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III RESULTS

III.1 ISOLATION AND ANALYSIS

III.1.1 Morphology

The SEM observations on the morphology of cuticular surface of female *S. digitata* are shown in Figs.1-6.

III.1.1.1 Body Wall

The transverse section (Fig.1) showed that the adult parasite has a thick outer body wall. The cross sectional views of uterus through mf region and alimentary canal are also seen. The body wall is made up of an outer cuticle and an inner muscle layer (Fig.2), consisting of well developed muscle cells arranged uniformly with a peripheral zone and an apical portion lining the pseudocoel. In between the muscle and cuticle is the hypodermal layer.

III.1.1.2 Cuticle

The cuticle comprises basically of three distinct layers, the outer, middle and inner ones (Fig.2). At a magnification of 20000 X, each layer could be resolved into three layers and thus more than 10 distinct subcuticular
Fig.1: TS of female S. *digitata* through mf region.
  bw - body wall, u - uterus, s - surface.

Fig.2: TS of the body wall of female S. *digitata*.
  c - cuticle, mc - muscle cell

Fig.3: TS of cuticle of female S. *digitata*.
  scl - subcuticular layers

Fig.4: Surface of female S. *digitata* showing striations.
  s - surface.

Fig.5: Surface of female S. *digitata* after treatment
  with TX100. s - surface.

Fig.6: Surface of female S. *digitata* after treatment
  with TX100. m - membrane denuded from the
  cuticular surface.
layers constitute the cuticle (Fig.3).

III.1.1.3 Surface

The parasite has a smooth surface topography with distinct uniform longitudinal striations (Fig.4). No regional difference was observed, except when treated with TX100 for the isolation of surface antigens (Se II.3.3.2), when it (Fig.5) showed uneven granular structures throughout, with denuded membrane particles (Fig.6).

III.1.2 Protein content of SAPs

The SAPs isolated by different techniques showed difference in their total protein content (Table I). In an adult parasite weighing about $30\pm5$ mg, the dissected cuticle was $6\pm1$ mg (20%). The remainder constituted mostly of muscle (26.7%), reproductive tissue (43.3%), pseudocoelomic fluid (3.3%) and alimentary canal (6.7%). The total protein content of whole worm was estimated to be $79.31$ mg/g body weight, of which $15.31$ mg (19.3%) was cuticular proteins. Homogenization of cuticle in PBS liberated 49.15% of the total cuticular protein. All the techniques involving detergents released considerable quantities of surface proteins. Among them, the SDS gave the maximum followed by TX100 and DOC techniques respectively (Table I).
Table I Protein content in SAPs isolated by different techniques

<table>
<thead>
<tr>
<th>SAP</th>
<th>Isolation technique</th>
<th>Protein mg/g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dissection</td>
<td>7.520 ± 0.30</td>
</tr>
<tr>
<td>2</td>
<td>TX100</td>
<td>0.670 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>DOC</td>
<td>0.593 ± 0.21</td>
</tr>
<tr>
<td>4</td>
<td>SDS</td>
<td>0.886 ± 0.35</td>
</tr>
</tbody>
</table>

Values are mean (n = 4) + SD

SAP, Surface antigen preparation

* PBS soluble cuticular protein

III.1.3 Marker enzymes in SAPs

The distribution of surface marker enzymes such as ALKPase, ATPase and 5'NA assayed in the SAPs are showed in histogram I. Significant differences were observed between the SAPs in their specific activities of all the enzymes assayed (Histogram Ia). Among them, the activity of ALKPase was higher in SAP2 than SAP1 followed by SAP3, SAP4 and in the whole worm homogenate. This distribution pattern was similar for ATPase and 5'NA also. While, among the enzymes, the specific activities of ATPase and 5'NA were almost similar but lesser than ALKPase in all the preparations.
Histogram 1

Activity of Marker enzymes in the surface antigen preparations of S. digitata

a. Specific activity

b. % Recovery

c. Enrichment

Legend:
- Whole worm
- PBS Cuticle
- TX100 Surface
- DOC Surface
- SDS Surface

Enzymes: ALKase, ATPase, 5'-NA
(Histogram la). The percentage recovery of enzyme activities based on assays of the whole worm homogenate (Histogram lb) was maximum in the SAP₁ which decreased in the order SAP₂, SAP₄ and SAP₃ respectively. While a comparison of the specific activity ratio of ALKPase, ATPase and 5'NA in the SAPs in relation to the enzymes in the whole worm homogenate (Histogram lc) showed that the enrichment with enzymes was significantly high (2 fold) in the SAP₂ followed by SAP₁, SAP₃ and SAP₄ respectively.

**III.1.4 Polypeptide profile of SAPs**

The polypeptide composition of SAPs is evident from the SDS-PAGE profile (Fig. 7). The cuticle homogenate (lane 1) has 12 prominent proteins where as the SAP₂ included only three dominant ones with molecular weights 17, 29 and 36 KDa (lane 4). The SAP₃ and SAP₄ (lane 3 and 2) also contain these three proteins along with few other minor ones which were more in the cuticle (lane 1).

A direct comparison of SAP₁ with SAP₂ by SDS-PAGE (Fig.10) revealed that the SAP₁ (lane 2) has three dominant proteins with molecular weight 17, 29 and 36 KDa which were in agreement with that seen in SAP₂ (lane 1). This observation indicated the similarity of the major proteins of PBS soluble cuticular proteins and TX100 released surface proteins.
Fig. 7: SDS-PAGE (reduced) profile of dissected cuticle (lane 1), SAP₄ (lane 2), SAP₃ (lane 3) and SAP₂ (lane 4).

Fig. 8a: ID pattern of SAP₁ (a), SAP₂ (b), SAP₃ (c) and SAP₄ (d) against anti SAP₂ antiserum.

Fig. 8b: ID pattern of cuticle (a) muscle (b) reproductive tissue (c) and pseudocoelomic fluid (d) against antiserum to cuticle.

Fig. 9: IEP profile of SAP₂ against anti SAP₂ antiserum.

Fig. 10: SDS-PAGE (reduced) profile of SAP₂ (S) and SAP₁ (C).

Fig. 11: CrIEP profile of cuticle against anticuticular antiserum.

Fig. 12: CrIEP profile of cuticle against anticuticular antiserum after purification by immunoadsorption with muscle (a) reproductive tissue (b) and pseudocoelomic fluid (c).
III.1.5 $^{125}$I Labelled SAPs

A significant incorporation of $^{125}$I was observed on the surface of *S. digitata* by Chloramine T method. The total counts in various antigen preparations isolated from $^{125}$I labelled female worms showed that the incorporation per ug protein basis was significantly high in the surface antigens (SAP$_2$) which was greater than in the dissected cuticle (three fold) and whole worm (five fold) (Table II). No significant incorporation was observed in the muscle or reproductive tissue. These observations clearly indicated that the TX100 has specifically liberated proteins from the surface of *S. digitata*.

Table II  Incorporation of $^{125}$I in the cuticular surface of female *S. digitata*

<table>
<thead>
<tr>
<th>No.</th>
<th>Antigen</th>
<th>cpm/ug protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Whole worm</td>
<td>481 ± 21</td>
</tr>
<tr>
<td>2</td>
<td>Dissected cuticle</td>
<td>698 ± 37</td>
</tr>
<tr>
<td>3</td>
<td>Surface (SAP$_2$)</td>
<td>2290 ± 42</td>
</tr>
</tbody>
</table>

Values are mean (n = 4) + SD
III.1.6 Antigenicity of SAPs

III.1.6.1 Comparison on the antigenicity of SAPs

All the SAPs produced visible precipitin bands in ID when challenged against antiserum raised against SAP₂ (Fig.8a). The SAP₁, SAP₂ and SAP₃ were similar in producing one distinct and one diffused bands each. Where as, the SAP₄ produced only diffused and faint bands. The reaction of identity between the SAPs were noted by the coalescing precipitin bands (Fig.8).

III.1.6.2 Antigenic complexity of cuticle

The CrIEP profile of cuticle homogenate against antiserum to it raised in rabbit is shown in Fig.11. There were 30 precipitin peaks formed in the anodic direction. According to their position, the peaks could be grouped under 4 regions. The first group of peaks numbered 1-7 appeared to have higher electrophoretic mobilities. The precipitin peaks in the second group numbered 8-16 and those of third group numbered 17-23 have moderate mobilities. Precipitin peaks numbered 24-30 seen around the sample well, form the fourth group. Significant variations in their height and intensities were observed between the precipitin peaks.
An interesting observation in this experiment was the presence of proteins responsible for the production of 5 precipitin peaks at the cathodic direction (Fig.11a-e). All these peaks stained with equal intensities and had identical positions.

III.1.6.3 Cross-reactivity of cuticle with somatic antigens

The ID profile of somatic antigen preparations against antiserum to cuticle is shown in Fig.8b. All the antigen preparations produced visible precipitin bands with definite difference in antigencity. The cuticle homogenate produced more than four precipitin bands, muscle and reproductive tissue produced three precipitin bands each, whereas the pseudocoelomic fluid produced only two precipitin bands.

III.1.6.4 Antigenic specificity of cuticle

In CrIEP, the anodic direction, the cuticle gave 10 precipitin peaks (Fig.12a) when challenged against cuticle antiserum from which the antibodies cross-reactive to muscle were precipitated out and eight precipitin peaks (Fig.12b) when challenged against cuticle antiserum from which the antibodies cross-reactive to reproductive tissue were precipitated out (Se II.5.2.1). While antiserum from which antibodies cross-reactive to pseudocoelomic fluid were
removed, produced 16 precipitin peaks (Fig.12c). In these three experiments, the retention of a high peak with high anodic mobility and other two peaks, one with moderate and other with low mobilities along with most of the cathodic migratory precipitin peaks (Figs.12a-c) were evident to show the presence of cuticle specific antigens.

III.1.6.5 Antigenicity of SAP2

The SAP2 when analysed in IEP against antiserum to it raised in rabbit (Se II.3.5.2) showed high antigenicity (Fig.9). There were three distinct precipitin arcs. The first one appeared to have high anodic mobility and the second one showed moderate mobility. The third precipitin arc showed low mobility and hence remained near the sample well.

III.1.7 Effect of temperature on the antigenicity of SAPs

The ID pattern of SAP1 treated at various temperatures when challenged against antiserum to cuticle is shown in Fig.13. The antigen treated at 0°C and 37°C produced three distinct precipitin bands. In the test using antigen treated at 50°C, the peripheral band was diffused, while it completely disappeared in that treated at 60°C. At 70°C, the middle precipitin band also became diffused and completely disappeared at 90°C. But one precipitin band
Fig.13: ID pattern of SAP₁ challenged against anticuticular antiserum after treatment for 30 min at 0°C (a), 37°C (b), 50°C (c), 60°C (d), 70°C (e), 80°C (f), 90°C (g) and 100°C (h).

Fig.14: CRIEP profile of challenged against anticuticular antiserum after treatment at 100°C for 30 min.

Fig.15: Dot-IFAT profile of SAP₂ against anti SAP₂ antiserum after treatment for 30 min at 37°C, 50°C, 65°C, 80°C and 100°C.

Fig.16: SDS-PAGE (reduced) profile of SAP₅ (lane 1) showing the naturally shed surface antigens and the SAP₂ (lane 2) showing the TX100 released surface antigens.

Fig.17: IEP profile of SAP₂ (a) and SAP₅ (b) against anti SAP₂ antiserum.

Fig.18: SDS-PAGE (reduced) profile of surface antigen preparations of egg (lane 1), embryo (lane 2), mf (lane 3) and adult (lane 4) S. digitata.

Fig.19: ID pattern of SAPs of egg (a), embryo (b), mf (c) and adult (d) against anti SAP₂ antiserum.
persisted in the SAP$_1$ treated even at 100°C (Fig.13h). A further analysis of SAP$_1$ treated at 100°C against antiserum to cuticle in CrIEP (Fig.14) gave five precipitin peaks with moderate, and three with low anodic mobilities. On the other hand, the disappearance (inactivation) of anodic migratory antigens which gave high precipitin peaks along with the entire cathodic migratory antigens all produced precipitin peaks in the control (Fig.11) was noted.

The reference spots of Dot-IFAT performed to study the effect of temperature on the antigenicity of SAP$_2$ is shown in Fig.15. The antigens treated at 37°C produced high fluorescence against antiserum to it raised in rabbit. In the SAP$_2$ treated at 50°C, the intensity of fluorescence was moderate, with decreasing intensities in SAP$_2$ treated at 65°C, and 80°C. A visible fluorescence was observed even in the antigen treated at 100°C.

III.1.8 Naturally shed surface antigens

The SDS-PAGE analysis of the culture supernatant of adult *S. digitata* (SAP$_5$) gave five prominent protein bands (Fig.16.1). While comparing with SAP$_2$ (Fig.16.2), the presence of a 29 KDa protein in agreement with that in SAP$_5$ was evident. The SAP$_5$ when challenged against antiserum to SAP$_2$ by IEP (Fig.17) produced visible precipitin arcs. The
formation of a distinct precipitin arc in the SAP$_5$ with high anodic mobility at the position identical to that present in SAP$_2$ was noted. Both of these experiments evidently indicated the possible shedding of 29 KDa surface antigen naturally into the environment.

III.1.9 Surface antigens of developmental stages

III.1.9.1 Polypeptide pattern

In SDS-PAGE, the surface antigens of the developmental stages of *S. digitata* (Fig.18) showed identical polypeptide patterns with 17, 29 and 36 KDa proteins as the predominant ones. Among them, the 17 KDa protein in the surface of egg was a relatively minor one that showed a steady increase on embryo, mf and adult surfaces. The 36 KDa protein, a major component of adult surface was present only in trace amounts on the egg, embryo as well as mf surfaces. On the other hand, the 29 KDa protein exists in an almost equal concentration in all these stages.

III.1.9.2 Antigenicity

In ID, the SAPs of egg, embryo, mf and adult (SAP$_2$) when challenged against antiserum to SAP$_2$ (Se II.3.5.2) produced visible precipitin bands (Fig.19). There
was one distinct and one diffused band each in the SAPs of all stages, of which, those against egg surface were faint. However, the results show the sharing of surface antigens in the developmental stages and adults of *S. digitata*.

### III.2 PURIFICATION AND CHARACTERIZATION

#### II.2.1 Non SDS-PAGE profile of SAP₂

Non SDS-PAGE of SAP₂ (Fig. 20) resolved six distinct native protein bands with relative mobilities (Rm) 0.09, 0.32, 0.41, 0.53, 0.61 and 0.76. Among them the proteins with Rm 0.09, 0.41 and 0.76 were the three major components, of which the Rm 0.76 protein was a predominant one. The other proteins with Rm 0.32, 0.53 and 0.61 were relatively the minor ones. Densitometric scanning of the non SDS-PAGE profile of SAP₂ (Fig. 26) showed a very high peak at Rm 0.76 protein band, a moderately high peak at Rm 0.09 protein band and a medium peak at Rm 0.41 protein band. Relatively very low peaks were observed at the minor protein bands such as Rm 0.32, Rm 0.53 and Rm 0.61. The PAGE analysis showed definite quantitative differences between the individual native proteins of SAP₂.
Fig. 20: Non SDS-PAGE (native) profile of SAP₂ with densitometric scan.

Fig. 21: SDS-PAGE (non reduced) profile of SAP₂ (lane 0) and SAFs 1 to 6 (lanes 1 to 6 respectively).

Fig. 22: SDS-PAGE (reduced) profile of SAP₂ (lane 0) and SAFs 1 to 6 (lane 1 to 6 respectively).

Fig. 23: Non SDS-PAGE (native) profile of SAP₂ (lane SA), SAF₁ (lane F₁), SAF₃ (lane F₃) and SAF₆ (lane F₆).

Fig. 24: SDS-PAGE (reduced) profile of SAP₂ (lane SA), SAF₁ (lane F₁), SAF₃ (lane F₃), and SAF₆ (lane F₆).
III.2.2 Purified SAFs

Electroelution of proteins from polyacrylamide gel slices corresponding to the six protein bands of SAP₂ resolved on non SDS-PAGE (native gel) gave six native surface antigen fractions such as SAF₁ (Rm 0.09), SAF₂ (Rm 0.32), SAF₃ (Rm 0.41), SAF₄ (Rm 0.53), SAF₅ (Rm 0.61) and SAF₆ (Rm 0.76). Quantitation of total proteins in the eluted SAFs indicated that the SAF₆ alone accounted for 40% of the total protein content of SAP₂ and only 25% and 15% respectively were shared by SAF₁ and SAF₃. The other minor fractions such as SAF₂, SAF₄ and SAF₅ accounted only for 8%, 6% and 6% respectively.

III.2.3 Protein composition of SAFs

On SDS-PAGE at reduced state, the SAP₂ showed the 17, 29 and 36 KDa as the three major polypeptide bands (Fig.22, lane 0). The purified SAF₁ (lane 1) is composed of the 17, 29 and 36 KDa protein subunits, where as the SAF₂ and SAF₃ (lane 2 and 3) both consisted only of the 29 and 36 KDa protein subunits. On the other hand, the last three fractions (SAF₄, SAF₅ and SAF₆, lane 4-6 respectively) were composed mainly of the 29 KDa protein only.

When analysed on SDS-PAGE at nonreduced state, the
SAP₉ (Fig. 21, lane 0) showed the 17, 29 and 36 KDa proteins. Moreover the presence of some other higher molecular weight proteins especially in between the range of 45 to 60 KDa was noted. The major portion of these higher molecular weight proteins were present in the SAF₁ (lane 1) and traces in SAF₂, SAF₃ and SAF₄ (lane 2-4). The presence of 17, 29 and 36 KDa proteins in SAF₁ (lane 1) were in low quantities and in SAF₂, SAF₃ and SAF₄ (lane 2-4) were of moderate quantities. However the SAF₅ and SAF₆ (lane 5 and 6) were composed almost exclusively of the 29 KDa protein.

The three major SAFs:– The non SDS-PAGE pattern of the three major SAFs are shown in the Fig. 23. The SAF₁ (lane F1) consists of only one protein of Rm 0.09, the SAF₃ (lane F3) showed only a protein band of Rm 0.41 and the SAF₆ (lane F6) showed only a protein of Rm 0.76 all remaining identical to the respective bands of SAP₂ (lane 0). No other protein band was observed in these three fractions indicating their purity.

The SDS-PAGE (reduced) pattern of these three major SAFs shown in Fig. 24 revealed that the SAF₆ (lane F6) is made up exclusively of the 29 KDa protein only, while the SAF₃ (lane F3) consists mainly of a 36 KDa protein and trace amount of the 29 KDa subunit. The SAF₁ (lane F1) comprised mainly of the 17 KDa protein with relatively lesser
quantities of the 29 and 36 KDa subunits.

III.2.4 Immunological characteristics of SAFs

III.2.4.1 Purified surface immunoglobulin G (SIGG)

The Igs precipitated from the antiserum to SAP₂ raised in rabbits when eluted using 0.0175 M SPB through DEAE-cellulose column (Se II.5.2.2), gave a high protein peak at 280 nm. Few minor peaks followed during successive elution with 0.2 M SPB. Dot-IFAT of SAP₂ using these peak Ig fractions showed that the Igs in the high peak produced high fluorescence. The other minor Ig fractions also showed visible but very low fluorescence against SAP₂. SIGG in the high peak was used for immunoaffinity purification of SIGGFs.

III.2.4.2 Surface IgG fractions (SIGGFs) purified by immunooaffinity technique

All the three major SAFs (SAF₁, SAF₃ and SAF₆) coupled on sepharose 4B beads showed considerable binding of SIGG on them which was evidenced from the visible fluorescence produced when tested few beads by IFAT (Se II.6.3.1). Elution with 0.1 M glycine-HCl buffer pH 2.5, yielded considerable quantities of bound IgG and the fractions were designated as SIGGF₁, SIGGF₃ and SIGGF₆.
III.2.4.3 Relative antigenicity of SAFs

Analysis on the antigenicity of SAFs against antiserum to SAP₂ tested by Dot-IFAT showed considerable activities in all the fractions. But the distinct difference in the intensities of fluorescence between the fractions indicated the difference in their antigenicity. According to the intensities of fluorescence as shown in the reference spot (Fig.25), these are graded as high, moderate, low and very low fluorescence. The data thus analysed (Table 3) showed that the SAF₆ (Rm 0.76/29 KDa) protein which was the most antigenic one, produced high fluorescence. The SAF₁, SAF₃ and SAF₅ showed moderate fluorescence where as SAF₂ and SAF₄ showed only low fluorescence.

III.2.4.4 Cross-reactivity/Avidity of SIgGFs

The SIgGFs purified by immuno-affinity technique when tested by Dot-IFAT against their corresponding antigen fractions (Table 3), the SAF₆ produced high fluorescence against SIgGF₆ and the SAF₁ as well as SAF₃ produced moderate fluorescence only against SIgGF₁ and respectively indicating their difference in avidity of the three major SAFs.
Fig. 25: Dot-IFAT of SAFs against SIGGFs showing very low (a), low (b), moderate (c), and high (d) intensities of fluorescence.

Fig. 26: IFAT of SAP₂ against anti SAP₂ antiserum showing membrane particles with difference in size, shape and intensities of fluorescence.

Fig. 27: TX100 treated male (a) and female (b) S. digitata when challenged against anticuticular antiserum by IFAT showing high surface fluorescence.

Fig. 28: IFAT of fresh egg cluster (a), embryo (b), TX100 treated mf (c) and female adult S. digitata (d) when challenged against anti SAP₂ antiserum showing surface fluorescence.
Table 3  Relative antigenicity and Cross-reactivity/specificity of purified surface antigen fractions in Dot-IFAT

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Antiserum to SAP$_2$</th>
<th>SIgG fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIgGF$_1$</td>
<td>SIgGF$_3$</td>
</tr>
<tr>
<td>SAP$_2$</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SAF$_1$</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SAF$_2$</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SAF$_3$</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>SAF$_4$</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SAF$_5$</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>SAF$_6$</td>
<td>++++</td>
<td>+</td>
</tr>
</tbody>
</table>

SAP$_2$ - surface antigen preparation isolated by TX100 technique; SAF - surface antigen fraction; SIgGF - surface immunoglobulin fraction.

+ very low fluorescence  ++ low fluorescence
+++ moderate fluorescence ++++ high fluorescence

But all the three antigen fractions (SAF$_1$, SAF$_3$, SAF$_6$) produced visible fluorescence in Dot-IFAT (Table 3) when challenged against any of the three SIgGFs indicating the presence of cross-reactive epitopes. The SIgGF$_1$ produced
low fluorescence with SAF2, SAF3 and SAF4 and only very low fluorescence with SAF5 and SAF6. The S1gGF3 produced low fluorescence with SAF1, SAF2 and SAF4 and only very low fluorescence with SAF5 and SAF6. The S1gGF6 produced moderate fluorescence with SAF5 and only very low fluorescence with all the other SAFs. However, among the SAFs, the occurrence of SAF6 (29 KDa) protein as the most antigenic one with less cross-reactivity as well as with high avidity was noted.

III.2.5 *In situ* localization of surface antigens

When IFAT was performed with antiserum to SAP2, followed by antirabbit IgG-FITC conjugate (Se II.6.3.1), the SAP2 under a fluorescent microscope showed discrete membrane particles with difference in their size, shape and intensity of fluorescence (Fig.26).

In IFAT, the fresh egg as well as embryo stages when challenged against antiserum to SAP2 followed by antirabbit IgG-FITC conjugate (Se II.6.3.1) showed high fluorescence on their surface (Figs.28a,b). But live mf or adult of *S. digitata* did not show any visible fluorescence on their surface in the IFAT against antiserum to SAP2 or cuticle indicating the failure of antisurface antibodies to bind on the surface of intact live mf or adult parasites.
Fig. 29: IFAT of TX100 treated female adult *S. digitata* when challenged against SIGGF₁ (a), SIGGF₃ (b), SIGGF₆ (c) and TX100 treated male against anti SAP₂ antiserum (d) showing surface fluorescence.

Fig. 30: The egg cluster (a), embryo (b), mf (c), female adult (d) and male adult (e) *S. digitata* exhibiting surface fluorescence when incubated with ConA-FITC.
On the other hand, when such IFAT was performed using these parasites after treatment with TX100 (Se II.6.3.1), there was high degree of fluorescence on the surface of mf (Fig.28c) as well as both male (Figs.27a & 29d) and female (Figs.27b,28d) adult parasites against antiserum to cuticle and SAP₂ respectively indicating the binding of surface antibodies.

III.2.6 Carbohydrate moities of surface

When live adult as well as developmental stages of _S. digitata_ were treated with ConA-FITC, a high surface fluorescence was observed in all the stages indicating the presence of exposed ConA binding carbohydrate moities on the surface of egg (Fig.30a), embryo (Fig.30b), mf (Fig.30c), female adult (Fig.30d) and male adult (Fig.30e) stages. No difference in the ConA binding property between the stages was observed. Similar ConA binding was observed even when the experiment was performed using TX100 treated _S. digitata_. The Fig.31 shows the ConA-FITC binding of the SAF₁, SAF₃ and SAF₆ respectively coupled on sepharose 4B beads (Se II.5.2.3.2). All the SAFs showed high fluorescence indicated that they possess ConA binding carbohydrate moities.
III.3 CROSS REACTIVITY AND APPLICATION

III.3.1 Cross reactivity of SAPs to *W. bancrofti*

The whole worm homogenate when challenged against sera from mf +ve patients with *W. bancrofti* infection to test the cross-reactivity, no visible reactions were obtained in ID, IEP or CrIEP. In CIEP analysis, there were visible precipitin bands (Fig.32a) which indicated the presence of cross reacting antigens in *S. digitata*. CIEP experiments conducted using SAP₁ and SAP₂ as antigens against the mf +ve human sera samples also produced visible precipitin bands (Figs.32b,c). A close examination on these three sets of results clearly indicated distinct differences between their banding patterns. However, a comparatively high reactivity was observed with the SAP₂ than the whole worm homogenate or SAP₁ (Fig.32).

III.3.2 CIEP analysis of SAPs against various human sera

The percentage positive reactions of different SAPs against various human serum samples tested in CIEP (Fig.33) is shown in the Table 4. All the antigen preparations such as whole worm homogenate, SAP₁ and SAP₂ were 100% sensitive in reacting with antibodies from mf +ve
Fig.31: The SAF1 (a), SAF3 (b) and SAF6 (c) coupled on sepharose 4B beads showing fluorescence when incubated with ConA-FITC.

Fig.32: CIEP profile of whole worm homogenate (a), SAP1 (b) and SAP2 (c) when challenged against human sera from mf +ve patients with bancroftian filariasis.

Fig.33A: CIEP profile of SAP2 showing different intensities of reaction (a,b,c) when challenged against different human sera from mf +ve patients with bancroftian filariasis.

Fig.33B: CIEP profile of SAP2 showing different intensities of reactions when challenged against human sera from mf +ve asymptomatic patients (d), symptomatic patients (e) a false positive reaction against endemic normal (f) and no reaction against cord blood (g).

Fig.34: Western blot analysis of SAP2 separated on non SDS-PAGE showing reaction bands when challenged against no serum (a), sera from symptomatic (b) and mf +ve asymptomatic (c) patients with bancroftian filariasis.

Fig.35A: Dot-ELISA of SAP2 showing reaction spots when challenged against different sera, mf +ve asymptomatic (N) and symptomatic (P) patients with bancroftian filariasis.

Fig.35B: Dot-ELISA of SAF1 (1), SAF3 (2) and SAF6 showing reaction spots from different mf +ve asymptomatic patients with bancroftian filariasis.
asymptomatic and symptomatic filarial patients.

Table 4  Positive reactions (%) of SAPs with human sera in CIEP

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cord blood (n = 20)</th>
<th>Endemic normal (n = 20)</th>
<th>Mf +ve (n = 55)</th>
<th>Symptomatic (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole worm</td>
<td>ND</td>
<td>25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAP₁</td>
<td>ND</td>
<td>15</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAP₂</td>
<td>ND</td>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

ND - Not detected. Values are in percentage.

There were no precipitin bands formed against any of these antigens when challenged against sera from cord blood, whereas, a proportion of endemic normal sera gave positive reactions in CIEP with all the antigens which was comparatively low with SAP₂ than the other two (Table 4).

III.3.3 Radio-immunoprecipitated SAP₂

The human sera from all the groups of patients have precipitated significant quantities of radio-iodinated surface antigens of *S. digitata* (Table 5).
Table 5  Radio-iodinated surface antigens precipitated by different sera samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Sera samples</th>
<th>CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Antisurface antiserum (n = 6)</td>
<td>7460 ± 130</td>
</tr>
<tr>
<td>2</td>
<td>Cord blood (n = 20)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Endemic normal (n = 20)</td>
<td>2064 ± 1542</td>
</tr>
<tr>
<td>4</td>
<td>Mf +ve asymptomatic (n = 55)</td>
<td>3302 ± 452</td>
</tr>
<tr>
<td>5</td>
<td>Symptomatic patients (n = 25)</td>
<td>3071 ± 146</td>
</tr>
</tbody>
</table>

Values are mean ± SD of the total counts in the precipitates when incubated 10 ug antigen (2290 CPM/µg protein) with 50 ul sera samples.

Among them the endemic normal showed minimum precipitation whereas the mf +ve gave the maximum. The symptomatic patients showed less counts than that of mf +ve and no counts were obtained in cord blood sera. The results revealed definite difference in the antibody titer of human subjects with various clinical status of filariasis.

III.3.4 Western blot analysis of the cross-reactivity of SAP₂

The reaction profile obtained from the western blot analysis of SAP₂ separated on non SDS-PAGE followed by
immunoblot against human sera from *W. bancrofti* mf +ve asymptomatic and symptomatic patients are shown in the Fig.34. Sera from both the groups of patients produced visible bands of immunoreaction. There were six distinct bands in both the groups with no marked difference between the banding patterns. Among these immunoreaction bands, the first, third and sixth (Rm 0.09, 0.41 and 0.76) were relatively prominent ones while the others (Rm 0.32, 0.53 and 0.61) were the minor ones. In general, the banding pattern (Fig.34) was identical to the non SDS-PAGE pattern of SAP₂ (Fig.20). These observations gave direct evidence showing that all the native protein components of SAP₂ have cross reacting epitopes with antibodies in the sera from patients with *W. bancrofti* infection.

### III.3.5 Dot-ELISA on the cross reactivity of SAFs

The Tables 4 and 5 show the cross reactivity of purified SAFs tested by Dot-ELISA (Se II.6.9) against sera from mf +ve patients with *W. bancrofti* infection. All the three SAFs (SAF₁, SAF₃ and SAF₆) produced visible reactions. Reference spots are given in Fig.35a. Analysis of the relative cross reactivity of SAFs at varying protein concentration ranged from 0.1 ng to 1000 ng per 0.25 ul/spot (Table 5), showed that up to 100 ng, visible reaction
spots were produced in all the SAFs. Below that concentration, the spots became faint and at 1 ng/0.25 µl/spot, the SAF₁ and SAF₃ failed to produce any visible reaction spots whereas the SAF₆ still produced distinct reaction even after dilution to 1 ng protein (Table 5), indicating its high avidity.

Table 5 Positive reactions (intensity) of mf +ve W. bancrofti patients sera against various protein concentrations of SAFs in Dot-ELISA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Concentration of protein (ng)/0.25 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>SAP₂</td>
<td>+++</td>
</tr>
<tr>
<td>SAF₁</td>
<td>+++</td>
</tr>
<tr>
<td>SAF₃</td>
<td>+++</td>
</tr>
<tr>
<td>SAF₆</td>
<td>+++</td>
</tr>
</tbody>
</table>

Analysed data from the tests with 55 mf +ve sera.

SAP₂ - surface antigen preparation isolated by TX100 technique; SAF - surface antigen fraction. + faint spot, ++ moderate spot, +++ bright spot, - no visible spot.

Dot-ELISA performed with a constant protein concentration of SAFs (100 ng/0.25 µl/spots) against human
sera from mf +ve patients with *W. bancrofti* infection at various dilutions (0-400 times) are shown in Table 6. All the SAFs produced visible reactions up to 100 times dilution. At 200 times dilution, the intensities became faint and that disappeared in 400 times diluted sera against SAF₁ and SAF₃. Where as the SAF₆ was the one retained to produce visible but faint reaction even at the dilution of 400 times.

Table 6 Dot-ELISA analysis of SAFs against human sera from *W. bancrofti* mf +ve patients at various dilutions

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilutions of human mf +ve sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SAP₂</td>
<td>+++</td>
</tr>
<tr>
<td>SAF₁</td>
<td>+++</td>
</tr>
<tr>
<td>SAF₃</td>
<td>+++</td>
</tr>
<tr>
<td>SAF₆</td>
<td>+++</td>
</tr>
</tbody>
</table>

Analysed data from the tests with 55 mf +ve sera.

SAP₂ - surface antigen preparation isolated by TX100 technique; SAF - surface antigen fraction; + faint spot; ++ moderate spot; +++ bright spot; - No visible spot.
Both these experiments confirm that all the major SAFs of *S. digitata* are cross-reactive to *W. bancrofti*, whereas the SAF$_6$ proteins was the one among them with highest cross-reacting avidity to *W. bancrofti* patients serum.

### III.3.6 Diagnostic utility of SAFs

The results of the evaluation of SAFs for the diagnosis of bancroftian filariasis is shown in the Tables 6 and 7. The Table 7 shows the percentage positivity of SAFs (100 ng protein per spot) when challenged against different human sera samples (100 times diluted) in the antibody detection test by Dot-ELISA. A cent percent positivity was observed in all the SAFs against both mf +ve asymptomatic as well as symptomatic subjects. None of these SAFs produced any visible reactions against cord blood sera samples indicating the absence of filarial antibodies in them. But a significant proportion of the endemic normals gave positive reactions with all the antigens tested. Among them, the SAP$_2$ showed the highest, the percentage decreased with SAF$_1$ and SAF$_3$ and comparatively lesser false positive reactions was observed in the SAF$_6$ with endemic normal (Table 7).
Table 7  Positivity (%) of SAFs in the antibody detection of human sera samples by Dot-ELISA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cord blood (n = 20)</th>
<th>Endemic normal (n = 20)</th>
<th>Mf +ve (n = 55)</th>
<th>Symptomatic (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP₂</td>
<td>ND</td>
<td>65</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAF₁</td>
<td>ND</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAF₃</td>
<td>ND</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAF₆</td>
<td>ND</td>
<td>40</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are in percentage, ND-not detected, SAP₂ - surface antigen preparation, SAF - surface antigen fraction.

Table 8  Positivity (%) of SIgGFs in the antigen detection of human sera samples by Dot-IFAT

<table>
<thead>
<tr>
<th>SIgG</th>
<th>Cord blood (n = 20)</th>
<th>Endemic normal (n = 20)</th>
<th>Mf +ve (n = 55)</th>
<th>Symptomatic (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIgG</td>
<td>ND</td>
<td>55</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SIgGF₁</td>
<td>ND</td>
<td>45</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SIgGF₃</td>
<td>ND</td>
<td>45</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SIgGF₆</td>
<td>ND</td>
<td>35</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are in percentage, ND - not detected, SIgGF - surface immunoglobulin G fractions.
The Table 8 shows the results of Dot-IFAT experiments conducted to evaluate the utility of SIgG or SIgGFs in the detection of circulating filarial antigens from human patients infected with *W. bancrofti*. A 100% positivity was observed in all the SIgGFs with mf +ve as well as symptomatic patients.

No visible reactions were observed with any of the SIgGFs against cord blood. But a significant number of false positive reactions were observed with sera from endemic normal which was high against SIgG and low against SIgGF<sub>6</sub>. In both the antibody and antigen detection tests, the observation on the 100% positivity with all forms of patients and relatively lesser false positive reactions with endemic normal with the SAF<sub>6</sub> (29 KDa)/SIgGF<sub>6</sub> draw attention in their utility for immunodiagnosis of bancroftian filariasis.