# The Identification of Eggs of Echinococcus by Immunofluorescence Using a Specific Anti-Oncospheral Monoclonal Antibody

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# THE IDENTIFICATION OF EGGS OF *ECHINOCOCCUS* BY IMMUNOFLUORESCENCE USING A SPECIFIC ANTI-ONCOSPHERAL MONOCLONAL ANTIBODY

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*Abstract.* A relatively simple and specific test has been developed to distinguish eggs of *Echinococcus* from those of other morphologically identical taeniid species. A specific anti-*Echinococcus* oncosphere monoclonal antibody was produced which binds in an indirect immunofluorescence test to egg-derived oncospheres of *E. granulosus* but not to those of other taeniid species, such as *Taenia hydatigena, T. saginata, T. pisiformis, T. ovis, T. multiceps, or T. taeniaeformis.* Specific fluorescence was obtained with oncospheres of *E. granulosus* derived from either hatch/activated viable eggs using artificial intestinal fluid or from hypochlorite/detergent treated eggs. The potential use of this test in the study of the transmission of *Echinococcus* in Turkana, Kenya, is discussed.

Hydatidosis of humans and domesticated animals caused by infection with the taeniid metacestode of *Echinococcus granulosus* is of significant public health and economic importance in many temperate and tropical areas.[1,2] The domestic dog is the main definitive host for the adult *Echinococcus* tapeworm and is thus responsible for the majority of environmental contamination with the infective and highly resistant egg stage. In addition, the dog may act as definitive host to other taeniid tapeworms and infection with more than one species is common. However, all mature eggs from tapeworms in the family taeniidae (which includes *Echinococcus*) are morphologically identical under light microscopy, thus sampling eggs from suspected *Echinococcus* transmission sites can give only limited information about gross taeniid contamination. The ability to distinguish eggs of *Echinococcus* from other common taeniids would be very useful in epidemiological studies on echinococcosis[3] particularly in the Turkana District of Kenya which has one of the highest prevalence rates of human hydatidosis[4,5] and where a pilot control project has been initiated.[6] A previous study in this semi-arid Turkana region has shown that well water and sand from Turkana living areas are contaminated with significant numbers of taeniid eggs.[7]

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Any method of distinguishing *Echinococcus* eggs from those of other taeniids should be highly specific, appropriate for identifying very small numbers of eggs and ideally amenable to use in a field laboratory. Immunofluorescence studies on the egg-derived oncosphere using specific polyclonal antisera has been shown to be a simple and rapid technique for the immunodifferentiation of eggs of *T. hydatigena* and *T. pisiformis* in a model system.[8] The present report describes the preparation of a species-specific anti-*Echinococcus* oncosphere monoclonal antibody to identify oncospheres of *Echinococcus* in an indirect immunofluorescence test.

## MATERIALS AND METHODS

### *Parasites*

Adult *Echinococcus granulosus* tapeworms were obtained postmortem from naturally infected dogs in Turkana. Dogs were killed by injection with sodium pentabarbitone and their small intestines removed, washed in tap water and opened longitudinally. Intestines containing *Echinococcus* tapeworms were soaked for 2 hr in saline (0.85% NaCl) at ambient temperature (30°C) to allow the majority of worms to drop off.[9] Whole worms and free proglottids were repeatedly washed in saline by sedimentation and decantation[10] and stored in saline containing 0.02% merthiolate.

Mature *T. pisiformis* and *T. hydatigena* tapeworms were obtained from dogs in Liverpool by purgation with arecoline acetarsol 12–15 weeks after experimental monospecific infection. Tapeworms of *T. ovis* and *T. multiceps* also were obtained after arecoline purgation, from naturally infected farm dogs in North Wales. A *T. saginata* tapeworm was obtained from a patient in the UK after treatment with niclosamide followed by a saline purge. Eggs of *T. taeniaeformis* were kindly given by Dr. L. Harrison (Centre for Tropical Veterinary Medicine, Roslin, Scotland).

Protoscoleces of *E. granulosus* were taken under sterile conditions from sheep hydatid cysts obtained from a UK abattoir and processed fresh or stored in cyst fluid at −30°C.

### Echinococcus *oncosphere preparations*

Approximately 50,000 *Echinococcus* tapeworms (plus free gravid proglottids) were washed in saline and incubated by continuous rotation in a solution of 1% pepsin and 1% HCl in saline (pH 2.0) (artificial gastric fluid [AGF]) for 2 hr at 37°C. Pepsinized material was centrifuged at 1,000 × *g* for 5 min, the pellet resuspended in 0.5 ml saline, layered onto neat Percoll and left for 5 min to allow dense debris and grit to sediment under gravity. The upper layer of eggs and pepsinized tissue was transferred to 5 ml of warm, filtered, artificial intestinal fluid (AIF), consisting of 1% sodium bicarbonate, 0.2% trypsin (Difco), 0.5% sodium choleate (Sigma), 0.4% sodium tauroglycocholate (BDH), 0.04% cholesterol (Sigma) and 5% whole sheep bile, in distilled water and incubated for 1 hr at 37°C with continuous rotation. This procedure resulted in a 50%–60% hatch (many immature eggs were present) and about 60% activation, i.e., freeing of the oncospheral membrane. The AIF-treated suspension was washed three times by centrifugation in 0.15 M phosphate buffered saline (PBS) pH 7.4, divided into 0.5-ml aliquots, layered onto 1-ml volumes of neat Percoll and centrifuged at 2,000 × *g* for 30 min at 4°C.[11] Oncospheres and immature eggs were carefully removed from the PBS-Percoll interface (the pellet consisted mainly of embryophoric blocks, hooks, unhatched eggs and some debris) and washed twice in PBS by centrifugation (1,000 × *g* 10 min). Aliquots of this suspension were frozen whole at −30°C for use in immunofluorescence tests, or frozen and thawed then sonicated on ice using a 3-mm probe (MSE) in 6 × 20 sec bursts (to give 95% disruption), allowed to stand for 2 hr at 4°C then stored frozen in aliquots (designated EgO) for use in hyperimmunization of mice.

### Taenia *oncosphere preparations*

Egg suspensions were obtained from gravid proglottids of the large tapeworms *T. hydatigena, T. pisiformis, T. saginata, T. multiceps* and *T. ovis* by sieving (80 μm) proglottis macerates to separate eggs from worm tissue.[12] Hatching and activation of eggs in AGF (1 hr) and AIF (30 min), and separation of oncospheres from unhatched eggs and embryophoric blocks by centrifugation on Percoll was carried out as above. Whole oncospheres were stored in PBS at −30°C.

### *Hypochlorite/detergent treatment of taeniid eggs*

Oncospheres of *E. granulosus, T. hydatigena* and *T. pisiformis* were also obtained from eggs by chemical means, using sodium hypochlorite solution[13,14] that did not rely on egg viability and enabled fluorescence to occur with the oncosphere still within the oncospheral membrane (nonactivated). The oncospheral membrane has previously been shown to fluoresce nonspecifically,[15] and provide a barrier to fluorescence of the oncosphere itself.[16] The dissolution of egg embryophores was achieved by incubating egg pellets in 1 ml of a solution of sodium hypochlorite in distilled water (to contain 1% available chlorine) by continuous pipette mixing for 2–4 min at room temperature and observing samples microscopically. When optimal numbers of eggs had disrupted to release oncospheres still within their oncospheral membranes but before the oncospheres themselves were affected, 20 ml of distilled water was added to stop the reaction and centrifuged at 500 × *g* for 5 min. Oncosphere pellets were purified by centrifugation on Percoll and washed as above and incubated in a 1-ml solution containing 1% w/v sodium deoxycholate (DOC) in 0.1 M Tris/HCl pH 8.3 for 1 hr at room temperature (C. Bursey and P. Craig, personal communication). The DOC-treated intact oncospheres were washed in distilled water and used in immunofluorescence tests.

### Echinococcus *protoscolex preparation*

About 30,000 protoscoleces (nonpepsinized) were washed by rotation over 4 hr in several changes of 0.2 M glycine/HCl pH 2.8 to dissociate bound sheep immunoglobulins. Protoscoleces were further washed in 0.1 M Tris/HCl pH 8.3 then resuspended in Tris/HCl containing 1% DOC and mixed overnight at room temperature, after which time all protoscoleces were lysed and showing tegumental damage. This suspension was sonicated on ice for 2 min, left overnight at 4°C and centrifuged at 2,000 × *g* for 30 min at 4°C. The supernatant was designated EgP and used to hyperimmunize mice, and as antigen to detect circulating antibodies in murine antisera.

### *Anti-oncospheral indirect immunofluorescence test*

Anti-oncospheral antibodies that bound with whole oncospheres, were detected using an indirect immunofluorescence test (IFAT). Oncospheres (AGF/AIF or hypochlorite/DOC-treated) were washed in distilled water and air dried overnight onto Teflon-coated multispot slides (Hendley-Essex, UK) at the rate of 20 per spot (in 15 μl vol). Murine antisera or hybridoma ascites fluid diluted in PBS with 0.05% Tween 20 (PBS-Tween) or neat hybridoma supernatants were incubated (15 μl/spot) with the unfixed oncospheres for 30 min at 37°C in a humidified box. Controls consisted of normal mouse serum, myeloma cell (X63) supernatants, neat fetal calf serum (FCS), and X63 myeloma ascites fluid. Slides were washed by soaking in PBS-Tween for 10 min, drained and incubated with fluorescein-conjugated sheep anti-mouse whole immunoglobulin (Ig) or anti-IgG, anti-IgM or anti-IgE diluted 1:60 in PBS-Tween (10 μl/spot) for 30 min at 37°C as above. Slides were finally washed in PBS-Tween, drained and then examined under glycerol (with 10% PBS added) using a Zeiss microscope with epifluorescence attachment.

### *Enzyme-linked immunosorbent assay (ELISA)*

The *Echinococcus* protoscolex extract (EgP) was used as antigen to assay for specific antibodies in murine antisera (diluted 1:50) and hybridoma supernatants using a peroxidase-ELISA as described previously.[16]

### *Hyperimmunization of mice*

Each of 5 BALB/c mice was injected intramuscularly, subcutaneously and intraperitoneally with 50 μl of EgO sonicate (represents products of 900 oncospheres per mouse) in 50 μl of incomplete Freund's adjuvant (IFA). Mice were boosted at 2-week intervals, exactly as above in IFA for the first boost, and without adjuvant for a subsequent 2 boosts. Mice were bled 1 week after the last boost and individual serum samples assayed for anti-oncospheral antibodies using IFAT with AGF/AIF hatched whole oncospheres, or by ELISA using "soluble" EgP extract. The two highest antibody reactors were given final boosts of 100 μl EgO intravenously and killed 3 days later to be used as spleen cell donors for hybridoma production.

Five BALB/c mice were also hyperimmunized using EgP extract (100 μg protein/mouse) and serum samples assayed similarly.

### *Hybridoma production and screening*

The X63 Ag8.653 myeloma cell line (Flow Laboratories, UK) was cultured ($5 \times 10^4$ cells/ml) in Dulbecco's modified Eagle's medium (DMEM) (plus 20 mM Hepes buffer) with 15% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 0.075% sodium bicarbonate and 0.2% gentamycin (DMEM/15% FCS). Donor spleen cells from an "anti-EgO" mouse were washed twice in DMEM mixed with $3 \times 10^7$ myeloma cells (grown up over the previous 2 days) and pelleted together at 400 × *g* for 8 min. Fusion was performed by adding 1 ml of 50% polyethylene glycol 4000 (PEG) containing 5% dimethylsulphoxide in PBS at 37°C, over 1 min shaking continuously. Cells were allowed to stand in PEG for 90 sec at 37°C and were then diluted over 3 min with 20 ml of DMEM. The fusion mixture was centrifuged (400 × *g* 8 min) and the pellet resuspended in 50 ml of DMEM/15% FCS and dispensed in the wells (4 drops/well) of 96-well microtiter trays and allowed to incubate overnight at 37°C in a humidified $CO_2$ (7%) incubator. An equal volume of DMEM/15% FCS with optimal concentrations of hypoxanthine, aminopterin and thymidine (HAT) was added to each well (double strength for first addition) and this feeding procedure was repeated at least twice over the next 10 days.[17]

Supernatants from wells showing cell growth

after 7 days were assayed by IFAT with fluorescein-conjugated anti-mouse Ig using *E. granulosus* oncospheres. Positive wells were re-screened by IFAT using *T. hydatigena* and *T. pisiformis* oncospheres in addition and specific *E. granulosus* oncosphere reactors transferred to 24-well trays and grown up in DMEM/15% FCS/HT containing 5% ascites from a X63 mouse myeloma (P. Bond and M. Hommel, personal communication). Selected hybridomas were cloned by limit dilution in the same medium using 96-well cloned microtiter plates. Supernatants from wells showing single cell colonies were reassayed to determine specific immunoglobulin isotype and then expanded to DMEM/15% FCS into 24-well trays and ultimately into 200-ml tissue culture flasks.

Hybridoma media containing monoclonal antibody (Mab) from bulk culture was concentrated (×40) under pressure through an $M_r$ 30,000 filter using an Amicon stirred cell (Amicon Corporation).

Expanded clones were also inoculated into Pristan-primed BALB/c mice for production of ascites fluid.

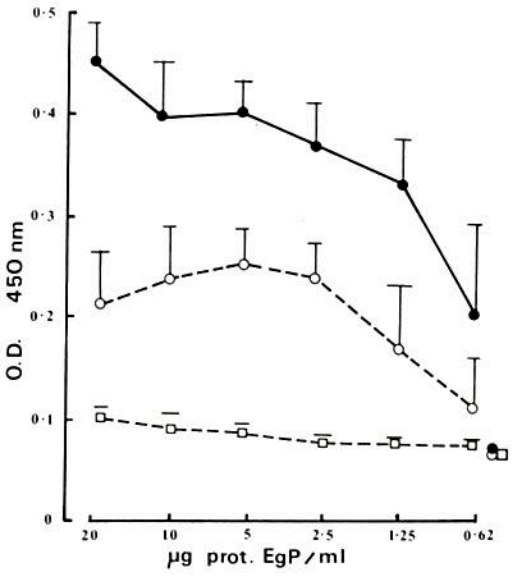


FIGURE 1. Binding of murine anti-protoscolex (●– – –●) and anti-oncospheral (○– – –○) antibody (IgG) in ELISA (mean OD ± SE) to an extract of *Echinococcus* protoscoleces (EgP). Normal mouse serum, □– – –□. No antigen controls ●, ○, □.

## RESULTS

### *Antibody responses in hyperimmunized mice*

The anti-EgO fluorescent antibody responses of individual mice varied from titers of 1:10 to 1:60 (2 mice) and were not *Echinococcus* oncosphere species-specific as fluorescence also occurred with oncospheres of *T. pisiformis, T. hydatigena* and *T. saginata.* Murine anti-EgO responses were also detectable in ELISA using EgP extract, and correlated with the high fluorescent antibody reactors, though ELISA responses were significantly below that obtained with the homologous anti-EgP group (Fig. 1). In addition, mouse anti-EgP hyperimmune sera bound very well to whole *Echinococcus* oncospheres, though again this was not a species-specific response.

### *Screening of anti-*Echinococcus *oncosphere hybridomas*

The rate of hybridoma colony production was about 30%–40% over 6 separate fusions, and 5% of these were positive antibody secretors when assayed with the *Echinococcus* oncosphere-IFAT. However, under binding selection pressure in IFAT using *Echinococcus, T. hydatigena* and *T. pisiformis* oncospheres, only one hybridoma was identified that secreted "species"-specific anti-*Echinococcus* oncosphere antibody, and was designated EgOH6.4E5. The EgP-ELISA did not detect any of the IFAT-positive anti-*Echinococcus* oncosphere antibody secretors.

### *Activity and specificity of anti-*Echinococcus *Mab*

The Ig isotype secreted in bulk culture (or in ascites) from two active clones B-1 and C-7 of EgOH6.4E5 was $IgG_1$. This Mab gave bright uniform fluorescence, both somatically and over the surface of oncospheres hatched from AGF/AIF-treated *Echinococcus* eggs. Negative fluorescence of oncospheres of the heterologous species tested was typified by a dull green, while nonspecific fluorescence appeared as a faint speckled pattern that occurred with the fluorescein-conjugated anti-mouse Ig alone (particularly with oncospheres of *T. hydatigena*). Thus the selection of Mab 4E5 during screening was already based on specificity of binding in IFAT to oncospheres of *Echinococcus* and not to those of *T. hydatigena*

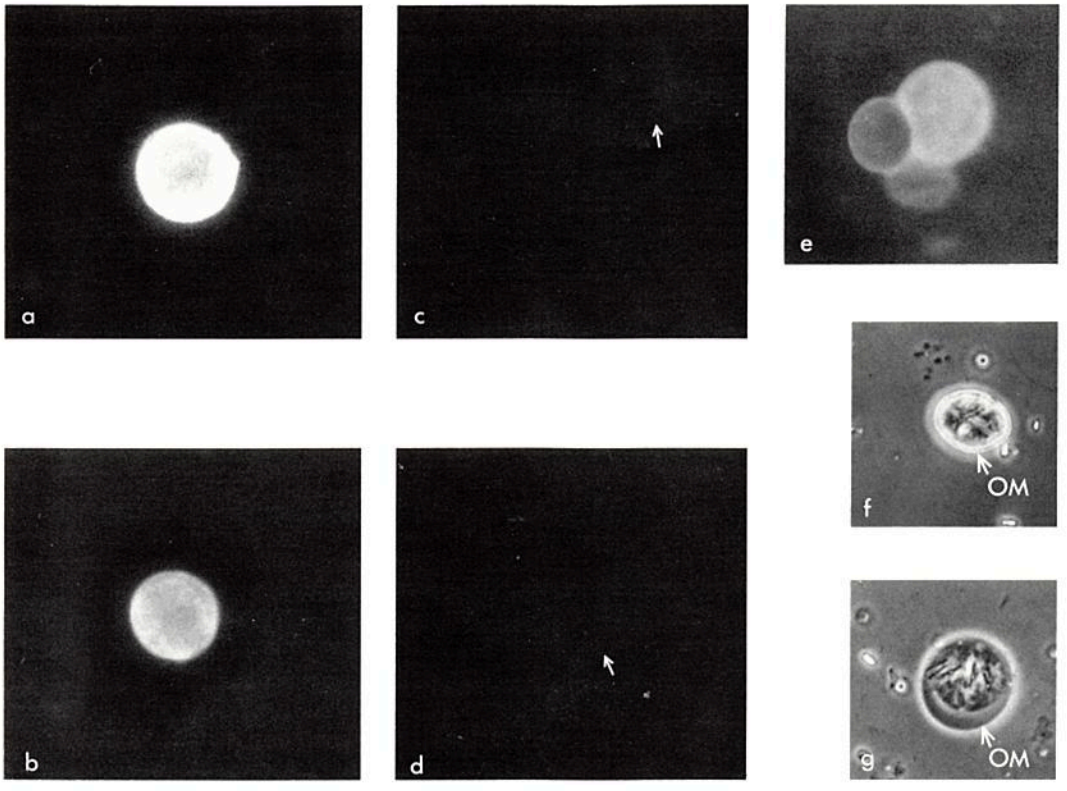


FIGURE 2. **a and b.** Specific fluorescence of *Echinococcus* oncospheres (AGF/AIF-hatched) with Mab 4E5.C-7 as the primary unlabeled antibody in an IFAT. **c.** Oncospheres of *Taenia hydatigena* incubated with Mab 4E5.C-7 in IFAT. **d.** Oncospheres of *T. pisiformis* incubated with 4E5.C-7 in IFAT **e.** Oncospheres of *Echinococcus* "hypochlorite-hatched" and incubated in 1% DOC prior to 4E5.C-7 Mab incubation in IFAT; note swollen oncosphere fluorescing, **f.** Oncosphere of *Echinococcus* (under phase contrast) within oncospheral membrane (OM). **g.** Oncosphere of *Echinococcus* within oncospheral membrane after incubation in 1% DOC (phase contrast).

or *T. pisiformis* (Fig. 2). The specificity of the Mab was reaffirmed using Amicon concentrates (whole or ammonium sulphate fractions) of bulk culture media. In addition, no specific binding was evident in IFAT with oncospheres derived from eggs of *T. saginata, T. ovis, T. multiceps* or *T. taeniaeformis* (data not shown). Specific fluorescence was obtained only with AGF/AIF-hatched *Echinococcus* oncospheres that had broken free of their oncospheral membrane (OM). A degree of nonspecific fluorescence was associated with the OM itself. However, it was also demonstrated that specific fluorescence of some (>60%) OM-bound oncospheres could be achieved after hypochlorite hatching of *Echinococcus* eggs, by preincubation of oncospheres in 1% DOC in TRIS/HCl pH 8.4 (Fig. 2e).

When 4E5 Mab was tested in an IFAT with air dried whole protoscoleces of *E. granulosus* some faint fluorescence was observed on the outer tegument but not within the protoscolex syncytium.

## DISCUSSION

Eggs of *Echinococcus* are morphologically indistinguishable from other taeniid eggs at least under light microscopy. The ability to reliably and practically differentiate not only *Echinococcus* but other taeniid eggs of public health importance (e.g., *T. solium* and *T. saginata*) would be very useful in epidemiological studies of these helminths. Previously the identification of *Echinococcus* eggs, (without resorting to in vivo infections) was possible only by the lengthy and skilled in vitro culture of oncospheres from ar-

tificially hatched eggs to their characteristic early metacestode stages.[3] Analysis of the parasite isoenzymes and DNA are already being applied to the problem of *Echinococcus* strain differentiation[18] and may be assessed for taeniid egg identification, though both these techniques (particularly the former) may have considerable problems in analyzing small quantities of eggs. The ideal test should be able to identify small numbers of eggs directly and it was considered that a specific immunological probe might be applicable. Specific monoclonal antibodies have already been shown to distinguish between New World species of the protozoan parasite *Leishmania*.[19]

The embryophoric blocks of the taeniid egg embryophore appear to be of low antigenicity,[8] and therefore the oncosphere within the egg which is known to be highly immunogenic[20] was the target for production of a specific anti-*Echinococcus* oncosphere Mab. An anti-oncospheral polyclonal antisera has been used previously in an immunofluorescence test to distinguish between egg-derived (AGF/AIF-hatched) oncospheres of *T. hydatigena* and *T. pisiformis*.[8] In the present study an *Echinococcus* anti-oncospheral Mab was produced that specifically bound, in an IFAT to oncospheres of *Echinococcus* and not to oncospheres obtained from eggs of 6 other taeniid species. Any effective immunodiagnostic test to identify eggs of *Echinococcus* which is based on specific oncospheral binding of antibodies, may be limited to identifying viable mature eggs unless oncospheres can be obtained intact from biologically inactive (but mature) eggs. The use of "chemical hatching" to remove the embryophore from taeniid eggs using hypochlorite solutions is not new.[13] However, specific fluorescence appears to be associated with liberated oncospheres and not those still enclosed in smooth OM. The ability to obtain oncospheral fluorescence of OM-bound hypoclorite-hatched oncospheres after DOC incubation in the present study may be useful. It is likely that incubation of OM-bound oncospheres in 1% DOC caused partial disruption or lysis of the oncospheral membrane to allow penetration of labeled or unlabeled anti-oncospheral antibodies. Microscopically, oncospheres incubated in DOC were observed to swell significantly within the OM which itself becomes expanded and more round (Fig. 2f, 2g). Hypochlorite/DOC treatment of taeniid eggs may be appropriate in practice for testing small numbers of eggs from fecal or environmental samples.

Protoscoleces from the mature metacestode (hydatid) of *E. granulosus* were investigated as a potential safe and relatively easily obtained source of putative specific antigens(s) for the development of a specific anti-*Echinococcus* oncosphere antibody reagent. Common antigens between the oncosphere and mature metacestode stages of taeniids have been demonstrated directly by immunoprecipitation techniques in *T. taeniaeformis*[21] and indirectly by ELISA using DOC extracts of *T. hydatigena* and *T. ovis*.[22] In the current study although both oncospheres and protoscoleces of *E. granulosus* appear to share some antigenic determinants, as mouse anti-EgP bound well in an IFAT to *Echinococcus* oncospheres, the EgP-ELISA did not detect any of the anti-*Echinococcus* oncosphere IFAT-positive hybridoma supernatants. However, 4E5 Mab did bind to the tegument of nonliving protoscoleces of *E. granulosus,* and thus may not be totally stage-specific as suggested by the result of the EgP-ELISA screen. The IFAT may be more sensitive than ELISA in detecting a low level of interstage cross-reaction of 4E5 Mab with the protoscolex tegument.

In conclusion, a species-specific anti-*Echinococcus* monoclonal antibody has been produced which binds specifically under indirect immunofluorescence to oncospheres of *E. granulosus* and not to those of six other taeniid species. This test is potentially amenable to specifically probing oncospheres derived from small numbers of eggs (<20) and will be assessed for the analysis of taeniid egg samples from suspected *Echinococcus* contamination/transmission sites in Turkana such as water holes,[7] and also in direct examination of dog feces for *Echinococcus* eggs.

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