Molecular and immunological characterisation of *Fasciola* species

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ABSTRACT

Fasciola hepatica and *F. gigantica* are polymorphic liver flukes that show considerable overlap between species, and various protein separation techniques have been used as alternative means of differentiation. Acid and alkaline polyacrylamide gel electrophoresis (PAGE) show differences between *F. hepatica* and *F. gigantica*. Following SDS-PAGE, *F. hepatica* proteins are characterised by the presence of eight major peptide bands, with molecular weights estimated as 48, 45, 43.5, 37, 33, 29, 27 and 25.5 kDa. In contrast, F. gigantica shows only five major protein bands of 57.6, 54, 48, 29 and 27 kDa. Isoelectric focusing (IEF) demonstrates 17 bands from *F. hepatica* and 22 bands from *F. gigantica* between pH 3.5 and pH 10. Although many bands appear common to both species, some are species-specific. Six cases of human acute fascioliasis diagnosed clinically, haematologically and immunologically are also studied. Gel immunodiffusion and immunoelectrophoresis, using adult *F. hepatica* and *F. gigantica* antigens, are used to determine the species, and indicate that the antisera are more specific for *F. hepatica*.

KEY WORDS: Electrophoresis, agar gel.
Electrophoresis, polyacrylamide gel.
Fasciola hepatica. Fasciola gigantica.
Hemagglutination tests.
Immunodiffusion.
Immunoelectrophoresis.

Introduction

Fascioliasis is a disease that infects a wide range of mammals including man. It is recognised as an important condition by the World Health Organization and recent reports estimate that 2.4 million people are infected. The disease is a serious public health problem in many areas of the tropics and subtropics.

Fascioliasis is caused by hermaphrodite trematodes of the genus Fasciola, with *Fasciola hepatica* and *F. gigantica* being the most common.[1-4] The fluke is a polytypic species which varies morphologically depending upon the particular host.[5,6] Also, a range of morphological types of fasciola occurs in south-east Asian countries such as Japan, Taiwan and the Philippines.[7-10]

F. hepatica and *F. gigantica* are easily diagnosed at the extremes of the morphological range; however, intermediate forms also occur and this has led workers to find alternative ways to differentiate them.

Molecular and immunological techniques have been used to differentiate species[11,12] and strains of the parasite,[13,14] and recently a soluble protein isoelectric focusing technique has been documented, providing simple and reproducible resolution.[14]

This study aims to differentiate *F. hepatica* and *F. gigantica* using native polyacrylamide gel electrophoresis (alkaline- and acid-PAGE), sodium dodecyl sulphate (SDS)-PAGE and isoelectric focusing (IEF) to characterise the body proteins of adult worms in order to identify those that are not typical of either species. In addition, species identification in human cases of fascioliasis will be performed by immunodiffusion and immunoelectrophoresis, using crude extracts of *F. hepatica* and *F. gigantica*.

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Material and methods

Choice of worms

Worms were obtained from the livers of slaughtered cattle at Alexandria abattoir, Egypt. They were classified as either *F. hepatica* or *F. gigantica* by macroscopic appearance and microscopic study of the branching of intestinal ceca. A total of 25 *F. hepatica* and 55 *F. gigantica* were studied.

Preparation of crude extract

F. hepatica and *F. gigantica* were washed in sterile saline and homogenised (10 worms/50 mL saline). The homogenate was centrifuged at 14,000 rpm at 4°C for 1 h. Supernatant protein concentration was measured by the Lowry method[15] and found to be 23.5 mg/mL for *F. gigantica* and 26.5 mg/mL for *F. hepatica*.

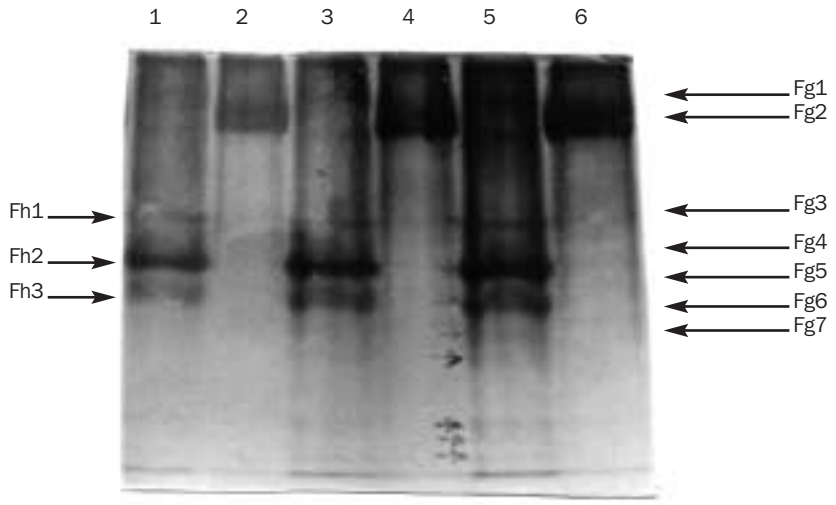

Fig. 1. Acid polyacrylamide gel electrophoresis (acid-PAGE) of *F. hepatica* and *F. gigantica* proteins prepared from whole worms.
Lanes 1, 3 and 5: *F. hepatica* protein samples at concentrations of 70, 140 and 210 μg protein per lane.
Lanes 2, 4 and 6: *F. gigantica* protein samples at the same concentrations per lane.
Three major protein peptides (Fh1, Fh2 and Fh3) faster in migration in *F. hepatica* versus two major protein peptides (Fg1 and Fg2) slower in migration in *F. gigantica*.

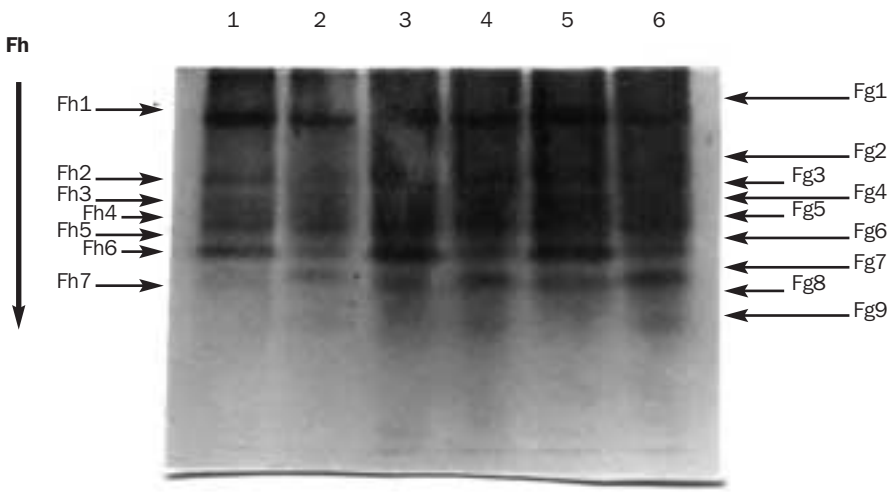

Fig. 2. Alkaline polyacrylamide gel electrophoresis (alkaline-PAGE) of *F. hepatica* and *F. gigantica* proteins prepared from whole worms.
Lanes 1, 3 and 5: *F. hepatica* protein samples at concentrations of 70, 140 and 210 μg protein per lane.
Lanes 2, 4 and 6: *F. gigantica* protein samples at the same concentrations.
Protein peptides Fg 8 and Fg 9 were present in *F. gigantica* only.

Gel electrophoresis

Native-polyacrylamide gel electrophoresis: Native-PAGE (at pH 8.6 and pH 4.0) was carried out using vertical electrophoresis apparatus (PROTEAN-II Cell, Bio-Rad, Richmond, CA, USA) at 120 V for 2 h. Gels used were 4.5% for stacking and 10% for separation.

For alkaline-PAGE (pH 8.6), TEMED and ammonium persulphate (final concentrations 0.04% [v/v] and 0.07% [w/v], respectively) were added to the stacking gel containing 3.6 mol/L urea and 7.5% (w/v) glycerol in 0.5 mol/L Tris-HCl buffer (pH 6.8). The running gel contained 6.1 mol/L urea with TEMED and ammonium persulphate (final concentrations 0.03% [v/v] and 0.07% [w/v], respectively) in 1.5 mol/L Tris-HCl buffer (pH 8.8). The electrode and running buffer consisted of 0.19 mol/L glycine and 0.24 mol/L Tris (pH 8.6).

For acid-PAGE (pH 4.0), the stacking gel contained 3.6 mol/L urea and 7.3% (w/v) glycerol in 0.5 mol/L KOH adjusted to pH 5.9 with acetic acid; TEMED and ammonium persulphate (final concentrations 0.05% [v/v] and 0.07% [w/v], respectively) were added. The separation gel contained 7.9 mol/L urea, TEMED and ammonium persulphate (final concentrations 0.04% [v/v] and 0.9% [w/v], respectively) in 0.5 mol/L KOH adjusted to pH 2.9 with acetic acid. The running buffer consisted of 0.19 mol/L glycine adjusted to pH 4.0 with acetic acid.[16]

Sodium dodecyl sulphate polyacrylamide gel electrophoresis: SDS-PAGE was conducted in polyacrylamide gel containing 0.1% SDS according to the method previously described[17] and involved denaturation of proteins by heating for 5 min in 1% SDS in a boiling water bath prior to applying them to the gel.

Gel staining

After electrophoresis (PAGE or SDS-PAGE), gels were stained for 2 h with 0.1% (w/v) Coomassie blue R-250 in methanol/acetic acid/water (50:7:43 by volume). Gels were destained with a solution of methanol/acetic acid/water (40:10:50 by volume).

Protein molecular weight determination

Molecular weights (kDa) of fractionated proteins on gels were estimated according to the method of Weber and Osborn[18] using standard protein markers (Bio-Rad).

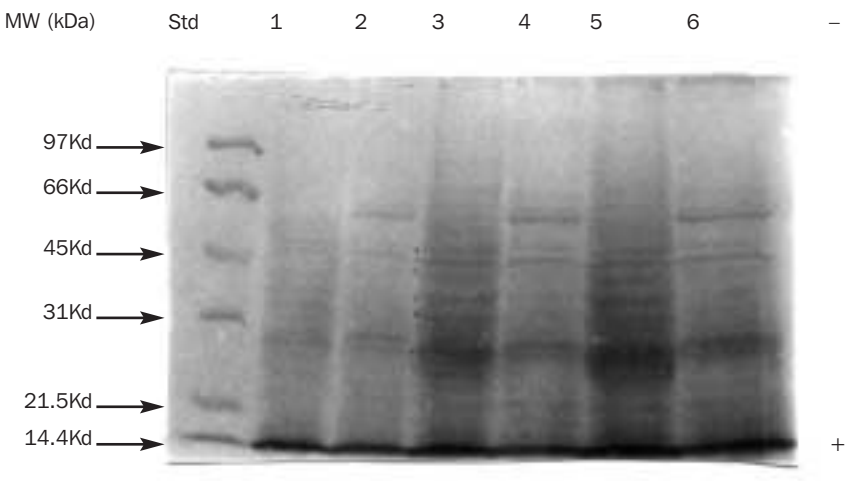

Fig. 3. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) of *F. hepatica* and *F. gigantica*. Proteins prepared from whole worms:
Std: Standard low molecular weight protein marker.
Lanes 1,3 and 5: *F. hepatica* different concentrations (23,46 and 69 μg protein). Lanes 2,4 and 6: *F. gigantica* at the same concentrations of their corresponding *F. hepatica* samples.
Anode is toward the bottom of photograph.
Eight major peptide bands were present in *F. hepatica* protein samples, their corresponding molecular weights were estimated at 48, 45, 43.5, 37, 33, 29, 27 and 25.5 kDa. *F. gigantica* had only 5 major bands of molecular weights 57.6, 48, 54, 29 and 27 kDa.

Isoelectric focusing

Six worms of each species were obtained from the livers of infected cattle, incubated at 37°C in PBS for 4 h and frozen at -20°C. When removed from the freezer they were thawed at room temperature, blotted with filter paper and weighed. Worms were then homogenised individually in double-distilled water (3 μL/mg worm) and the homogenate was centrifuged at 11,000 rpm for 12 min.[19] Supernatant protein concentration was estimated by the Lowry method.[15]

IEF was performed on a microprocessor-controlled electrophoresis unit (Phastsystem, Pharmacia Biotechnology, Uppsala, Sweden) as described by Olsson *et al*.,[20] using gels at pH 3.5-10, run at 1500 V, 5 mA and 3.5 W at 15°C. Gel (1-2 mm thick) was prepared using 30 g acrylamide, 0.8 g bis-acrylamide in 100 mL H_2O (w/v), 1% TEMED, 0.1% (w/v) riboflavine and 40% Ampholene (pH 3.5-10) (Pharmacia).

Protein concentration of all samples was adjusted to 2.2 mg/mL and 0.5 μL each sample was applied to each lane. Isoelectric point (pI) standard of pH 3.5 to pH 10 was applied with each sample so that a pI value could be assigned to selected bands on the gel. After electrophoresis, the gel was fixed in 5% TCA for 30 min and stained with Coomassie blue R-250 for 2 h. Isoelectric point values of each protein were then estimated.[20,21]

Immunodiagnosis of **Fasciola** *species*

Patient sera: Blood samples were obtained from six cases of acute fascioliasis that showed a high indirect haemagglutination (IHA) titre (≥ 1 in 2560). Serum was separated and used in immunodiffusion analysis and immunoelectrophoresis.

Immunodiffusion analysis: Analysis was carried out in 1% Noble agar (Difco, Detroit, MI, USA) in 0.1 mol/L Tricine buffer (Bio-Rad; pH 8.6) containing 3% polyethylene glycol (6000 K), as described by Harrington *et al*.[22]

Immunoelectrophoresis: Electrophoresis was carried out at 0.8 V/mm for 2 h using 1% agarose (Bio-Rad) in 0.1 mol/L veronal buffer (pH 8.6), as described by Mayer and Walker.[23]

Results

Electrophoretic patterns of native proteins prepared from worms fractioned at low pH (acid-PAGE) and high pH (alkaline-PAGE) are shown in Figures 1 and 2, respectively. Acid-PAGE showed three major protein peptides for *F. hepatica* (Fh1, Fh2 and Fh3) and two major protein peptides for *F. gigantica* (Fg1 and Fg2), separated by the speed of migration. Minor peptides were also found in *F. hepatica* (Fh4 to Fh8) and *F. gigantica* (Fg8 and Fg9), and some protein peptides were common to both species.

Figure 3 shows SDS-PAGE electrophoretic patterns of *F. hepatica* and *F. gigantica* proteins according to their molecular weights. *F. hepatica* samples were characterised by eight major peptide bands, with molecular weights estimated at 48, 45, 43.5, 37, 33, 29, 27 and 25.5 kDa. In addition, several minor bands were present. *F. gigantica* had only five major bands, with molecular weights 57.6, 54, 48, 29 and 27 kDa.

Isoelectric focusing patterns of *F. hepatica* and *F. gigantica* body proteins are shown in Figure 4. Seventeen bands were demonstrated for *F. hepatica* and 22 bands for *F. gigantica* in the pH range of 3.5 to 10. *F. hepatica* protein bands were located between pI 4.6-7.2, and in *F. gigantica* the range was pI 4.2-9.5.

Six patients diagnosed with acute fascioliasis were included in the study. Figure 5A shows the results of immunodiffusion. Serum samples from acute patients showed two dense precipitin lines between the *F. hepatica* antigen and the corresponding antiserum, while two light precipitin lines were observed in the case of *F. gigantica* antigen. Figure 5B shows the results of immuno-electrophoresis. Several precipitin arcs were seen with the *F. hepatica* antigen, three of which were more dense than the others (one towards the anode, one towards the cathode and the third one towards the well of application). Only two preciptin arcs were seen with the *F. gigantica* antigen, one of which was dense.

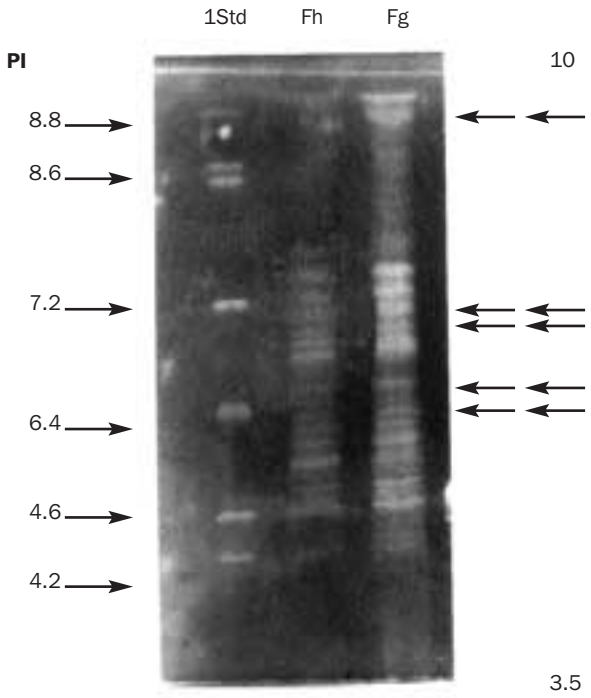

Fig. 4. IEF of whole body protein of *F. hepatica* and *F. gigantica*, showing 17 bands from *F. hepatica* and 22 bands for *F. gigantica* in the pH range of 3.5-10. The right arrows indicate the extra five bands of *F. gigantica*. Fh: *F. hepatica*, Fg: *F. gigantica*, and Std: Standard protein markers.

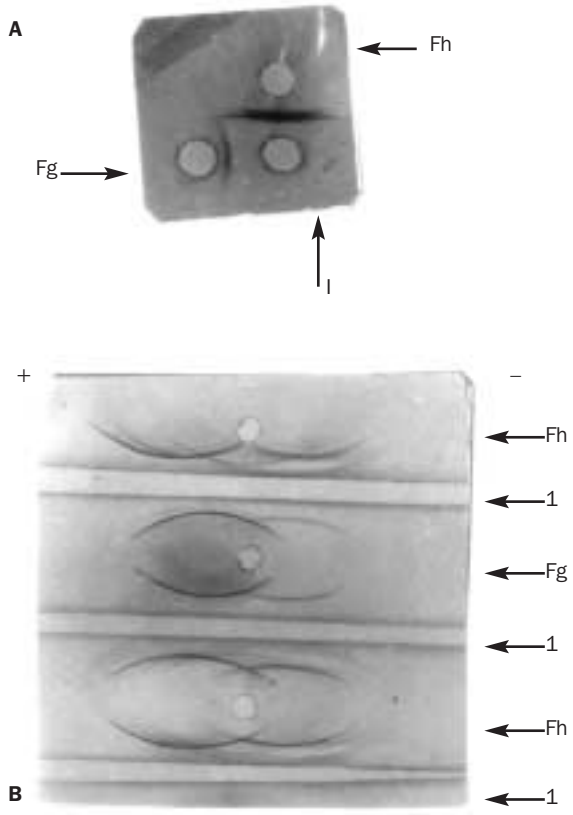

Fig. 5. Immunodiffusion analysis (A) and immunoelectrophoresis (B). *F. hepatica* and *F. gigantica* antigens prepared from crude extract of adult worms reacted with acute Fasciola patient serum with IHA titre ≥ 1 in 2560 Fh: *F. hepatica* Fg: *F. gigantica* 1: Acute patient serum.

Discussion

In Egypt, buffalo, cattle, sheep and goats are infected with *F. gigantica*.[24] However, Farag *et al*.[25] and Mansour *et al*.[26] have reported that most human fascioliasis is caused by *F. hepatica*, and interestingly Curry *et al*.[27] found *F. hepatica* in Egyptian mummies. Therefore, the present study attempted to characterise *Fasciola* species in animals and humans using molecular and immunological techniques.

Acid-PAGE, alkaline-PAGE, SDS-PAGE and isoelectric focusing were considered to be the most reliable of such methods for the differentiation of the two species. When protein samples of each species were fractionated at high pH (alkaline-PAGE) a number of peptides were isolated, some of which were common to both species

Using SDS-PAGE, a number of peptides were separated from each species on the gel and they differed in their migration position and thus their molecular weights. In the present study, *F. hepatica* showed bands with molecular weights ranging from 25.5 to 48 kDa, while *F. gigantica* showed bands that ranged from 27 to 54 kDa.

Rivera Marrero *et al*.[28] studied the SDS-PAGE electrophoretic pattern of the E/S product of *F. hepatica* and found fractions of 25 to 48 kDa, 12 to 14 KDa and 150 to 160 kDa. Sampaio Silva *et al*.[29] identified 11 peptide bands in *F. hepatica*, with molecular weights ranging from 12.4 to 116 kDa. Maleewong *et al*.[30] reported SDS-PAGE analysis of both crude and fractionated E/S product of *F. gigantica* that revealed three major bands with molecular weights ranging from 14.4 to 27 kDa.

In the present work IEF identified approximately 17 bands from *F. hepatica* and 22 bands from *F. gigantica* in the pH range from 3.5 to 10. All protein bands were located between pI 4.6-7.2 and pI 4.2-9.5 in *F. hepatica* and *F. gigantica*, respectively.

Automated IEF has been used to distinguish *F. hepatica* from *F. gigantica* species by Lee and Zimmerman.[19] *F. hepatica* body protein showed 15 dominant peaks in the pH range of 4.6 to 9.3, whereas *F. gigantica* showed 18 dominant peaks in the pH range of 4.6 to 9.2, the majority of which shared between the two species.

In the study reported here, gel diffusion and immunoelectrophoresis using crude adult worm antigens were used to diagnose human fascioliasis; however, the antiserum proved more specific for the *F. hepatica* antigen. The results were confirmed by immunoelectrophoresis, in which more precipitin arcs were seen with *F. hepatica* antigen than with *F. gigantica* antigen. □

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