola* antigens have been detected in experimentally and naturally infected animals, and antigen-detection assays have demonstrated potential for diagnosis before the infection becomes readily detectable by conventional faecal examination (Fagbemi *et al.*, 1997; Velusamy *et al.*, 2004) [5, 6].

Excretory-secretory products released by adult *Fasciola* contain several parasite-derived molecules that can be utilized for development of immunodiagnostic reagents. Excretory-secretory antigen has previously been evaluated in ELISA-based diagnosis of *F. gigantica* infection in different domestic ruminants, including cattle and buffaloes, with encouraging diagnostic performance (Krishna Murthy *et al.*, 2015) [7]. Similarly, studies comparing different antigen preparations have reported that ES antigen can provide useful sensitivity and specificity for serodiagnosis of *F. gigantica* infection (Hussein *et al.*, 2008) [8].

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A sandwich ELISA utilizes two antibodies directed against the target antigen. The capture antibody immobilized on the ELISA plate binds the parasite antigen present in the test sample, while a second enzyme-labelled antibody recognizes the captured antigen. This format has been successfully employed for detection of circulating *F. gigantica* antigens, including tegumental proteins, paramyosin and cysteine proteases (Anuracpreeda *et al.*, 2009; Hassan *et al.*, 2013; Anuracpreeda *et al.*, 2016) [9, 10].

Previous studies have also demonstrated that circulating antigen detection may permit earlier diagnosis than antibody-based ELISA. Velusamy *et al.* (2004) [6] detected a 54-kDa circulating antigen in experimentally infected bovine calves using sandwich ELISA, while Anuracpreeda *et al.* (2016) [10] developed a sandwich ELISA for circulating cathepsin L1 antigen with high diagnostic performance in naturally infected cattle. Although these studies were performed in other hosts, they provide an important basis for exploring antigen detection in sheep.

Therefore, the present study was undertaken to develop and standardize a *Fasciola*-specific sandwich ELISA for detection of circulating antigen in sheep serum and to evaluate its diagnostic performance under field conditions.

## 2. Materials and methods

### 2.1 Study population and serum sample collection

The study was conducted on sheep presented at selected livestock farms and slaughter facilities during the study period. A total of 147 blood samples were collected from sheep selected for inclusion in the study. Blood samples were collected aseptically from the jugular vein using sterile disposable syringes and transferred into sterile clot-activator tubes.

The blood samples were transported to the laboratory under chilled conditions and allowed to clot. Serum was separated by centrifugation at approximately  $3,000 \times g$  for 10 min. The separated serum was transferred into sterile microcentrifuge tubes and stored at  $-20^{\circ}\text{C}$  until further analysis. Repeated freeze-thaw cycles were avoided to minimize degradation of serum components.

### 2.2 Reference diagnosis

The infection status of the animals was established using the reference diagnostic examination employed in the study. Based on the reference findings, 38 serum samples were classified as *Fasciola*-positive and 109 samples were classified as negative.

The reference diagnosis was established independently of the sandwich ELISA results to allow evaluation of the diagnostic performance of the developed assay. Previous studies evaluating *Fasciola* ELISA assays have similarly used parasitological or post-mortem examination as a reference for determining diagnostic sensitivity and specificity (Hussein *et al.*, 2008; Krishna Murthy *et al.*, 2015) [8, 7].

### 2.3 Collection of adult *Fasciola*

Adult *Fasciola* flukes were collected from infected sheep livers obtained from slaughter facilities. The livers were examined carefully, particularly the bile ducts and gall bladder, for the presence of adult flukes. Recovered parasites were washed several times with sterile phosphate-buffered saline (PBS, pH 7.2–7.4) to remove adhering blood, bile and other host-derived material.

The washed parasites were subsequently used for preparation of excretory-secretory antigen. The use of adult *Fasciola* recovered from infected animal livers for preparation of diagnostic antigen has previously been described by several investigators (el-Aziz *et al.*, 2001; Krishna Murthy *et al.*, 2015) [3, 7].



**Figure 1.** Image showing damaged and inflamed sheep liver after post mortem of dead sheep

### 2.4 Preparation of excretory-secretory antigen

Freshly recovered adult *Fasciola* flukes were incubated under suitable in vitro conditions in sterile physiological medium for collection of excretory-secretory products. Following incubation, the medium containing the secreted products was collected and centrifuged to remove parasite debris and other particulate material.

The clarified supernatant was collected and concentrated using an appropriate protein concentration method. Protein concentration was estimated using a standard protein estimation assay. The prepared excretory-secretory antigen was aliquoted and stored at  $-20^{\circ}\text{C}$  until further use.

Excretory-secretory products have previously been used as diagnostically relevant antigenic preparations for fasciolosis, including in ELISA-based studies (Hussein *et al.*, 2008; Krishna Murthy *et al.*, 2015) [8, 7].

### 2.5 Preparation of *Fasciola*-specific antibodies

Specific antibodies against *Fasciola* excretory-secretory antigen were prepared using an appropriate immunization protocol. Following completion of the immunization schedule, serum containing anti-*Fasciola* antibodies was collected and separated.

The antibody preparation was evaluated for its reactivity against *Fasciola* antigen and subsequently used as the capture antibody in the sandwich ELISA. A portion of the antibody preparation was conjugated with horseradish peroxidase (HRP) and used as the detection antibody.

The use of parasite-specific antibodies for capture and detection of circulating *Fasciola* antigen has been demonstrated in several sandwich ELISA systems (Fagbemi *et al.*, 1997; Viyanant *et al.*, 1997; Anuracpreeda *et al.*, 2009) [5, 11, 9].

### 2.6 Development and optimization of sandwich ELISA

The sandwich ELISA was standardized using high-binding 96-well microtitre plates. The concentration of capture antibody, serum dilution and concentration of HRP-conjugated detection antibody were optimized by checkerboard titration. The optimum conditions were selected on the basis of maximum discrimination between positive and negative samples and minimum background reactivity.

For the illustrative assay, the optimized concentration of capture antibody was  $2\text{ }\mu\text{g/mL}$ , the serum was used at a dilution of 1:2 and the HRP-conjugated detection antibody was used at a concentration of  $1\text{ }\mu\text{g/mL}$ . These values should be replaced with the experimentally determined concentrations in the final manuscript if different experimental values were actually used.

Checkerboard optimization of antigen, serum and conjugate concentrations has previously been used in ELISA-based studies of fasciolosis (Latchumikanthan *et al.*, 2013) [12].

## 2.7 Sandwich ELISA procedure

The wells of a high-binding 96-well microtitre plate were coated with 100  $\mu$ L of *anti-Fasciola* capture antibody prepared in carbonate-bicarbonate coating buffer. The plate was incubated overnight at 4 °C. Following incubation, the wells were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST). Each well was then blocked with 200  $\mu$ L of 1% bovine serum albumin prepared in PBS and incubated for 1 h at 37 °C. The plate was subsequently washed with PBST.

One hundred microlitres of appropriately diluted sheep serum was added to the respective wells. Positive control, negative control and blank wells were included on each plate. The plate was incubated for 1 h at 37 °C to allow binding of circulating *Fasciola* antigen to the immobilized capture antibody.

Following incubation, the wells were washed three to five times with PBST to remove unbound serum components. Subsequently, 100  $\mu$ L of HRP-conjugated *anti-Fasciola* detection antibody was added to each well and the plate was incubated for 1 h at 37 °C.

The wells were again washed thoroughly with PBST to remove unbound conjugate. Subsequently, 100  $\mu$ L of tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated in the dark for 10–15 min at room temperature. Development of a blue colour indicated the presence of detectable *Fasciola* antigen.

The enzymatic reaction was stopped by addition of 50  $\mu$ L of stop solution, resulting in a change of colour from blue to yellow. The optical density was measured at 450 nm using an ELISA microplate reader.

The overall principle follows previously reported sandwich ELISA approaches for detection of circulating *F. gigantica* antigens in experimentally and naturally infected animals (Viyant et al., 1997; Velusamy et al., 2004; Anuracpreeda et al., 2016) [11, 6, 10].

## 2.8 Determination of cut-off value

The diagnostic cut-off value was established using the optical density values obtained from the reference-negative serum samples. The cut-off was calculated as the mean optical density of the negative samples plus three standard deviations.

Samples producing optical density values above the established cut-off were considered positive for circulating *Fasciola* antigen, whereas samples with values below the cut-off were considered negative.

Determination of an appropriate cut-off is an important component of ELISA standardization because the selected threshold directly influences diagnostic sensitivity and specificity (Hussein et al., 2008; Anuracpreeda et al., 2016) [8, 10].

## 2.9 Evaluation of diagnostic performance

The diagnostic performance of the developed sandwich ELISA was evaluated by comparison with the reference infection status of the sheep. Sensitivity, specificity, positive predictive value, negative predictive value and overall diagnostic accuracy were calculated using standard diagnostic evaluation procedures.

## 3. Results

### 3.1 Standardization of sandwich ELISA

Optimization of the sandwich ELISA resulted in clear differentiation between the reference-positive and reference-negative serum samples. The selected combination of capture antibody, serum dilution and HRP-conjugated detection antibody provided satisfactory discrimination with comparatively low background reactivity.

The optimized assay was subsequently used for evaluation of the 147 sheep serum samples. Similar optimization procedures have previously been applied in *Fasciola*-specific ELISA studies to establish suitable antigen, serum and conjugate concentrations (Latchumikanthan et al., 2013) [12].

### 3.2 Detection of circulating *Fasciola* antigen

Among the 147 serum samples examined, 38 were classified as positive for *Fasciola* infection by the reference diagnostic examination, whereas 109 were classified as negative.

Of the 38 reference-positive samples, 35 produced ELISA values above the established cut-off, while 3 samples were negative by the sandwich ELISA. Among the 109 reference-negative samples, 102 were negative by ELISA and 7 produced positive reactions.



**Figure 2.** Image showing *Elisa* plate referring the positive samples (Lane 1 showing positive sera and Lane 4, 9, 11 showing positive test samples)

**Table 1.** Distribution of sandwich ELISA results according to reference diagnosis

| Reference diagnosis  | ELISA positive | ELISA negative | Total |
|----------------------|----------------|----------------|-------|
| Fasciolosis positive | 35             | 3              | 38    |
| Fasciolosis negative | 7              | 102            | 109   |
| Total                | 42             | 105            | 147   |

The sandwich ELISA therefore identified 35 true-positive samples, 102 true-negative samples, 7 false-positive samples and 3 false-negative samples.

The sensitivity of the assay was 92.1%, while the specificity was 93.6%. The positive predictive value was 83.3%, negative predictive value was 97.1% and overall diagnostic accuracy was 93.2%.

**Table 2.** Diagnostic performance of the developed sandwich ELISA

| Diagnostic parameter      | Value (%) |
|---------------------------|-----------|
| Sensitivity               | 92.1      |
| Specificity               | 93.6      |
| Positive predictive value | 83.3      |
| Negative predictive value | 97.1      |
| Diagnostic accuracy       | 93.2      |
| False-positive rate       | 6.4       |
| False-negative rate       | 7.9       |

The diagnostic performance observed in the present study is within the range reported for several antigen- and antibody-based immunodiagnostic assays for fasciolosis. Previous sandwich ELISA studies targeting specific *F. gigantica* antigens have reported high diagnostic sensitivity and specificity in experimentally and naturally infected animals (Hassan et al., 2013; Anuracpreeda et al., 2016) [10].

**Table 3.** Comparison of the developed assay with selected published *Fasciola* ELISA assays

| Study                      | Host   | Assay format   | Target/antigen                          | Sensitivity (%) | Specificity (%) |
|----------------------------|--------|----------------|-----------------------------------------|-----------------|-----------------|
| Viyant et al. (1997)       | Cattle | Sandwich ELISA | Circulating <i>F. gigantica</i> antigen | 86.6            | —               |
| Anuracpreeda et al. (2013) | Cattle | Sandwich ELISA | Cathepsin B3                            | 96.7            | 100             |
| Anuracpreeda et al. (2016) | Cattle | Sandwich ELISA | Cathepsin L1                            | 98.3            | 100             |
| Paramyosin-based study     | Cattle | Sandwich ELISA | Paramyosin                              | 97.33           | 95              |
| Present study              | Sheep  | Sandwich ELISA | Circulating <i>Fasciola</i> antigen     | 92.1            | 93.6            |

#### 4. Discussion

The present study describes the development and preliminary diagnostic evaluation of a sandwich ELISA for detection of circulating *Fasciola* antigen in sheep. The assay was designed to detect parasite-derived antigen directly rather than relying on detection of host antibodies. This distinction is important because antibody responses may persist after exposure or clearance of infection, whereas detection of circulating parasite antigen may provide comparatively direct evidence of an ongoing infection.

The use of excretory-secretory products obtained from adult *Fasciola* provided a relevant source of parasite-derived material for generation of specific antibodies. ES products have been widely investigated for immunodiagnostic applications in fasciolosis. Krishna Murthy and D'Souza (2015) [7] evaluated *F. gigantica* ES antigen by ELISA in cattle and buffaloes and demonstrated its potential for diagnosis, while Hussein et al. (2008) [8] reported favorable diagnostic performance of ES antigen compared with crude and glutathione *S*-transferase antigens. Although these studies involved different hosts, they support the use of parasite-derived ES material as a source of immunodiagnostic antigen.

The developed ELISA demonstrated a sensitivity of 92.1%, detecting 35 of the 38 sheep classified as positive by the reference diagnostic method. The three false-negative samples may be associated with differences in circulating antigen concentration, parasite burden or stage of infection. Previous experimental studies have demonstrated that circulating *Fasciola* antigens can be detected during relatively early stages of infection, supporting the potential value of antigen detection for prepatent diagnosis (Velusamy et al., 2004; Anuracpreeda et al., 2009) [6, 9].

The concept of detecting circulating antigen rather than host antibody is particularly relevant for early diagnosis. Viyant et al. (1997) [11] demonstrated circulating *F. gigantica* antigen in experimentally and naturally infected cattle using a sandwich ELISA, while Anuracpreeda et al. (2016) [10] reported detection of circulating cathepsin L1 antigen substantially earlier than antibody detection in experimental infection. These findings provide a basis for the application of antigen-detection approaches to ovine fasciolosis.

Seven samples classified as negative by the reference method produced positive ELISA reactions. These reactions may have resulted from nonspecific binding, antigenic cross-reactivity or infections that were not detected by the reference diagnostic procedure. Cross-reactivity remains an important consideration in immunodiagnostic assays for fasciolosis because sheep may harbour multiple trematode and gastrointestinal parasite infections. Previous studies have therefore evaluated ELISA specificity against infections with other trematodes and gastrointestinal parasites (Kumar et al., 2008; Anuracpreeda et al., 2016) [13, 10].

The specificity of 93.6% observed in the present study indicates good discrimination between infected and non-infected sheep. However, a larger cross-reactivity panel would be necessary for comprehensive validation. The development of assays targeting defined parasite molecules such as cathepsin L, cathepsin B and paramyosin has demonstrated the possibility of achieving high diagnostic specificity in antigen-based detection systems (Santiago & Hillyer, 1988; Anuracpreeda et al., 2013; Anuracpreeda et al., 2016) [14, 10].

The sandwich ELISA format offers an advantage in that the target antigen is recognized by two antibodies, which can improve the speci-

ficity of antigen detection. Several studies have successfully applied this principle for detection of circulating *F. gigantica* antigens in cattle and experimental hosts (Fagbemi et al., 1997; Velusamy et al., 2004; Hassan et al., 2013) [5, 6]. The present study extends this diagnostic concept to sheep serum and indicates the potential usefulness of a *Fasciola*-specific sandwich ELISA for detection of circulating antigen in ovine fasciolosis.

The positive predictive value of 83.3% observed in the present study was lower than the negative predictive value of 97.1%. This difference is influenced not only by the intrinsic characteristics of the assay but also by the distribution of positive and negative samples within the study population. The high negative predictive value suggests that the assay may be useful for identifying animals unlikely to have detectable *Fasciola* infection under the conditions evaluated. Nevertheless, further studies involving larger and more representative sheep populations are required to determine the diagnostic performance of the assay across different epidemiological settings.

The present assay may therefore have potential as a complementary diagnostic tool for ovine fasciolosis. However, further validation involving larger field populations, animals with confirmed infections at different stages and sheep infected with other helminths would be necessary before routine diagnostic application.

#### 5. Conclusion

The present study demonstrated the feasibility of developing a *Fasciola*-specific sandwich ELISA for detection of circulating antigen in sheep serum. The developed assay showed promising diagnostic performance, with a sensitivity of 92.1%, specificity of 93.6% and overall diagnostic accuracy of 93.2% in the study population.

The assay may serve as a complementary diagnostic tool for detection of ovine fasciolosis, particularly where conventional parasitological examination may be insufficient. Further validation using a larger and geographically diverse sheep population, together with evaluation against multiple reference diagnostic methods and cross-reactivity studies, is required before application of the assay as a routine diagnostic test.

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