

## THE DIAGNOSIS OF SCHISTOSOMIASIS IN MODERN AND ANCIENT TISSUES BY MEANS OF IMMUNOCYTOCHEMISTRY

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Although *Schistosoma* worms infect millions of people today they were evident in ancient Egyptian times, with one of the classic symptoms "haematuria" being described in various medical papyri. A current epidemiology study means diagnostic tools that can be applied to ancient dehydrated tissues are now needed. To overcome this immunocytochemistry has been used, producing positive staining to *S. Mansoni* and *haematobium* antigens in both modern and ancient tissues, suggesting that *Schistosoma* antigens may still be present after thousands of years. Initially, the presence of silica particles enmeshed within ancient tissues made sectioning difficult but this has been overcome by soaking the tissue in a weak solution of hydrofluoric acid which does not disrupt the epitopes whilst the silica dissolves. The application of Immunocytochemistry to ancient tissues has proved to be relatively cheap to perform and may preclude other more complicated tests.

**Key words:** Histology, paleopathology, paleoparasitology, mummies.

*Hoy en día las lombrices de schistosoma infectan a millones de personas, pero esto también ocurrió en el antiguo Egipto ya que los papiros médicos describen los síntomas clásicos de "haematuria". Para realizar estudios epidemiológicos se necesitan elementos diagnósticos que se puedan aplicar a tejidos deshidratados antiguos. Esto se solucionó utilizando la inmunocitoquímica la que produjo una tinción positiva para los antígenos de S. Mansoni y haematobium, tanto de tejidos modernos como de los antiguos. Inicialmente, la presencia de partículas de sílica dificultó los cortes histológicos, pero esto se solucionó al someter las muestras a un baño de ácido hidrofúrico diluido, el que no daña los antígenos epitopos mientras que la sílica se disuelve. La aplicación de la inmunocitoquímica a tejidos antiguos es relativamente barata y puede servir de prelude a otros análisis más complejos.*

**Palabras claves:** Histología, paleopatología, paleoparasitología, momias.

Schistosomiasis is not a new disease and although first described in depth in the early 1900's (Bilharz 1852; Logan 1905; Sambon 1907), it was evident in ancient Egyptian times, with one of the classic symptoms "urine in the blood" being described in the Ebers papyrus, as the "ahaa" disease (Farooq 1973). Such haematuria is also mentioned some 50 times in various other medical papyri (Contis and David 1996). The ancient Egyptians also wrote of boys becoming men when blood was seen in their urine, as this was likened to the young female's first menstruation (Despommier et al. 1995). Also archaeological evidence such as wall reliefs, hieroglyphs and papyri all confirm that their lifestyle encompassed activities such as bathing, fishing and playing in the Nile, and this combined with bad sanitation habits, would make almost everyone susceptible to this infection.

Scientific evidence also supports the above as the disease has been positively identified in ancient tissues using histological methods (Ruffer 1910). More recently, the presence of *S. haematobium* antigens have been found in the tissues taken from the shin of a predynastic mummy, some 5000 years old, by means of an enzyme-linked immunosor-

bent assay (ELISA) (Deelder et al. 1990; Miller et al. 1993). Radiology has also suggested the presence of schistosomiasis in mummies by showing one of the classic symptoms of *S. haematobium*, namely the calcification of the bladder (Isherwood et al. 1979).

As a consequence of the schistosomiasis tissue bank established at the Manchester Museum by Dr. A.R. David, previously described in *Parasitology Today* (Contis and David 1996) a need for diagnostic tools that can be applied to ancient tissues has occurred in order to study the distribution and epidemiology of this disease. However, as many diagnostic tools are often very expensive, low in sensitivity, and often need body fluids to work they are impractical when working with ancient, dehydrated tissues. Therefore to overcome such problems immunocytochemistry has been used, and successfully applied to both modern and ancient tissues. The use of Immunocytochemistry to diagnose disease in ancient tissues is not common practice, although applying it to tissues embedded in wax, to show cellular components and neurotransmitters has recently been reported (Fulcheri et al. 1992; Appenzeller 1998).

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However, preparing tissue sections in wax often has deleterious effects on the tissue, causing diffusion, loss, masking and even chemical alterations to the antigens of interest, after being subject to high temperatures. To ensure that tissue sections are not subject to high temperatures, an alternative embedding material can now be used called glycol methacrylate (GMA) which not only polymerizes at low temperatures but also allows the tissue sections to be cut a lot thinner, which also enhances sensitivity and often produces intense reactions (Heryet and Gatter 1992). This embedding protocol is more suitable to this type of study as high sensitivity is required in order to detect antigens thousands of years old.

To combat the fact that the schistosome antigens may be present in the ancient tissues but somewhat degraded, and therefore at smaller levels, a very sensitive immunostaining procedure that is easily visualized had to be used. Using an indirect fluorescence staining protocol with antisera directed against the epitopes of *S. Mansoni* antigens, visualisation of positive staining to *S. Mansoni* and *haematobium* antigens in modern mouse tissues and a tissue sample some 50 years old from an Egyptian cadaver has been achieved respectfully.

Positive immunostaining upon ancient Egyptian tissues has also been achieved suggesting that schistosoma antigens may still be present after thousands of years. Particularly promising results have been achieved with the liver samples of a 4,000 year old mummy and the urinary/bladder tissue of a 3,000 year old mummy.

### Selecting the most Appropriate Tissues

Four ancient Egyptian tissue types were selected for investigation from the Manchester Museum collection as follows:

#### The Bladder / Urinary system

This is a specific site of infection by *S. haematobium*. It was taken from the headless mummy known as the Glasgow mummy. In a previous autopsy performed for the Manchester Museum the outer wrappings had been removed from the body (Wilkinson 1996). The tissue taken for immunocytochemistry purposes was therefore easily accessible and came from the pelvic area, where the blad-

der and urinary system are situated. Removed manually with a scalpel, this was very hard to remove as the tissue was brittle and hard in texture. The Glasgow mummy has been dated by Dr. A.R. David to (c. 1000 B.C.) making this tissue c. 3000 years old.

#### The intestines

This was taken from the viscera packages left on the upper thigh area of a female mummy whose coffin bears the name Asru dating to the XXV<sup>th</sup> Dynasty (c. 750 B.C.), making the tissue c. 2700 years old. Earlier studies of this tissue had shown it to be intestinal material and that parasitic eggs of some kind were present possibly laid by the

*Strongyloides* worm (Tapp et al. 1979). The tissue was very spongy and sticky to touch, and a strong spicy aroma was also present, suggesting that the tissue was heavily impregnated with the resins used to mummify the dead in ancient times. Again, the intestines are usually a site of heavy infection by either *S. Mansoni* or *haematobium*. The presence of eggs which have never been definitely identified also made this an excellent tissue to study, especially as many worm infections can successfully coexist, and if her lifestyle had exposed her to *Strongyloides*, *Schistosoma* infection may have also occurred.

#### The liver

The liver is another organ affected by schistosomiasis, especially *S. Mansoni*. A small section of liver held within a canopic jar was used. The jar belonged to an Egyptian male dating to the XII<sup>th</sup> dynasty (c. 1991-1785 B.C.), making the tissue c. 4,000 years old. Unlike Asru's tissue, there are no other foreign materials present which in the past has made this an excellent specimen to reconstitute, section and stain. Not only did the tissue display clear cellular characteristics with histological staining, but after utilizing the electron microscope, the presence of an unknown species of worm was found. Although it was decided that the fluke was of the genus *Fasciola hepatica*, this was by no means conclusive (Tapp et al. 1979). An added bonus to using this tissue is the absence of resins usually found enmeshed within mummified tissues as this means no external artifacts will interfere with sectioning or immunological reactions when immunostaining.



## Unspecified Tissue

The unspecified tissue was taken by means of an endoscope from the intact mummy known as 1766. Tissue from this particular mummy was chosen as previous X-ray studies showed that calcification of the bladder was present, a classic symptom of the disease schistosomiasis, especially if caused by *S. haematobium* (Isherwood et al. 1979). However, although the outer wrappings of the mummy have a hole in the left shoulder area where the endoscope can enter, it has proven difficult to access the pelvic area in which the calcification is present. Taking into account that previous research (Deelder et al. 1990; Miller et al. 1993) confirms that, schistosoma eggs and antigens may be present in many different tissue types to varying degrees, although the ideal tissue would be the bladder, tissue fragments were obtained from the thoracic cavity. The material collected from 1766 dates to the Roman period, c. 1<sup>st</sup>/2<sup>nd</sup> century AD, making the material c. 2700 years old.

## Modern tissues

The reactivity of the different antisera to be used (Alvesbrito et al. 1992; Curtis et al. 1996; Doenhoff et al. 1978; Dunne et al. 1981; Dunne et al. 1984; Dunne and Bickle 1987; Dunne et al. 1986; Dunne et al. 1991; Grzych et al. 1986) were tested upon mouse livers uninfected and infected with *S. Mansoni*. Also, when applying the antisera to the mummy tissues, they served as negative and positive controls respectively. Both the antisera and tissues were provided by Dr. M Doenhoff from the University of Bangor. In order to minimize non-specific background staining a blocking serum was applied to the tissue sections prior to immunostaining. To ascertain whether the antisera would react with *S. haematobium* epitopes, infected bladder tissue that had been blocked in wax and badly stored for some 50 years, taken from an Egyptian cadaver was used. This was provided by F. Barnett, University of Manchester.

## The reaction of the antisera upon modern tissues

Without primary antiserum, no apple green fluors were seen although the outer shell of the eggs could be seen under the fluorescent microscope as

the shell has a natural fluorescence. The positive staining achieved by all the antisera upon infected mouse liver confirmed that any of the antiserum could be used as a diagnostic tool. However, before applying any of the antisera to the ancient tissues they were applied to the Egyptian bladder infected with *S. haematobium*. Each antiserum produced positive staining, confirming that the antiserum reacts with either species epitopes and also that even after 50 years of being exposed to fluctuating temperatures and conditions, the epitopes were still present to some degree.

## The reaction of antisera upon ancient tissues

The majority of particles suctioned from the inside of 1766 thoracic cavity were probably resin as this had a natural yellow glow under the microscope. No real tissue integrity could be seen and it is therefore not surprising that there was very little positive staining. However, a few reactions occurred, where a few very pale green, oval shapes were seen. Attempts have now been made to obtain more precise tissue samples using radiology and the endoscope together. Part of the calcified bladder has now been obtained for future experiments.

The liver of Nekht Ankh showed promising results. Within the tissue, clear green staining can be seen which is distinct from the rest of the tissue. As there is no background staining these results suggest that schistosoma may have been present. However, it must be noted that, as previous studies have shown the presence of a parasitic worm that may be *Fasciola hepatica*, (Tapp et al. 1979) the positive reactions may be due to the presence of epitopes belonging to this species, as *Schistosoma* and *F. hepatica* share several antigenic epitopes. Future work will therefore have to eliminate this possibility, by applying *F. hepatica* serum to sections before the schistosoma antiserum in order to mask any such epitopes.

As the intestinal material from Asru was difficult to cut the immunostaining results were limited. Tissue was clearly seen, with fluorescent material overlapping it. Only one section showed any positive reaction, and no eggs were seen within any of the sections. This was particularly disappointing, as it is known that eggs of some kind are present in Asru's intestine (Tapp et al. 1979). However, the



positive result obtained from this study indicates that further testing will be worthwhile and therefore a lot more sections must be tested before any conclusions can be drawn.

The urinary/bladder tissue from the Glasgow mummy has produced very interesting results, with several shapes, some of which have cellular characteristics positively staining. Every section either showed general positive staining or oval shapes similar to eggs. To reinforce these results some sections had no primary antiserum applied to them and these did not stain positively. The positive results seen in the liver of Nekht Ankh and the Glasgow mummy must now be reinforced in order to confirm the presence of schistosomiasis. Reinforcement of the above results are now being pursued, utilizing several alternative protocols and equipment such as the electron microscope.

Such a test was predominately dependent upon the successful sectioning of both the modern and ancient tissues, the majority of which were embedded in immunoresin for higher sensitivity. Although there were no problems cutting the modern and ancient unembalmed tissues, the presence of resins used in the mummification process and silica particles enmeshed within certain ancient tissues made sectioning difficult. It is not known whether the silica (sand) particles are simply contamination from mining the natron which was used to desiccate the body, or simply the desert environment. It may have been deliberately placed into the resin to help with the grinding and mixing of the plant materials, such as juniper and myrrh, that made the

resins used for mummification, just in the same way it was used to quicken the grinding of corn plants to make bread (Leek 1972).

To overcome such cutting problems, such tissues are now soaked in a very weak solution of hydrofluoric acid. Experiments with both modern and ancient tissues have shown that, at the correct concentration the acid does not disrupt the antigenic epitopes as positive staining still occurs, and the ancient tissues can be cut easily as the silica is slowly dissolved. Although unembalmed tissues are preferable, if silica and resins are present this acid treatment is now an option in future studies.

### Conclusion

The application of immunocytochemistry as proved to be relatively cheap, easy to perform and has several advantages, for example, the use of immunoresin to embed the tissue allows very thin sections to be cut, thus increasing sensitivity. Also, the low temperature at which the resin polymerizes means the epitopes present in the tissues are not subject to high, often damaging temperatures needed for wax preparations. Immunocytochemistry has added a new dimension to diagnosing diseases in ancient tissues and may eventually be used to diagnose other diseases such as malaria.

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