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Schistosomiasis in modern and ancient tissues

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Abstract

Schistosoma worms are ancient, infecting man in both in the past and modern times. Today they infect more than 300 million people, mainly in the developing world where lifestyle is still similar to their ancestors. As part of an epidemiology study of Schistosomiasis, ancient tissues from the Manchester Museum and collections around the world are now being tested for the disease. Many problems have arisen whilst working with the ancient tissues, ranging from accessibility to its preparation for tests. However, many of the problems encountered have now been overcome enabling immunocytochemistry to be successfully applied to infected modern and ancient tissues, suggesting that schistosoma antigens can survive thousands of years. Immunocytochemistry has continued to be the predominant test used for this study, although DNA, ELISA and microsomal strips are also being explored

Introduction

Schistosomiasis is endemic today, infecting more than 300 million people, mainly in the developing world. Although most *Schistosoma* only infect animals, humans are infected by certain species, particularly *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*.

Both literary and physical evidence have shown that this is not a new disease. Such examples are the classic symptom, blood in the urine, described in the Ebers Papyrus as the *aaa* disease (Farooq, 1973), ancient Egyptian boys described as becoming men when blood was seen in their urine (Despommier et al., 1995), histological work showing worms (Millet et al., 1980), the ELISA (Miller et al., 1993) and radiology (Isherwood et al., 1979).

The aim of this study is therefore to create an epidemiological profile of schistosomiasis in Egypt over the past 5,000 years. However, as the aforementioned techniques can be impractical when working with ancient tissues on a large scale as they may be very expensive (radiology and ELISA), or low in sensitivity (histology) an alternative test was sought that would be robust, cost effective, reproducible and sensitive. Immunocytochemistry meets all the aforementioned criteria, and has been successfully applied to both modern and ancient tissues in this study (Rutherford, 1997, 1999, 2000, 2002, Lambert-Zazulak et al., 2003). The application of immunocytochemistry to ancient tissues often only reports the demonstration of cellular components (Fulcheri et al., 1992; Nerlich et al., 1993). Most studies also embed ancient samples in paraffin wax. In contrast, this study embeds both modern and ancient tissues in an alternative medium called glycol methacrylate (GMA, Taab, UK) in order to detect schistosoma antigens. GMA resin was chosen as preparation in hot wax often has deleterious effects upon the tissue, causing diffusion, loss and even chemical alterations to the antigens of interest. As the ancient schistosoma antigens will already be degraded and only present in very small quantities, such high temperatures are avoided as (GMA) polymerizes at 4°C. The hardened resin also allows much thinner tissue sections to be cut (2µm) which enhances the sensitivity of the test, often producing intense reactions (Heryet and Gatter, 1992).

Although immunocytochemistry is now used upon samples thought to harbor the ova and worms, other diagnostic tests have also been investigated, namely the Enzyme linked immuno sorbent assay (ELISA) (Miller et al., 1993). As the ELISA target is a circulating anodic antigen (CAA) regurgitated from the worms' gut, it should be present in all vascular tissues of the body. The test, however, does have limitations as the CAA's are only present during the occurrence of an active infection as they are degraded each day by the liver (Miller et al., 1993).

Other tests such as histology, EITB (Al Sherbiny et al., 1999) and DNA analysis were also explored as additional techniques if immunostaining results were positive. Histology was used to show the general morphology of tissue samples, whereas the EITB targeted antibodies rather than antigens in

the tissue. DNA has been successfully extracted from several ancient samples, from which a small region of the schistosome's Cytochrome oxidase C (COI) gene has been amplified. Sequencing of the ancient *S. haematobium* DNA has also been successful, producing a 97% match to the modern sequence found today.
Although several procedures have been explored, the mainstay of the research is the development of immunocytochemistry, with ELISA and DNA work used to support initial results. Immunocytochemistry is now being used extensively at the University of Manchester by Rutherford (2002) on a large number of ancient samples as part of the epidemiological study of schistosomiasis. The immunostaining results obtained from the initial study and the protocol now used is briefly outlined below.

Materials and methods

Modern tissues

The liver of both *S. mansoni* infected and uninfected mice, *S. haematobium* infected and uninfected hamster livers and *S. mansoni* and *S. haematobium* worms were used as positive and negative controls.

Mummified tissues

The initial 50 samples were collected in context of merely establishing what experimental formats were the most appropriate. Several of the samples had no provenance but this was not relevant at this point in the research process as the main aim was to perfect and establish research guidelines for the large-scale study. As bladder, liver and visceral samples were the preferred target for immunocytochemistry only 24 of the 50 samples were suitable for immunostaining.

Antisera

Polyclonal antisera directed towards an array of epitope sites found on *S.mansoni* (raised in rabbit) and *S. haematobium* (raised in hamster) antigens were used. These were supplied by Prof. M.Doenhoff, University of Bangor, Wales. The secondary antibody was conjugated to biotin. To visualize the reaction, the protein streptavidin conjugated with fluorescein, FITC was used (Amersham, UK).

Preparation of modern and ancient tissues for blocking in immunoresin

The modern tissue samples were prepared according to the manufacturer's instructions (Taab, UK). In contrast, small pieces of ancient tissue (2mm) were placed into 1 part conditioning solution (Surfacem, UK) to 99 parts distilled water. The conditioner contains no perfume, preservatives or color which may interfere with the immunocytochemistry in some way. After being left for 7 days at 4°C the tissues were gently rinsed 3 times with distilled water and placed in 50% alcohol for a further 5 days at 4°C. This was then replaced with 70% then 90% alcohol for a minimum of 1 day each time. Two changes of acetone were then used; firstly overnight at 4°C, followed by a further 8 days at 4°C. GMA immunoresin was then used (X3) for 3 days each time at 4°C. The samples were then blocked in hardened resin. Tissues impregnated with sand and resin proved difficult to section unless the conditioning solution was replaced with a dilute solution (2.5%) of hydrofluoric acid (Sigma, UK). The samples were left to soak at room temperature for 4 weeks. Once removed from the acid the samples were washed carefully with distilled water then placed into 50% alcohol. The remainder of the aforementioned protocol was followed from this point.

Immunostaining

Before any antiserum was applied to the sections, a blocking serum was applied to each section (10mls PBS pH 7.4, 0.05g Bovine Serum Albumin, 150µl donkey serum (*S. mansoni*) or goat serum (*S. haematobium*), 50µl Triton X). An indirect immunostaining protocol was then followed. Staining of any DNA present in the tissue sections was also carried out whilst the tertiary streptavidin was incubating on the sections. This was achieved with the application of Hoechst-bisbenzimide stain, (Sigma, UK) which was applied to each section 2 minutes before the streptavidin was washed off.

Results

Reaction of antisera upon modern samples

The antisera produced excellent immunostaining results on *S. mansoni* infected mouse liver, *S. haematobium* infected hamster liver and both species of worms. The Hoechst stain bound strongly between the A and T residues within the nuclei of the modern tissue, ova and worms.

Examples of positive reactions upon mummified tissues

As well as producing positive results for schistosome antigens the DNA specific stain (Hoechst) also bound in the same areas as the antiserum, which warrants DNA analysis.

Manchester Museum 7700/1766, c. 1,800BP, Fayoum Oasis (Middle Egypt), bladder

Discrete staining to both ova and worms has been seen. Some worms retained their form enabling them to be identified as the *S. haematobium* species. The ELISA supports these results and indicates an active infection (CAA 80ng/ml).

Manchester Museum 7700/1777 Asru, c.2,750BP, Luxor (Upper Egypt), bladder/Intestine

Immunostaining is precise to oval bodies thought to be eggs. The ELISA showed that the intestinal tissue also taken from

Asru was positive for CAA (455ng/ml) and the EITB showed a pale positive reaction for *S.haematobium* antibodies.

Manchester Museum 7700/13010/5, No Provenance, bladder

Discrete staining to several oval shapes was seen. All sections from this mummy have been consistently positive in this study suggesting that the bladder had a high yield of deposited schistosoma ova.

Manchester Museum 7700/9430, No Provenance, Canopic jar material

Positive staining to clusters of oval shapes was achieved. ELISA results supported this by showing a positive result for CAA (22ng/ml).

Leicester Museum 528/1981.1885, mummy 1, c.2,700BP, Akhmim (Middle Egypt), pelvic tissue

Positive staining to small clusters of ova was seen. DNA analysis has amplified a small fragment of the *S.haematobium* Cytochrome oxidase C subunit 1(COI) from this sample.

Leicester Museum 528/1980.1882, mummy 2, c.2,700BP, Akhmim (Middle Egypt), pelvic tissue

Positive staining to clusters of ova was achieved. This is unlike the solitary ova and worms found in other mummy samples.

Discussion

Only a few uncontaminated ancient samples dating to the 12th Dynasty were prepared with the same ease as the modern tissues. The remaining ancient samples had varying amounts of mummification resins and gritty silica particles enmeshed within them. A dilute solution of hydrofluoric acid, enabled (2µm) intact sections to be cut with ease. Experiments with both modern and ancient tissues have shown that at the (2.5%) dilution, the acid does not disrupt the antigenic epitopes, as positive staining still occurs. Although this pre treatment is now an option, because of its hazardous nature tissues without resin or sand are sought first, but if not available, any samples with sand present are soaked in an acid solution.
Although a few examples of worms found in 7700/1766 still resembled modern *S. haematobium* worms the majority of ova and worms were often distorted in shape. However, this is not surprising as most of the samples were in excess of 1,000 years old. Regardless of shape, positive reactions did still occur suggesting schistosoma antigens are present after many centuries. The DNA specific stain also indicated that DNA was still present to some degree in the ancient tissues, particularly within the immunostained ova. One could hypothesise that the chitin shell had played some role in protecting the DNA within the ova. Further research is now underway to investigate this hypothesis.

Although the results reported in this paper do confirm the presence of schistosome parasites in both Upper and Middle Egypt no real pattern can be seen. The only conclusion that can be made is that the *S. haematobium* parasite was present in Middle Egypt over 2,700 years ago at Akhmim as seen in the pelvic tissue samples taken from the Leicester mummies. Leicester 1, known as Bes en Mut was a priest in the great temple of Min, whereas Leicester 2, named Ta Bes was a teenage girl. The amount of ova was markedly different between these two mummies with the teenage girl having a high distribution whereas the priest had only small clusters. This mirrors the patterns seen in patients today as children and teenagers are heavily infected as their activities often entail more contact with water and acquired immunity is thought to occur with adulthood. The *S. haematobium* species was also present in the Fayoum Oasis 1,800 years ago as seen in the bladder tissue of 7700/1766. The mummy called Asru also confirms the presence of *S. haematobium* in Luxor (Upper Egypt) some 2,700 years ago. Today *S. haematobium* is still prevalent in both Middle and Upper Egypt whereas *S.mansoni* is almost absent in the upper region. As the EITB results display only a pale band for *S.haematobium* antibodies, further tests at the DNA level are needed to reinforce which species is present in Asru's bladder. The positive results seen in the cases of mummy 7700/13010/5 and the canopic jar material numbered 7700/9430 has limited impact regarding the epidemiology study. This is because the only information known about each sample is that mummy 7700/13010/5 may have been a scribe and the inscription on the canopic jar suggests that this person was a steward. The overall results do highlight the fact that in contrast to today where mainly village dwelling people are affected by schistosomiasis, there was probably no real differences between the classes as they would have used the river and even had fresh water pools in their gardens.
Now standard procedures have been established sample collection has shifted in context, to only provenanced samples that could be mapped to a certain place and time. Such provenanced samples are seen in the author's continuing work, where medieval samples collected from Sudanese Nubia dating to circa 1,500 BP have now been immunostained. Two samples have shown positive results and are being investigated further. In addition, preparations for immunocytochemistry tests to be carried out on a large group of samples from the 48 Greco-Roman mummies found in the Dakhleh Oasis are also underway. This particular group is an excellent source of material as many of the samples have been taken from the liver, colon, intestines and coprolites, all of which harbor both the schistosome worms and ova. After many provenanced samples have been tested, a distribution pattern should emerge, which in turn will contribute valid information in the field of anthropology.

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