

Value of Serum and Milk ELISAs in Diagnosis of Fasciolosis in Lactating Buffaloes Using *Fasciola gigantica* Partially Purified Somatic Antigen

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Abstract: A developed ELISA was established to diagnose *Fasciola gigantica* infection in lactating buffaloes by detecting antibodies in both serum and milk samples. An immunogenic fraction was obtained from crude somatic antigen after fractionation on CNBr-Sepharose 4 B affinity column chromatography and used in detection of fasciolosis in lactating buffaloes. The prevalence of infection in 61 lactating buffaloes was established using serum and milk ELISAs compared with detection of *Fasciola* eggs in fecal samples. Serum ELISA gave the highest diagnostic value (68.9%) followed by milk ELISA (65.6%), while parasitological examination gave 36.1%. By SDS-PAGE, the isolated fraction resolved into five bands of molecular weights 97, 84, 65, 21 and 17 KDa. Immunoreactive bands of the isolated fraction were detected by immunoblot using naturally infected buffalo sera and positive defatted milk. Two bands of molecular weights 97 and 84 KDa were detected by positive sera while other two bands of molecular weights 65 and 17 KDa were detected by positive defatted milk. In conclusion, a partially purified *Fasciola gigantica* somatic antigen is potent antigen in the diagnosis of fasciolosis in buffaloes using serum and milk ELISAs. Moreover, milk ELISA is suitable as a mean of routine veterinary diagnosis of *Fasciola gigantica* infection and an alternative to testing sera.

Key words: *Fasciola gigantica* • Lactating Buffaloes • Affinity Isolated Fraction • Serum and Milk ELISAs

INTRODUCTION

Fasciolosis caused by the trematode *Fasciola hepatica* and *Fasciola gigantica* is a worldwide parasitic disease common in ruminants, especially cattle, buffaloes, sheep and goats. It was estimated that there were over 300 million cattle and 250 million sheep were exposed to these parasites in the globe [1]. Fasciolosis causes sudden death in acute cases [2, 3], while diarrhea, jaundice, ascitis and bottle jaw are predominant features in chronic cases. *Fasciola* spp. are also capable of causing disease in humans [4]. Fasciolosis can be diagnosed by classical coprological techniques to reveal parasite eggs in faeces. Although this method is 100% specific, it is not adequate for early diagnosis because eggs are not found in the feces until 10–12 weeks after

infection, when flukes reach maturity and hepatic injury has been produced [5]. In contrast, fluke specific antibodies are detectable in the serum of most animals by 14 days post infection [6, 7].

Crude adult somatic antigen was a key player in the diagnosis of fasciolosis where, it showed higher efficacy in diagnosis of *F. hepatica* in sheep compared with excretory-secretory antigen [8]. In addition, it proved more diagnostic activity compared with egg antigen in *F. gigantica* infection among cattle and buffaloes [9]. Recently, crude somatic worm antigen was utilized to identify seroprevalence of human fasciolosis in Iran [10].

The use of milk for diagnosis and surveillance of different diseases has become routine and milk antibody testing plays a significant role in cattle disease control and eradication programmes in many countries [11, 12].

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It has been shown for many infections that there is generally a good qualitatively correlation between milk and serum antibody titers [11-14], but milk sampling is easier, cheaper and non-invasive compared to blood sampling.

With regard to the diagnosis of parasitic diseases by detection of antibodies in lacteal secretion, the total IgG concentration is 20-35 times lower in bovine milk than in serum [15, 16]. Moreover, the sensitivity of immunoassay and specificity of utilized antigen are of utmost importance, especially for testing milk samples. Consequently and based on the hypothesis that the diagnostic potency of crude antigen can be optimized by isolation of its immunogenic fraction, the objective of the present study was to isolate the most immunogenic fraction of *F. gigantica* adult worm somatic antigen using affinity column chromatography. Moreover, determination the sensitivity of this fraction in the diagnosis of fasciolosis using serum ELISA compared with milk ELISA was another target.

MATERIALS AND METHODS

***F. gigantica* Adult Worms:** Adult worms were collected from bile ducts of naturally infected liver of slaughtered bufaloes from a local abattoir at Cairo, Egypt, identified as *F. gigantica* by its characteristic morphological features

Animals: A total number of 61 lactating buffaloes located at El-Sharkia governorate, Egypt during a year 2012 were used to perform this study.

Fecal Samples: Rectal fecal samples were collected in polyethylene sacs and coprologically examined for the detection of *Fasciola* eggs according to Soulsby [17].

Serum and Milk Samples: Blood and milk samples corresponding to fecal samples were collected from the lactating buffaloes. After centrifugation at 3000 rpm for 15 min at 4°C, serum and defatted skim milk samples were separated, labeled and stored at -20°C until analyzed by ELISA.

Preparation of Whole Worm Antigen: Worms were washed thoroughly with distilled water and homogenized in 0.15 M phosphate buffer saline (pH 7.2) supplemented with 2mM phenyl methyl sulphonyl fluoride (PMSF) and 0.02 % NaN₃ in a Ten Broeck tissue grinder. The homogenate was sonicated, centrifuged at 14000 rpm for

30 min, supernatant was collected and stored at - 20°C until use as described by Abdel- Rahman *et al.* [18]. The protein content of supernatant was determined according to the method of Lowry *et al.* [19].

Preparation of Hyperimmune Serum: Antibodies against *F.gigantica* adult worm antigen were raised in rabbits according to Fagbemi *et al.* [20]. Rabbits were initially injected subcutaneously with protein of adult worm extract (40 µg /Kg body weight) in Freund's complete adjuvant and booster dose of extract in incomplete adjuvant was injected on day 14. Second and third booster doses were given on day 21 and 28, respectively. Blood samples were collected 4 days after final injection. Antiserum was aliquoted and stored at -20°C until use.

Affinity Column Chromatography: Affinity purification of *F. gigantica* adult worm antigen was performed using Cyanogen-bromide Sepharose 4B (CNBr-Sepharose 4B). In brief, *F. gigantica* hyperimmune rabbit serum was dialyzed against 0.1 M NaHCO₃ containing 0.5 M NaCl and 0.02% NaN₃ and coupled to swollen beads by strictly following the manufacturer instructions. Bound fraction was eluted with 50 mM glycine and 500 mM NaCl pH 2.3.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): The crude extract and its isolated fraction were mixed with reducing buffer samples and electrophoresed on SDS-PAGE slab gels as described by Laemmli [21]. After separation, the gel was fixed in 50% methanol and stained with silver nitrate according to Wray *et al.* [22]. Molecular weight standards were resolved on the same gel to calculate the relative molecular weights of the examined antigens (Amersham Pharmacia, Biotech, Uppsala, Sweden).

Immunoblotting Assay: The assay was carried out to identify the immunoreactive components in crude antigen and isolated fraction. The assay was carried out as the method described by Towbin *et al.* [23]. Nitrocellulose membrane was incubated separately with *F. gigantica* positive buffaloes sera and defatted skim milk obtained from lactating buffaloes, from which infection with *F. gigantica* was confirmed by detection of eggs in their feces.

Enzyme Linked Immunosorbent Assay (ELISA): The assay was utilized to assess the diagnostic potentials of the isolated fraction against 61 serum and defatted skim

milk samples collected from *F. gigantica* infected and non-infected buffaloes. The optimum antigen concentration, serum and horse radish peroxidase conjugated antibodies dilutions were determined by checkerboard titration. The test procedures were carried out according to Santiago *et al.* [24]. The cut off point of optical density values was determined according to Allan *et al.* [25]. Sera were diluted 1:100, while defatted skim milk samples were utilized without dilution. Milk and serum samples of buffaloes from which fasciolosis was confirmed by detection of eggs in feces were used to estimate sensitivity. Estimation of sensitivity was according to Fleiss [26].

RESULTS

Electrophoretic Profile of *F. gigantica* Crude Extract and Isolated Fraction: Electrophoretic profile of *F. gigantica* crude extract showed 15 bands with molecular weights ranged from 165- 14.3 KDa. Five of them were detected in isolated fraction at 97, 84, 65, 21 and 17 KDa (Fig. 1).

Immuoreactive Bands: Eight immuoreactive bands of molecular weights 97, 84, 65, 38, 24, 23, 21 and 14.3 KDa were detected in crude extract using positive serum. Only two of them (97 and 84 KDa) were detected in isolated fraction (Fig. 2). Meanwhile, four bands of molecular weights 65, 23, 17 and 14.3 KDa were detected in crude extract using positive defatted skim milk. Two bands of molecular weights 65 and 17 KDa were identified in the isolated fraction (Fig. 3).

Efficacy of *F. gigantica* Isolated Fraction in Diagnosis of Fasciolosis

Prevalence of *F. gigantica* Infection in Lactating Buffaloes: Parasitological and serological examinations for detection of infection were performed in a total of 61 lactating buffaloes. Results depicted in table (1) showed that *F. gigantica* infection was detected in only 22 (36.1%) buffaloes by fecal examination compared with 42 (68.9%) and 40 (65.6%) by serum and milk ELISAs, respectively (Figs. 4 & 5).

Diagnostic Sensitivity of Serum and Milk ELISAs in the Detection of Fasciolosis: Serum and milk ELISAs recorded 100% diagnostic sensitivity using affinity isolated fraction. Whereas, in all testing buffaloes, none of the animals that yielded eggs in feces failed to show a positive ELISA results (Table 2).

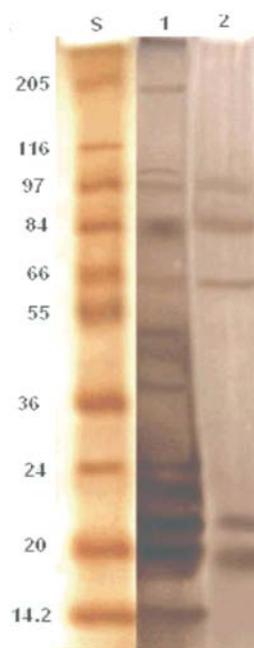


Fig. 1: Comparative electrophoretic profile of *F. gigantica* crude extract (Lane1) and isolated fraction (Lane2). Molecular weight standards in KDa (Lane S)

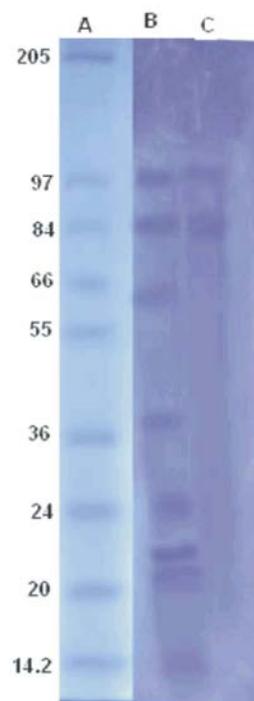


Fig. 2: Immuoreactive bands identified by positive buffaloes sera in *F. gigantica* crude extract (Lane B) and in isolated fraction (Lane C). Molecular weight standards in KDa (Lane A)

Table 1: Detection of antibodies against *F.gigantica* infection in lactating buffaloes by serum and milk ELISAs using *F.gigantica* isolated fraction compared with parasitological examination

| No. of examined animals | Parasitological examination | | | | Serum ELISA | | | | Milk ELISA | | | |
|-------------------------|-----------------------------|------|----------|------|-------------|------|----------|------|------------|------|----------|------|
| | Positive | | Negative | | Positive | | Negative | | Positive | | Negative | |
| | No | % | No | % | No | % | No | % | No | % | No | % |
| 61 | 22 | 36.1 | 39 | 63.9 | 42 | 68.9 | 19 | 31.1 | 40 | 65.6 | 21 | 34.4 |

Table 2: Sensitivity of serum and milk ELISAs in diagnosis of fasciolosis in lactating buffaloes using *F.gigantica* isolated fraction

| Parasitological <i>Fasciola</i> -positive buffaloes | Serum ELISA | | Milk ELISA | |
|---|-------------------|-----------------|-------------------|-----------------|
| | Positive reactors | Sensitivity (%) | Positive reactors | Sensitivity (%) |
| 22 | 22 | 100 | 22 | 100 |

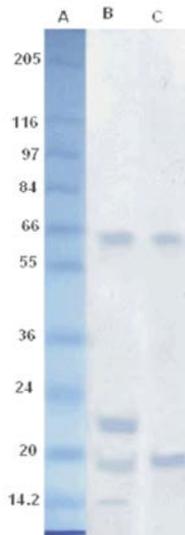


Fig. 3: Immunoreactive bands identified by positive defatted skim milk in *F. gigantica* crude extract (Lane B) and in isolated fraction (Lane C). Molecular weight standards in kDa (Lane A)

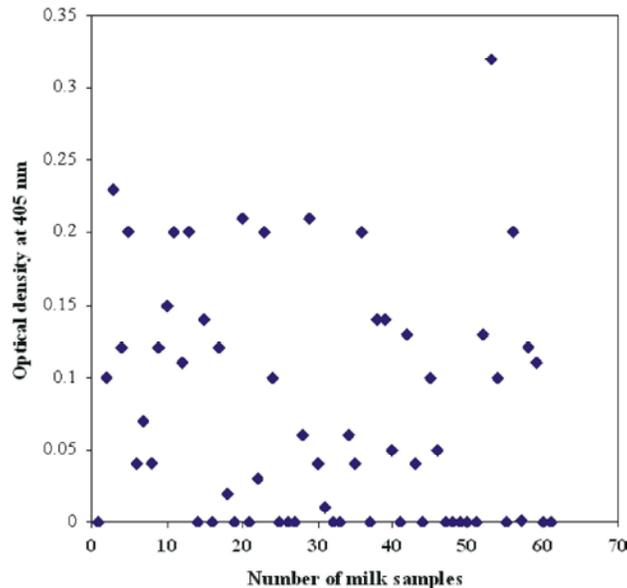


Fig. 4: Potency of isolated fraction in the diagnosis of fasciolosis using milk samples

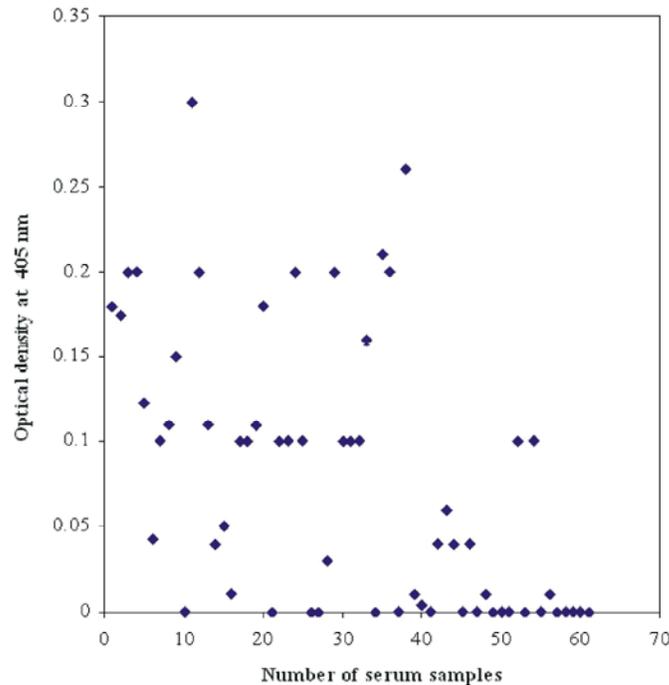


Fig. 5: Potency of isolated fraction in the diagnosis of fasciolosis using serum samples

DISCUSSION

Fasciolosis has detrimental effect on milk yield and fertility [27, 28] and therefore must be controlled in dairy buffaloes in endemic areas. A prerequisite for the proper implementation of animal health control programs is to have available reliable and simple to use diagnostic techniques for screening and monitoring large numbers of animals at affordable price. Many immunological techniques have been developed over years using different *Fasciola* antigens for diagnosis of parasitic infection and to replace the parasitological techniques, which are time consuming and usually lack sensitivity and reproducibility [29]. In addition, the key to the success of any diagnostic assay which detects antibodies to a given organism is the development of a satisfactory antigen (s). In the current research, the selection of *F. gigantica* somatic antigen and ELISA was based on the previous studies suggested the somatic antigen as satisfactory in the diagnosis of *F. gigantica* among cattle and buffaloes [9] and ELISA as a good tool for epidemiological surveys of fasciolosis [30].

In present study *F.gigantica* somatic extract was purified by CNBr-Sepharose 4B affinity column chromatography. Purification process yielded a fraction with protein content 360 µg/ml and resolved in to five bands of molecular weights 97, 84, 65, 21 and 17 KDa by

reducing SDS-PAGE. In recent study in our laboratory one band of molecular weight 65 KDa was detected in *F.gigantica* isolated fraction that is partially responsible for cross-reaction with *Toxoplasma gondii*, using the same purification technique [31]. Moreover, another isolated fraction with also a single band but with molecular weight 97 KDa was obtained using ion exchange chromatography [29]. This difference is probably due to different utilized purification approach, where they used ion exchange chromatography while in the current research a CNBr-Sepharose 4 B affinity column Chromatography was utilized.

Concerning the immunodiagnostic potentials of the isolated fraction, immunoblot analysis revealed two bands of molecular weights 97 and 84 KDa using *F.gigantica* positive buffalo sera. A band of 97 KDa was previously detected in paramyosin antigen using Western blot analysis with antiserum raised against *F. hepatica* [32]. Different immunodiagnostic bands of lower molecular weights (7-40 KDa) were detected by Western blot analysis in *F. hepatica* ES fraction against infected sheep sera [33]. While, [34] concluded that *F.gigantica* infected sheep serum reacted with lower and higher molecular weight antigens (7.8-119.2KDa). Reasons behind these differences could be partially due to different adopted method of purification and type of purified antigen.

In the current research two different bands of molecular weights (65 and 17 KDa) were detected by positive milk samples from naturally infected lactating buffaloes. A band of molecular weight 17 KDa was exclusively detected by positive milk sample in *F. gigantica* ES fraction [35]. This band is probably the same band that was identified in the current study (17KDa). This assumption is based on the fact proving cross-reaction between different developmental stages of parasites and their products [36].

In the current research, an ELISA, which originally developed to detect serum antibodies was adopted for use with milk samples. The use of milk for diagnosis and surveillance of different diseases has become a routine and milk antibodies testing now plays a significant role in bovine diseases control and eradication programs in many countries [11, 12]. So, the other objective of the current study was to investigate whether ELISA can be applied to milk samples as an alternative to serum samples for assessing individual status in relation to *F. gigantica* infection.

In the current research, detection of antibodies reactive to isolated fraction in buffaloes serum and milk samples by indirect ELISA revealed that 42 from 61(68.9 %) and 40 from 61 (65.6%) were *F. gigantica* positive reactors, respectively. Comparing these results with that of coprological examination (36.1 %) performed in the current research demonstrated the advantage of antibodies detection assay. This observation was previously reported by [9, 13, 35, 37]. Although, as reported above, IgG concentration is 20-35 times lower in bovine milk than in serum [15, 16], milk ELISA recorded a comparable diagnostic activity (65.6%) to serum ELISA (68.9 %). It has been shown for many infections that there is generally a good qualitatively correlation between milk and serum antibody titers [11-14], but milk sampling is easier, cheaper and non-invasive compared to blood sampling.

In the present study, serum and milk ELISAs recorded 100% diagnostic sensitivity using affinity isolated fraction. Whereas, in all testing buffaloes, none of the animals that yielded eggs in feces failed to show a positive ELISA results. Our results coincided with Rokni *et al.* [38], although they used *F. hepatica* ES product purified by gel filtration followed by ion exchange chromatography and the isolated fraction exhibited 100% diagnostic sensitivity using sera from coprologically positive patients with fasciolosis. In addition, a comparable result was detected by Ghazy *et al.* [35] who reported that serum and milk ELISAs using ES-F5 antigen

proved high sensitivity (99.2 and 97.7 % respectively), but they purified *F. gigantica* crude extract using gel filtration chromatography on sephacryl S-200 column. This information strongly supported the selection of ELISA in the present study and leads us to believe in the accuracy of milk ELISA in the diagnosis of fasciolosis as well as serum ELISA. Also, it proved the diagnostic efficacy of the isolated fraction. Meanwhile, the present results were conflicted with that of Kumar *et al.* [39]. They suggested that *F. gigantica* purified somatic antigen (27 KDa) proved only 81% sensitivity in detection of *F. gigantica* and *F. hepatica* infections in buffaloes by indirect ELISA. This difference could be attributed to differences in antigen preparation procedures.

CONCLUSION

The current research introduces a partially purified antigen which could be successfully utilized in diagnosis of fasciolosis in lactating buffaloes and proved high sensitivity in serum and milk ELISAs. In addition, milk ELISA is an effective alternative to serum ELISA for diagnostic and surveillance purposes; where milk collection is non invasive, easy and cost effective since veterinarians are not required to collect milk samples and farmers can submit samples directly to regional laboratories. Lastly, based on the current studies it is recommended to use milk tank samples in the preliminary diagnosis of fasciolosis.

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