Brachylaima cribbi (Digenea: Brachylaimidae): scanning electron microscopical observations of the life-cycle stages

A.R. Butcher1*, J.K. Brealey2, D.I. Grove1 and R.B. Dymock2
1Department of Clinical Microbiology and Infectious Diseases and 2Department of Histopathology, Institute of Medical and Veterinary Science, The Queen Elizabeth Hospital, Woodville 5011, South Australia, Australia

Abstract

Brachylaima cribbi is a recently described species of terrestrial trematode that infects mammals and birds with helicid land snails as its first and second intermediate hosts. The adult worm is 2.5–6.0 mm long by 0.5–0.8 mm wide being a long slender cylindrical worm with oral and ventral suckers in the anterior quarter and genital pore in the posterior quarter. Scanning electron microscopy shows that there is a dense covering of tegumental spines at the anterior end which diminishes towards the posterior extremities of the worm. Development of spines was observed in juvenile and mature adult worms. In young worms 1–3 weeks post infection (wpi) spines appear as buds with a serrated edge each having 1–4 spikes per spine. As the worm ages the spines broaden and by 5 wpi the number of spikes per spine increases to an average of 8.1. The serial development of oral sucker papillae in the cercaria, metacercaria and adult worm was observed with the finding of an elongated papilla with a bifurcated tip on the cercaria becoming a shorter and thicker elongated papilla with a large central stoma on the metacercaria. In the adult worm, this papilla becomes dome-shaped with a small central stoma. For some of these papillae a cilium could be seen extended from the central stoma. Other life-cycle stages illustrated were the hatched egg with an extruded egg membrane minus an operculum and a portion of the branched sporocyst dissected from the digestive gland of the land snail Theba pisana showing a terminal birth pore. Scanning electron microscopy morphological features of the adult worm observed for the first time in a Brachylaima were the unarmed cirrus extended from the genital pore with released sperm present and the Laurer’s canal opening visible in tegumental folds on the dorsal surface approximately 300 μm posterior to the genital pore.

Introduction

Brachylaima cribbi Butcher & Grove, 2001 is a recently described terrestrial trematode of the family Brachylaimidae that infects mammals and birds with land snails as the first and second intermediate hosts (Cribb, 1990; Cribb & O’Callaghan, 1992; Butcher & Grove, 2001). This intestinal fluke has been reported to infect humans in South Australia (Butcher et al., 1996, 1998) and a laboratory life-cycle has been established with eggs recovered from human faeces (Butcher & Grove, 2001). This life-cycle has allowed the study of the morphological features, the host–parasite relationship of the parasite...
and the naming of the new species. The genus Brachylaima Dujardin, 1843 is a large and confused genus with many species lacking complete morphological and life history data (Ubelaker & Dailey, 1966; Butcher & Grove, 2001).

Scanning electron microscopy (SEM) is an important tool for the study of the surface topography of life-cycle stages of worms and can assist in taxonomic classification as well as provide an insight into the development and functionality of structures. There have been numerous studies of the surface structure of digeneans since the pioneering work of Threadgold (1963) on the tegument and surface features of Fasciola hepatica. Brachylaima aequans and B. fascatus are currently the only species of the 70 or more belonging to the genus Brachylaima having any reports of SEM studies of their surface topography (Zdarska & Soboleva, 1980; Zdarska et al., 1988; Zdarska, 1994). Many of the adult worms and larval stages in the genus are morphologically similar and species separation is often based on host specificity, geographical location and minor differences in morphological features. A considerable number of the species were described and named in the 1800s and early 1900s and lack complete morphological detail of all life-cycle stages, host range, cercarial chaetotaxy and SEM morphology. There has been no definitive study undertaken to resolve these uncertainties in many of the species in the genus. Consequently, progress has been stilted in the characterization of new species and the accurate identification of known species of Brachylaima from different hosts and geographical location as well as studies of the host–parasite relationship.

Since the reports of human infections with Brachylaima cribbi in South Australia (Butcher et al., 1996, 1998) there has been a need to increase our understanding of this parasite and the genus. Improvements in our current knowledge of the taxonomic characteristics of B. cribbi will assist the ability of other investigators to correctly identify this organism in animals and humans from other locations throughout the world. The aim of the present study was to observe the surface features of all the life-cycle stages of B. cribbi in order to further the understanding of this significant genus of terrestrial digeneans as well as assist in the future diagnosis of this newly described parasite.

Materials and methods

Life-cycle stages of Brachylaima cribbi were obtained from a laboratory life-cycle established by using eggs recovered from faeces of an infected human (Butcher & Grove, 2001). Cercariae were collected from infected helicid land snails, Theba pisana Müller, by placing the snails in a Petri dish with a thin film of distilled water. Emerging cercariae were collected and washed with distilled water. Metacercariae were dissected from the kidneys of infected laboratory-reared Helix aspersa Müller and washed with distilled water. Adult worms were dissected from the small intestine of C57BL/6j mice at weekly intervals from 1 to 5 weeks post infection (wpi) with B. cribbi metacercariae. Worms were washed with sterile phosphate buffered saline before fixation.

Cercariae, metacercariae and adult worms were fixed at room temperature for a minimum of 1 h in 4% formaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Cercariae were placed on 200 nm polycarbonate filters (Nucleopore, Pleasanton, California) to prevent their loss during fixation and dehydration. To reduce the effect of fixation contraction on the metacercariae and adult worms, selected specimens were heat-killed by rapid application of near boiling saline followed immediately by washing with excess cold saline. These specimens were then fixed with 4% formaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. All specimens were post-fixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 for 30 min, followed by dehydration through a graded series of ethanol concentrations then critical point dried in CO₂ using a Hitachi HCP-2 critical point drier. Dried specimens were mounted on aluminium stubs using double sided tape and coated with gold at 3.5 mA for 5 min in an ion-sputtering apparatus (Eiko Engineering, Mito City, Japan). Specimens were examined in a Hitachi S-520 scanning electron microscope at an operating voltage of 25 kV.

Tegumental spine development was observed in randomly selected adult worms at 1, 2, 3 and 5 wpi. Individual spines were viewed on SEM photomicrographs taken at a magnification of 3000 x. The morphology of the spines and the number of spikes per spine were recorded for spines on the lateral margin of the worms at the level of the oral and ventral suckers and slightly anterior to the genital pore. Statistical differences for spikes per spine versus worm age and location on the body surface were compared using one-way ANOVA with Tukey’s post test using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, California, USA).

Results

Adult worm (figs 1–3)

The general surface features of B. cribbi are of a long slender cylindrical worm ranging in length from 2.5–6.0 mm by 0.5–0.8 mm wide. The oral and ventral suckers are located in the anterior quarter of the worm with the genital pore in the posterior quarter (fig. 1A). In a mature worm (greater than 3 wpi) there is a dense covering of tegumental spines on the anterior body but the number of spines diminishes towards the posterior extremity and they are almost absent distal to genital pore. Spines on the anterior body form concentric rings around the tegument but are absent from the lateral and ventral surfaces of the oral and ventral suckers and the surrounding surface of the genital pore (fig. 1B,C). Below the genital pore the tegument has a scaly appearance with two further orifices present, the Laurer’s canal pore and the excretory pore (fig. 1D,E). The Laurer’s canal pore is located on the ventral surface approximately 300 μm below the genital pore. It is seen in the tegumental folds as a pore of 2–4 μm in diameter with sperm or other unidentified material occasionally visible in the orifice. The excretory pore is located at the posterior extremity of the worm with the tegument forming tight concentric bands around the pore.
Fig. 1. A–E. Brachylaima cribbi adult worm. A. ex C57BL/6J mice 5 weeks post-infection showing oral (os) and ventral suckers (vs) and genital pore (gp). Bar = 500 μm. B. Ventral view of the oral and ventral suckers showing rows of tegumental spines. Bar = 100 μm. C. Genital pore showing extended cirrus (c) releasing sperm (s). Bar = 100 μm. D. Laurer’s canal pore (lp) on the dorsal surface approximately 300 μm posterior to the genital pore. Bar = 10 μm. E. Excretory pore at the posterior extremity of the worm. Bar = 10 μm.
The oral sucker (fig. 1B) is sub-terminal to the anterior margin of the worm, being a muscular ovoid disc with a tight constriction at the anterior aspect. Numerous dome-shaped papillae with a small central stoma (fig. 2C,D) or without a stoma (fig. 1C) are seen on most areas of the body but are predominantly found around the oral and ventral suckers and the genital pore. A single cilium can be seen extending from the central stoma of some of the dome-shaped papillae (fig. 2D). The ventral sucker is seen as a large raised muscular disc devoid of spines and with a scattering of dome-shaped papillae around the circumference and on the ventral surface. The physical size of the sucker results in a broadening of the worm at this point and is generally the widest aspect of the worm.

The tegumental spines project from the worm’s surface as buds to form plates with a serrated edge each having a varying number of spikes per spine depending on the age of the worm (fig. 3A–E). This is quantified graphically in fig. 4. The average number of spines examined was 120 at the oral sucker level, 69 for the ventral sucker level and 26 at the genital pore level. At each of the worm ages there was no significant difference in the number of spikes per spine at any of the levels evaluated. The only observed difference was that the density of the spines at the level of the oral and ventral suckers was greater than that at the genital pore level. Spines of worms 1 wpi are seen as tightly packed buds with an average of 4.1 spikes per spine. As the worm ages to 2–3 wpi the existing spines broaden and new spines appear which have a single spike. The average number of spikes per spine decreases to 3.8 and 3.6 spikes per spine respectively due to the increase in the number of single spiked spines. In mature adult worms 5 wpi the spines become fan-shaped, are firmly held by the surrounding tegument, and have an

Fig. 2. A–D. Series showing the development of papillae on the oral sucker in cercariae, metacercariae and adult worms of *Brachylaema criibi*. Bars = 2 µm. A. Cercaria: elongated papilla with bifurcated tip. B. Metacercaria: ventral view of elongated papilla with central stoma. C. Adult worm: dome-shaped papilla with central stoma. D. Adult worm: cilium extending from the central stoma of a dome-shaped papilla.
Fig. 3. A–E. Series showing development of tegumental spines in *Brachylaima cribbi* adult worms from 1 to 5 weeks of age. A–C. Spines at the level of the oral sucker at (A) 1 week post-infection (wpi), (B) 3 wpi and (C) 5 wpi. Bars = 10 μm. D–E. Spines at the level of the ventral sucker at (D) 3 wpi and (E) 5 wpi. Bars = 10 μm. F–G. *B. cribbi* eggs. Bars = 10 μm. F. Egg showing an absent operculum. G. Extruded egg membrane after hatching of the miracidium. H. Highly branched sporocyst removed from the digestive gland of *Theba pisana* 12 wpi. Bar = 100 μm. Inset showing cercarial birth pore (bp) at the terminal end of a sporocyst branch. Bar = 50 μm.
average of 8.1 spikes per spine. There was a significant difference in the number of spikes per spine in the mature adult worms at 5 wpi when compared with young adult worms at 1–3 wpi (P < 0.01).

Egg (fig. 3E,G)

Eggs of *B. cribbi* are asymmetrical having one side slightly flattened and measure 26–32 \( \mu m \times 16–17.5 \mu m \). The shell has a smooth surface with no patterning and has a raised knob or roughening of the shell at the abopercular pole. The operculum is inconspicuous; it is high on the anterior end and the join is seen as a fine etching in the shell. After the miracidium has hatched, the operculum is generally absent and extruded egg membrane may protrude from the opercular aperture.

All attempts to examine the miracidium from *in vitro* hatched eggs were unsuccessful.

Sporocyst (fig. 3H)

The sporocyst is a highly branched network of tubules which are intertwined throughout the connective tissue of the digestive gland of the infected snail. Scanning electron microscopy of a portion of sporocyst dissected from an infected *T. pisana* showed a network of tubules with no distinctive surface patterns or structures. Bulges are present along the tubules where cercariae are present and a birth pore can be seen at the terminal end of the sporocyst branches.

Cercaria (fig. 5A–D)

Cercariae have an elongated spinose body with prominent oral and ventral suckers of approximately equal size and a short simple tail (fig. 5A). The prominent surface feature of the cercaria is an abundance of papillae clustered around the anterior extremity, on the margins and ventral surfaces of the oral and ventral suckers, in the buccal cavity, along the lateral margins and on the anterior dorsal surface. There are three types of papillae identifiable on the cercariae. The most prevalent is an elongated papilla with a bifurcated tip. These are seen on the oral and ventral suckers and lateral, ventral and dorsal body surfaces. The second papilla is dome-shaped, being mainly confined to the anterior body at the level of the oral sucker. The third papilla is crater-shaped and has a raised rim with a hollow centre. Only three pairs of these crater-shaped papillae were seen. They correlate with light microscopical studies of cercarial chaetotaxy patterns with one pair at the AIL (anterior lateral) level (fig. 5B) and two pairs at the ML (medial lateral) level (see Butcher & Grove, 2001). Other surface features include secretory gland ducts, which are seen as pits in the tegument on each side of the anterio-lateral area of the oral sucker (fig. 5C), and two excretory pores at the posterior tip of the tail (fig. 5D).

Metacercaria (fig. 5E–I)

Metacercariae are leaf-shaped with oral and ventral suckers of approximately equal size (fig. 5E). The tegument is smooth and has a covering of fine spines which diminishes towards the posterior extremities. Oral and ventral suckers are round to ovoid muscular discs which can be seen relaxed or tightly contracted. In older larvae (greater than 40 wpi), the oral sucker becomes...
SEM of Brachylaima cribbi
constricted at the anterior margin and resembles that of an adult worm. Also, tegumental spines of older metacercariae are seen as buds on the tegument surface (fig. 5I) resembling those seen in young adult worms at 1 wpi. The genital pore is visible on the ventral surface in the posterior third of the body.

The two predominant types of papillae seen on the metacercariae are dome-shaped papillae and elongated papillae with a central stoma (fig. 5E,G). These types are found primarily on and around the oral and ventral suckers. It is possible that there are two other types of papillae. These are crater papillae with and without a central projection. They are confined to the oral sucker particularly around the anterior aspect and could be contracted elongated papillae.

**Discussion**

This study of the SEM surface features of *B. cribbi* has shown for the first time the development of tegumental spines in adult worms, the changes in papillary structure from cercaria to adult worm, and the morphological features of the cirrus and Laurer’s canal pore of the adult worm.

When the development and growth of the tegumental spines on the adult worm is considered, it can be seen that there is a significant change in spine morphology as the worm grows from 1–3 wpi compared with worms at 5 wpi. It is apparent that for the first three weeks after infection there is considerable growth and development of the worm’s surface structures, especially the tegumental spines and therefore these worms are considered to be juvenile adults. This correlates with the allometric variation reported on light microscopical measurements of adults 1–3 wpi that were compared with adult worms greater than 3 wpi (Butcher & Grove, 2001). An adult worm can not be considered fully developed and therefore provide accurate morphological data until it is greater than 3 weeks old at which time the spines are multi-pointed and have an average of 8 spikes per spine. Growth of tegumental spines and the changes seen in their morphology has been reported for many other trematodes such as for the heterophyid *Stictodora tridactyla* where the spines transform from single to multi-pointed as the worm ages (Abdul-Salam et al., 2000). Spines having 1–3 ribs with sharp points have been described by Zdarska et al. (1988) in 6–8-day-old *B. aequans*. That report represents the only description of spines of an adult *Brachyaima* and is clearly that of a juvenile rather than a mature adult worm. The size, shape and number of points corresponds to a 1-week-old *B. cribbi* but further comparison is not possible as the authors did not observe older worms.

Knowledge of the morphology and growth of spines could be used to determine the age of a population of worms in naturally infected hosts. In the case of *B. cribbi*, if the host has been recently infected, the spine morphology will show an average of 3–4 spikes per spine indicating the presence of young worms. Conversely, if the worms present have multi-pointed spines with greater than 8 spikes per spine, the worms can be considered to be fully developed mature adults. This information could be useful in determining if *B. cribbi* can develop to maturity in a particular definitive host and maintain an infection for at least 5 weeks in that host. Furthermore, this method of assessing worm maturity can be used to ensure that mature adults are being studied when comparing morphometric data from various naturally infected definitive hosts.

Different types of papillae were seen and described on the cercariae, metacercariae and adult worms. The serial development of oral sucker papillae from cercaria to adult worm was observed with the finding of an elongated papilla with a bifurcated tip on the cercaria becoming a shorter and thicker elongated papilla with a large central stoma on the metacercaria. This can only be appreciated by comparing figures 2B and 5G insert which are photomicrographs of the same papillae seen from en face and lateral views, respectively. In the adult worm, this papilla becomes dome-shaped with a small central stoma. Occasionally, a cilium could be seen extended from the central stoma. We speculate that the cercarial elongated papilla represents a sensory cilium covered with tegument to protect it from the potentially damaging effects of their terrestrial environment. As metacercariae mature, these papillae become shorter with a thickening of the tegument but still retaining an opening to the cilium within. In the transformation from metacercaria to adult worm, the oral sucker papilla changes from an elongate papilla to a tegument-covered, dome-shaped papilla with a small stoma through which the cilium can protrude, presumably to receive chemical and or mechanical stimuli. This is consistent with the hypothesis put forward by Zdarska & Soboleva (1980) that cercariae of terrestrial trematodes lack free cilia but appear to protect the sensory cilium by covering it with tegument to withstand mechanical and dehydration damage. Zdarska & Soboleva (1980) did not pursue their investigation to the adult worm stage but we have and our findings support their thesis.

Ciliated and non-ciliated dome-shaped papillae have also been described in other adult trematode worms (see Chai et al., 1998, 2000; Woo et al., 1998; Abdul-Salam et al., 2000; Dangprasert et al., 2001). The dome-shaped ciliated papillae of *B. cribbi* adult worms appear similar to those described in other trematodes except that the cilium is rarely seen extended from the stoma. The extended cilium could be the result of processing or fixation of the worm but what it does reveal is that a cilium is present within these papillae. In *B. aequans* adult worms, Zdarska et al. (1988) described the presence of two types of papillae; those with very short finger-like processes and papillae with no opening or cilium. It is difficult to know if the papillae with short finger-like processes seen on *B. aequans* are the same as the dome-shaped papillae with central stoma of *B. cribbi*. Further studies of the papillary structure of *B. aequans* and other *Brachyaima* spp. are required.

The metacercarial papillae of *B. cribbi* and *B. aequans* appear to have few similarities. The finger-like papilla, described by Zdarska & Soboleva (1980) for *B. aequans*, appears equivalent to the elongated papilla of *B. cribbi* but does not have a central stoma. Also, there are no dome-shaped papillae on *B. aequans* and the possible crater-like
papillae seen on B. cribbi appear to have been described on B. aequans as elevations with openings and small depressions laterally to the oral sucker. Zdarska (1994) has described the surface structures of B. fuscatus metacercariae. Seven different types of papillae were found on the body surface and oral and ventral suckers and the tegument is described as being an interconnected network of ridges. In comparison, B. cribbi has a strikingly different tegumental structure, being smooth and devoid of any folds or ridges. Further, of the papillae present on B. cribbi, only the dome-shape and hollow pit or craters with and without projections appear to show any similarity to those of B. fuscatus.

In summary, this study of B. cribbi surface structures has highlighted the lack of comparable data for other species of the genus. We have shown that the morphology of the spines can be used to determine the level of maturity of an adult worm and have described the development of oral sucker papillae from the cercarial stage to the adult worm. Other significant structural features seen were the hatched egg, sporocyst and the Laurer’s canal opening and extended cirrus releasing sperm in adult worms.

References


(Accepted 11 January 2002) © CAB International, 2002