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STRUCTURAL CHARACTERIZATION AND IMMUNOLOCALIZATION OF EGG ANTIGENS CROSS - REACT WITH *TOXOCARA VITULORUM*, *FASCIOLA GIGANTICA* AND *MONIEZIA EXPANSA* MATURE FLUKES

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Abstract

A structural homology between eggs of *Toxocara vitulorum*, *Fasciola gigantica* and *Moniezia expansa* was proved by the use of SDS-PAGE. In immunoblot, 9,11 and 7 polypeptides were recognized in *F. gigantica, M. expansa* and *T. vitulorum* eggs respectively by their respective rabbit anti-adult antisera. Moreover, components of 240 KD and 206 KD were recognized in the three eggs by different anti-adult antisera. The anatomic localization of the crossreactive epitopes in eggs was determined by indirect immunofluorescence microscopy. The cross - reactive epitopes were mainly associated with embryonted cells of *F. gigantica*, egg shell, larvae and vitelline membranes of *T. vitulorum* and egg shell and granular layer of *M. expansa*.

Introduction

The components of helminth eggs are partially responsible for eliciting host immune response. Development of granulomatous pathology is thought to be the result of an immunogenic response to antigens released from maturing schistosome eggs lodged in pre-sinusoidal hepatic capillaries (Steven *et al.*, 1992). In fascioliasis, potential candidate antigens are found in eggs and adult worms (Reddington *et al.*, 1984). Consequently, protection against fascioliasis was proved utilizing both stages. Either by subcutaneous surgically implanted adult flukes (Eriksen and Flagstad, 1974), subcutaneous injection of eggs in vitro (Rajasekariah and Howell, 1978) or immunization of animals with adult purified antigens (Sexton *et al.*, 1990; Wijfels *et al.*, 1994 and Antonio *et al.*, 1997). The various components of eggs such as soluble antigens, shells and developing larvae, can all serve as potential reagents for use in a wide array of immunologic investigations. *Toxocara vitulorum* fully embryonated eggs contain second larva which is the infective stage and the antigenic targets of host immune response (Amerasinghe *et al.*, 1994; Ghosh, 1996 and Usha-Saini *et al.*, 1997). This notion was further confirmed by the 92% protection against toxocariasis conferred by *T.vitulorum* larvae compared to 100% protection produced by adult extracts (Amerasinghe *et al.*, 1992). As in fascioliasis and toxocariasis, immunization of animals with adult homogenate of *Moniezia expansa* exhibited significant increase in antibodies production and immunized lambs showed the lowest infection with *Moniezia sp.* (Polec, 1991). Antigen conservation between egg and adult flukes was proved (Smith *et al.*, 1983; Aronstein *et al.*, 1985 a and b and Abdel-Rahman and Derbala, 1999). The common antigens could be that responsible for eliciting host immune response and significance in immunodiagnosis and immunoprophylaxis against parasitic infections.

The present study was designed to identify common antigens between egg and adult of *Fasciola gigantica, Toxocara vitulorun* and *Moniezia expansa*. In addition to those common between egg and adult of different species, and immunolocalize egg antigenic targets recognized by anti-adult antibodies.

Materials and Methods

Parasites: Adult worms of *F.gigantica, T.vitulorum* and *M. expansa* were collected from slaughtered animals (buffaloes, and sheep) at Cairo abattoir. Eggs of *F.gigantica* were collected from gall bladder of slaughtered buffaloes. While both *T.vitulorum* and *M. expansa* eggs were collected from their mature worms. After washing extensively with distilled water, both *T.vitulorum* and *F. gigantica* eggs were incubated until full embryonation.

Antigen preparation: Whole worm antigens of the flukes were prepared as described by Abdel-Rahman and Abdel-Megeed (2000) with slight modifications and designated as *T. vitulorum* antigen (TvA), *F. gigantica* antigen (FgA), and *M. expansa* antigen (MeA).

Egg antigens were prepared by homogenizing separately, in 0.15M pBS, pH 7.5 supplemented with 2mM phenyl methyl sulphonyl fluoride (PMSF) and 0.02% NaN_3 in a tissue grinder held in ice bath. Homogenates were sonicated at 28 µ at 60 pulses in MSE sonicator. The sonicates were centrifuged at 18.000 rpm for 1h at 4°C. Supernatants were separately collected to give *T.vitulorum* egg antigen (TvEA), *F.gigantica* egg antigen (FgEA) and *M.expansa* egg antigen (MeEA) were assayed for protein content by the method of Lowry *et al.*, (1951), aliquoted and stored at-20°C until use.

Rabbits immunization with whole worm antigens: Performed as described by Abdel-Rahman and Abdel-Megeed (2000). The three antisera were rabbit anti- *T. vitulorum* antigen (RATvA), rabbit anti-*F. gigantica* antigen (RAFgA) and rabbit anti *M. expansa* antigen (RAMeA).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): After treatment with reducing sample buffer, egg antigens of the three helminths were separately electrophoresed on SDS-polyacrylamide slab gels according to Laemmli (1970). Bio-Rad high and low molecular weight markers were included in each gel. After separation, gels were fixed in 50%, methanol and stained with silver stain according to Wray *et al.* (1981).

Immunoblotting: Egg antigens were separated by SDS-PAGE and then electroblotted onto nitrocellulose papers according to Towbin *et al.* (1979) in a blotting system at 10v/gel overnight. Nitrocellulose papers were incubated with the three diluted anti-adult antisera raised in rabbits. After washing, papers were incubated with alkaline phosphatase –conjugated anti-rabbit IgG. Then exposed to substrate solution for 30 min. Nitrocellulose sheets were rinsed with distilled water to stop the reaction.

Indirect immunofluorescence: Eggs of the three helminths were fixed with acetone for 5 min. After washing with 0.01 M PBS pH 7.4, eggs were incubated in PBS containing 10% (w/v) normal rabbit serum for 30 min. Eggs were then covered with the appropriate dilution of the primary homologous and heterologus rabbit anti-adult antibodies and incubated for 1h in humid chamber. After washing with PBS, eggs were incubated with FITC conjugated anti-rabbit IgG for 1h. Then they were washed with PBS and mounted in buffer glycerol, transferred to a microscope slide and examined under fluorescence microscope. In negative control test, antisera were replaced with normal rabbit sera. Fluorescence photographs were taken to record the results.

Results

Electrophoretic make-up of egg antigens: Structural homology between the eggs of *T.vituloum, F. gigantica* & *M. expansa* was apparent specially in terms of 116 KD, 76 KD and 66 KD (Fig 1 Lanes A, B and C). Nevertheless, eggs differed in a number of less predominent polypeptides at both high and low molecular weight ranges (Fig 1 lanes A, B and C). Among the exclusive molecules to *T.vitulorum* eggs those of 123 KD, 29.5 KD and 13 KD (Fig 1 Lane A). While a component of 195 KD was selectively expressed by *F.gigantica* eggs (Fig 1 Lane B). A component of 155 KD was associated only with *M. expansa* eggs (Fig 1 Lane C).

Egg molecular targets recognized by homologous and heterologous anti-adult antisera:

***F. gigantica* egg target antigens.** RAFgA recognized nine polypeptides in *F. gigantica* aggs of molecular weights 240, 206, 195, 178, 141, 117, 45, 26 and 15 (Fig2 Lane B). Only three of 240 KD, 206 KD and 15 KD were identified by RAMeA (Fig 3 Lane B). While RATvA detected only two polypeptides of 206 KD and 66 KD (Fig 4 Lane B).

***M. expansa* egg target antigens.** *M.expansa* antigenic targets recognized by RAMeA were eleven polypeptides of molecular weights 240, 206, 178, 116, 102, 79, 63, 58, 52, 45 and 15.5 (Fig 3 Lane C). Only three of them (206 KD, 178 KD and 45 KD) were recognized by RAFgA (Fig 2 Lane C). Meanwhile RATvA reacted with components of 178 KD, 116 KD and 15.5 KD (Fig 4 lane C).

***T. vitulorum* egg target antigens.** Seven cross-reactive components between egg and adult *T.vitulorum* were detected by RATvA. The apparent molecular weight of these polypeptides were 206 KD, 178 KD, 116KD, 89 KD, 55KD, 54KD and 16.6 KD (Fig 4 lane A). RAFgA reacted with three polypetides of 240 KD, 206 KD and 141 KD (Fig 2 Lane A). A component of 240 KD was also identified by RAMeA in addition to 116 KD and 13 KD (Fig 3 Lane A).

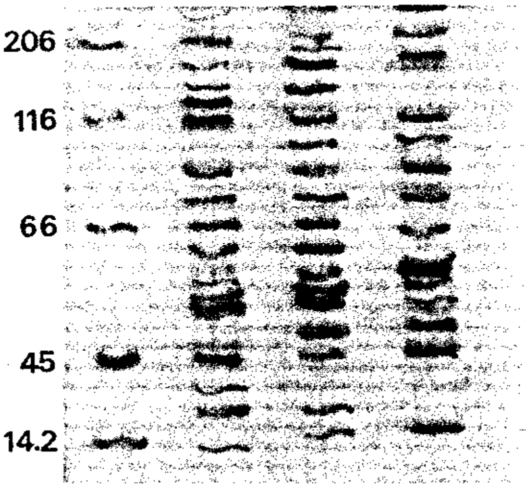

Fig1. SDS PAGE pattern of egg antigens. Lane A. TvEA, Lane B. FgEA and Lane C. MeEA. Molecular weight standards (Lane S)

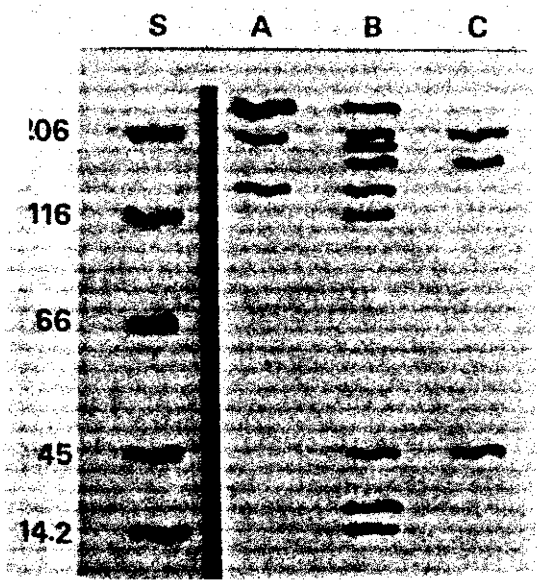

Fig 2. Identification of egg antigenic targets recognized by RAFgA. A. TvEA, B. FgEA and C. MeEA. Molecular weight standards (Lane S)

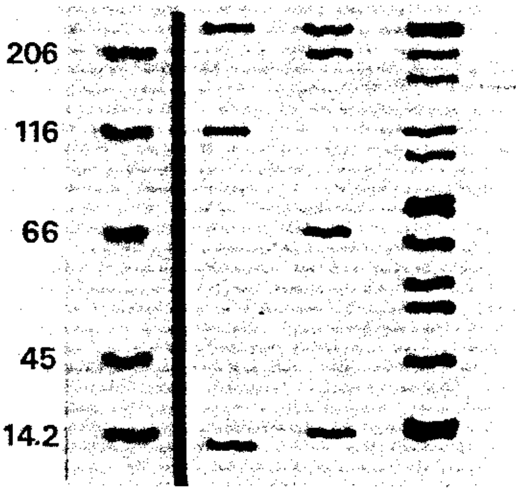

Fig 3. Identification of egg antigenic targets recognized by RAMeA. A. TvEA, B. FgEA and C. MeEA. Molecular weight standards (Lane S)

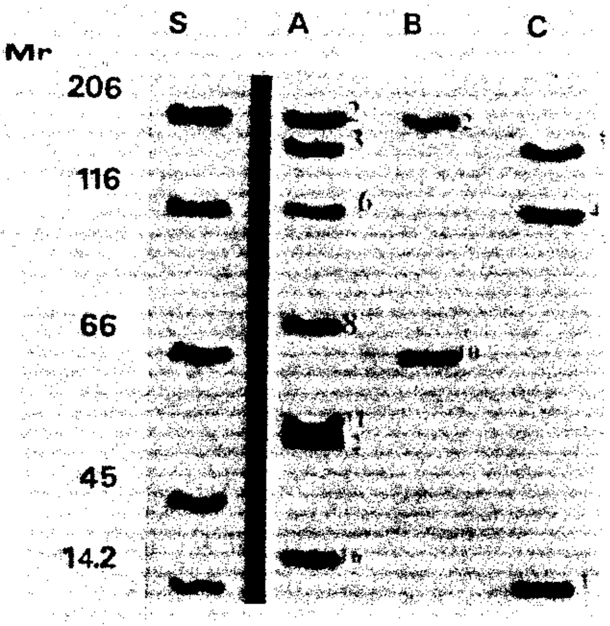

Fig 4. Identification of egg antigenic targets recognized by RATvA. A. TvEA, B. FgEA and C. MeEA. Molecular weight standards (Lane S)

Anatomical localization of egg antigenic targets: In *F.gigantica* eggs, IFA in which RAFgA was utilized indicated intraoval staining of embryonated cells without the involvement of egg shell globules (Fig 5A). Similar distribution but weaker staining patterns were observed with RAMeA (Fig 5 B) and RATvA (Fig 5c). In *T.vitulorum* eggs, RATvA bound to antigens in the egg shell, vitelline membranes and embryo developing cells (morula, gastrula and cleavage) (Fig 6 A). While RAMeA reacted with antigens associated with an organized larva not with egg shell or vitelline membranes. (Fig 6 B). Moreover, RAFgA gave a moderate reaction within the developed larva, vitelline membranes and egg shell (Fig 6C). *M.expansa* eggs consist of substanial shell includes hexacanth embryo provided with pyriform apparatus. A granular layer lies between the shell and the embryophore. Of these structures, the antigenic targets recognized by RAMeA were associated with egg shell and the granular layer. While the embryo and pyriform apparatus were stained negatively (Fig 7 A). The same structures were also reacted with RAFgA but the reaction is less intense and dotted in some areas (Fig 7 B). Meanwhile, the egg shell is the only structure that stained with RATvA in an irregular distribution pattern (Fig 7C).

Discussion

In the current research, a structural homology in *T.vitulorum*, *F. gigantica* and *M. expansa* eggs was observed by the use of SDS-PAGE. This homology reside in components of similar molecular weights between the three eggs as 116KD, 76KD and 66 KD. These components may be responsible for a possible crossreactivity between egg extracts. They were detected despite of differences in egg morphology, structure and constituents of each one. On the other hand, comparison of the egg electrophoretic profiles revealed that eggs were still qualitatively and quantitatively distinct in terms of some molecular entities which seemed to be exclusive to each egg. Electrophoretic analysis has long been used to better clarify the homogenity and heterogenity of the protein composition of helminths (Siles - Lucas and Bandera, 1996). Inter and intra specific variations of *Echinococcus granulosus* were probed by employing SDS-PAGE (Derbala, 1998). Common as well as specific antigens of protoscoleces, hydatid fluids and mature worms of *E. granulosus* isolated from camel and equine origins were recorded. Moreover Ashour *et al.* (1995) introduced a comparative electrophoretic profile of four adult ascaridid nematodes. They observed, by the use of SDS-

PAGE, 13 common bands among the four species. While species specific bands were also detected. These observations together with the data presented in the current research proved the successful use of SDS-PAGE in determining helminths protein composition which reflect their genetic constitution. This advantage could be utilized in discrimination between morphologically similar species (Lee and Zimmerman, 1993).

Cross-reaction between egg and adult stages of each helminth species was proved in the present study by the use of immunoblot in which rabbit anti-adult antisera were utilized. RAFgA identified nine polypeptides in FgEA in the range of 15KD-240KD. While RAMeA reacted with eleven molecules of molecular weights ranged from 15.5 - 240KD in MeEA. RATvA recognized seven *T. vitulorum* egg components ranged from 16.6KD-206KD. This antigenic conservation was previously described in *F. hepatica* fluke by Dalton *et al.* (1985). They identified a common glycoprotein of 260 KD from mature and immature worm extracts. This component has a molecular weight close to that of cross-reactive polypeptide of 240 KD described in the present study between egg and adult *F. gigantica.* The antigenic continuity between larval stages and adult worm was also observed in *Trichostrongylus colubriformis* by Milner *et al.* (1987).They indicated that the majority of proteins synthesized by adult worm were also present in larval stages with minor qualitative differences being indicative of differences in protein expression or turn over. These observations along with the present data suggest that continuity of expression is the rule rather than the exception. Cross-reaction was not only observed between egg and adult of the same species but also of different species. A component of 206 KD was recognized in the three egg extracts by RAFgA. It was also recognized in TvEA and FgEA by RATvA and in FgEA and MeEA by RAMeA. This observation proved the existence of this component in the three egg and adult extracts. Furthermore, a component of 240 KD was detected in the three egg extracts by RAMeA and in both TvEA and FgEA by RAFgA. These data lead us to belive in the importance of these common molecules as successful vaccine candidates against mixed parasitic infections. Since crossreactive antigens appear to have common protective features as previously proved between *F. hepatica* and *S. mansoni* (Haroun and Hillyer, 1988 and Hillyer, 1995) and between *F. gigantica* and *Trichinella spiralis* (El-Azzouni and Hegazy, 1993).

The anatomic localization of the crossreactive antigenic targets in the three eggs recognized by anti-adult antisera were studied by IFA. In *F. gigantica* eggs, the cross-reactive antigens were confined to the embryonated cells, however absent in egg shell. This staining pattern was previously observed in *F. hepatica* eggs by monoclonal antibodies raised against schistosome antigens (Aronstein *et al.*, 1985 a and b). Interestingly these monoclonal antibodies recognized, in addition to egg epitopes, antigenic targets in both mature and immature flukes of *F. hepatica*. These observation along with the distribution pattern of cross-reactive epitopes in *F. gigantica* eggs, suggest the wide spread existence of these epitopes in helminths.

Fluorescent labelling of *T. vitulorum* crossreactive egg antigens, as revealed by RATvA , were mainly associated with embryo developing cells, vitelline membranes and egg shell. The vitelline membranes did not react with RAMeA but reacted with FAFgA together with developed larva. This differential labelling pattern could be attributed to the developmental stage of embryonated cells. In earlier fluorescent studies of *Toxocara canis*, eggs with adult derived antisera, crossreactions to the egg shell, to the fluid surrounding the larva, to larval cuticle and to internal tissues of the larva were observed (Smith *et al.*, 1983). Despite of the generic differences, some of these structures were also recognized in *T. vitulorum* eggs by adult antisera utilized in the present study as egg shell and larva. However, *M. 'expansa* egg embryo did not react with anti-adult antisera. The cross reacting antigens with RAMeA and RAFgA were associated with egg shell and granular layer. While RATvA reacted only with egg shell. The strong labelling of the granular layer is probably due to the excretory - secretory antigens of the embryo.

Collectively, cross-reaction between egg and adult stages of each helminth or of different helminths was proved in the current research. Isolation of cross-reactive epitopes will facilitate the evaluation of its potency as useful reagents for the development of subunit vaccine (s) for helminths.

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