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IMMUNOLOGICAL AND HISTOLOGICAL REACTIONS OF PATIENTS VERSUS FERTILE AND STERILE *ECHINOCOCCUS GRANULOSUS SENSU LATO* CYSTS

By

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Abstract

Hydatid cyst or cystic echinococcosis (CE) is a metacestode of dog *Echinococcus granulosus* a worldwide zoonotic disease. The study evaluated parasite interaction between patient and CE using 25 cysts of Egyptian patients before therapeutic interventions. These cysts were divided in two groups: G1: fertile with protoscoleces and G2: non-fertile (sterile) without protoscoleces. Patients' body reaction differed due to type of CE; fertile one stimulated the local immune defense mechanism in form of granulomatous immune reactions and significant immune cell infiltration. Histologically, this led to increase in the adventitial layers thickness with thick fibrous tissue as compared to non-fertile, and guarded against the hydatidosis sustainability. Besides, fertile CE cyst GATA-3 remained dominant keeping the infection in control status. But, if CD-4^+ & CD-8^+ T-cell numbers became boosting, CE didn't sustain fertility as shown by using panel of GATA-3, CD-4^+, CD-8+ antibodies that represented Th2, Th subset, and cytotoxic T cells.

Keywords: Egypt, Patients, Hydatidosis, CD-4^+, CD-8^+, fertility, GATA-3, Viability

Introduction

Echinococcosis/hydatidosis is worldwide zoonotic parasitic disease (WHO, 2021). Carnivores including dogs are the definitive hosts and produce eggs containing infective oncospheres (Eck- ert and Deplazes, 2004) and herbivorous animals including man are the intermediate hosts accidentally ingested the infective eggs, which can stay viable for one year but do not play a role in the natural cycle. The greatest prevalence of hydatidosis in man and animal was found in countries of the temperate zon-es, including several parts of Eurasia (the Me-diterranean regions, southern and central parts of Russia, central Asia, China), Australia, America (especially South America) and north and east Africa (Grosso *et al,* 2012).

Besides, in rare occasion man may acquire the eggs from spectrum of aberrant hosts or pet-dogs (Dyab *et al,* 2005) or even its fears (Altintas, 2003). Hence, the risky cycle sheep-dog-man (Haridy *et al,* 2000). Globally, the commonest human cases were caused by G1 sheep strain of *E. granulosus* (Haridy *et al,* 2008). After 5 days post eggs ingestion, metacestode (60 to 70µm in diameter) consisted of an internal cellular layer (germinal layer) and an outer acellular, laminated layer, which gradually induced a granulomatous host reaction, followed by a fibrous tissue reaction and formation of a connective tissue layer (Galindo *et al*, 2008). Human cysts size is highly variable and usually ranges between 1 & 15cm, or even up to >20cm in diameter. Infection starts without symptoms for years. Signs and symptoms occur depend on cyst's location and size. Cysts may be single or multiple (at least 25%) liver being the first filter in eggs' way as 50%-75% were found and 20%-30% in lung (Ibrahim and Morsy, 2020). Also, kidney, spleen, heart, bones, muscles, CNS (Wilson, 1991), spinal cord (Mazyad *et al,* 1998) and less commonly, brain, musculoskeletal system, retroperitoneal organs, pancreas, and thyroid gland (Yilmaz *et al,* 2013). Once a cyst reached a

diameter of 1 cm, its wall differentiates into a thick outer, non-cellular membrane, which covers the thin germinal epithelium. From this epithelium, cells begin to grow within the cyst. Then become vacuolated, and are known as brood capsules, which are the parts of the parasite from which protoscolices bud. Often, daughter cysts also form within cysts (CDC, 2010). Adventitial layer formed by IH immune response is mainly cell-mediated, involved infiltration of eosinophil cells and macrophages, and low-level polarized Th1 reactions. Antibody responses are weak and are, normally, indiscernible in the early 2 to 3 weeks after infection. Its thickness is always variable with some focal fibrosis (Hidalgo *et al,* 2019). The GATA-3 is a member of transcription factors termed by its capacity to bind to the DNA sequence GATA essential to shift the differentiation of Th0 to the Th2 while suppressing their diversity toward Th1 (Yagi *et al,* 2011). The CD-8^+ glycoprotein plays a critical role in T cell receptor binding for cytotoxic T cells activation by MHC-I and is a co-signaling receptor that couples TCR occupancy to second messenger pathways (Daniels and Jameson, 2000). Also, the role of corecceptor molecules, CD-4^+ augment TCR signaling by stabilizing interactions between TCR & MHC-II ligands and by assisting recruitment of a kinase to the TCR-MHC complex that is crucial for initiating signaling (Artyomov *et al*, 2010). In the intermediate host, there were 2 types of CE, fertile cyst that produce protoscoleces (protoscolex) from cyst germinative layer and non-fertile (sterile) one without protoscolex and unable to infect dogs (Daryani *et al*, 2009), however, beyond variability in CE fertility was not clear (Paredes *et al*, 2007).

In cross-sectional studies, humans were associated with variability in CE fertility (Cabrera *et al*, 2008). So, it is an appropriate model to study cyst infertility influencing factors, by working in defining causes of non-fertile CE in humans, recognizing both increased apoptosis levels in the germinative layer of the non-fertile cysts and various immunoglobulin profiles (Paredes *et al*, 2011). From many studies implemented in hydatid patients and experimental trials, commonly accepted that CE cyst establishment is chiefly mediated by impairment of dendritic cells (DC) differentiation and maturation, and by the differentiation of specialized T regulatory cells and associated cytokines such as transforming growth factor-beta (TGF-β) and Interleukin-10 (IL-10) (Riganò *et al*, 2007). Besides, a feature of *E. granulosus* infection can stimulate T helper 2-like (Th2-like) type response, which is useful for the parasite to survive, rather than T helper 1 (Th1) cytokines, proficient in its destruction (Mourglia-Ettlin *et al*, 2011). This adventitial layer is composed of collagen fibers, epithelioid cells, eosinophils and lymphocytes. To establish itself inside the host, the germinal layer produces the laminated layer, and to continue its life cycle, generates protoscoleces (Hidalgo *et al,* 2020).

The present work aimed to study the morphological and structural difference in FCE & SCE as well as the ability of both to stimulate the immune-mechanism of patients' body to spot some light on the suspected life span of these cysts in the patient body.

Materials and Method

Ethical Statement: Ethical approval was obtained from the ethical committee of Cairo Faculty of Medicine on 1/10/2014 and its consecutive adjustments and analogous ethical standards. All procedures related to the human samples were covenant with the ethical standards settled by the National Research Committee and the 1964 Helsinki Declaration.

The present cross-sectional study investigated reaction of patient body versus FCE & SCE as to relationship between T-cell populations in adventitial layer formed by patients' defiance immune mechanism.

Hydatid cysts: Twenty-five samples were collected from clinical, parasitological and radiological proved hydatidosis adults from the

Tropical Medicine Research Institute and Nasser Institute for Research and Treatment and the National Hepatology, from January 2020 to December 2020. The study objectives were clarified to all patients. Pregnant women, children less than five years old, those with cancers, hepatitis, nephritis, and abnormal hepatic or renal function tests were excluded. Besides, small cysts were viewed as still developing and without commenced protoscolex production. Pathological examination was kindly carried at the Department of Pathology, Cairo Faculty of Medicine.

Characterization of CE: Cysts were obtained from various hepatic segments. HCF was emptied into sterilized test tubes and the shreds of the germinative layers and the hydatid sand were allowed to precipitate. The metacestodes were washed three times in a clean normal saline. Thus, the large remnants of the parasite were eliminated using sterilized wide porous mesh and the filtrate was precipitated at room temperature for five minutes and the supernatant was smeared for microscopic examination (El-Aal *et al*, 2015). Viability was confirmed by using a urine strip test, where high glucose and low protein measurements indicated fertility and vice versa with those non-fertile ones (El Saftawy *et al*, 2017).

CE processing: Considering an unpublished work, the RFLP analysis of CE samples proved to be *Echinococcus granulosus sensu lato*. Walls from both fertile and non-fertile cysts were fixed in formalin and processed paraffin-embedded, sectioning and H & E staining (Alturkistani *et al,* 2016). Thickness of germinative, laminated, and adventitial layers was evaluated using an ocular micrometer calibrated with a stage micrometer in an Olympus CX23 microscopy. Measurements in µm were performed in 20consecutive areas by a mean value and standard deviation.

Histochemical staining with Masson's Trichrome was done for a better demonstration of germinative layer (Das *et al*, 2014). Estimation of CD-8^+, CD-4^+, & GATA-3 Immune-Reactive Cells: To differentiate between host T-cells by using 3 antibodies (CD-4^+, CD-8^+, & GATA-3). Paraffin blocks specimens were cut to 5µm thickness sections and then deparaffinization and rehydration antigen retrieval was done with 10mM sodium citrate, 0.05% Tween-20, pH 6.2.

Primary antibodies for distinctive biomarkers were used: 1-Murine anti-human (GATA binding protein-3) GATA-3 antibody; a purified and recombinant protein conveyed in *E. coli*, 2- CD8-Ab: 13 aa synthesized peptide targeting alpha chain of human CD-8 molecule's cytoplasmic domain, & 3- CD4 (IgG): recombinant monoclonal antibody against CD4 molecules (Bancroft and Gamble, 2002).The primary antibody was incubated overnight at room temperature at a dilution of 1:200. Horse radish peroxidase-conjugated secondary antibody was supplied from the Ultra Vision Detection System (Thermo Fisher Scientific, USA). Finally, antigen detection was done by using DAB-Plus Substrate Kit and hematoxylin stain was used for counterstaining the manipulated slides. In control slides, the step of immune-staining with primary immunoglobulin was omitted.

Quantitation of intra-adventitial immune-reactive lymphocytes in CE: Host immune cells were recognized by the pathologist based on their morphological criteria and staining pattern in H&E. The total intra-adventitial lymphocytic count/20 high power field was calculated using IHC staining control slides. Additionally, a comparative analysis was performed between fertile and non-fertile CE for positive CD-4^+, CD-8^+, & GATA-3. This was done by imaging the adventitial layer for each patient microscopically at high power. Images were entered into the Image J software; where the grid-tool was chosen to assign and count the positive cells within certain squares with an automatic calculated pixel area per point (Russ and Russ, 2017). Cells were manually selected and logged on the image directly by the software. Areas, where the nuclei of the

host T cells can't be differentiated, were dismissed. Mean number of cells (C/N), and mean pixel area of manipulated squares (A/N) were calculated in the form of an average number of lymphocytes per *pixel*^{2} using the following equation (El Saftawy *et al*, 2020).

$$\frac{C/N}{A/N} = C/A$$

Where: C= no. of cells; N= no. of squares; A= area per point.

Statistical analysis: Data were analyzed after Hidalgo *et al*. (2019) where a score index was assigned to the with a score from 0 to 3 according to these criteria: mild=1, up to 30 inflammatory cells/ high power field (HPF) moderate = 2, ranging from 30 to 100 inflammatory cells per HPF; severe =3 where more than 100 inflammatory cells/ HPF were calculated. The assessment was performed in an average of 20 HPF. Inflammation scores were classified into low (0.5 to 1.5) & high (2 to 3). Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp., Armonk, N Y, USA) was used Data were expressed as mean+/-S D, median, minimum & maximum in quantitative. Quantitative variables were compared using non-parametric Kruskal-Wallis with adjusted Mann-Whitney test (Chan, 2003).

Results

Patients 15/25 (60%) were of fertile CE with high viability (≥85%) while 10(40%) were non-fertile. Fertile and non-fertile CE from livers were characterized grossly by a whitish laminated layer and a thick adventitial layer (AL) composed of collagen fibers detached from laminated layer, without significant differences between patients and age or sex.

Thickness of CE layers according to cyst fertility: Gross examination of CE layers in fertile and non-fertile CE was confirmed with microscopic micrometry; fertile CE possess almost a thicker fibrous layer than non-fertile CE with two folds (660µm+/- 55.2 vs. 160µm +/- 15.4), with significant differences (P-value <0.05). There were variations in thickness of the laminated layer based on CE cyst fertility, complete measurements of CE cyst layers in the two groups were shown. Other significant variances were between germinated layers were thinner or absent in non-fertile cysts.

Characterization of adventitial layer: Tissue sections of CE cyst wall showed pathogenesis of laminated and adventitial layer. Fertile CE features showed the same adventitial and laminated layer characteristic of non-fertile CE, with an adventitial layer composed of inflammatory cells tightly attached to the laminated layer. Collagen fibers were around the sinusoids. Non-fertile CE with little or no germinated layer with a thin laminated layer, the adventitial layer composed of palisading foamy macrophages, lymphoid follicles, and multinucleated inflammatory cells. A laminated layer was demonstrated as control tissue.

Characterization of germinative layer: Morpho-histological analysis of fertile and non-fertile cysts showed that between germinative layer cells, there were several mononuclear cells that was not present in every non-fertile one. Immune cells were seen as single cells or as big sheets.

Quantification of intra-adventitial inflammatory cells in fertile and non-fertile CE: Inflammatory infiltrates established several sheet-like collections in the adventitial layer. As much as 80% of examined adventitial layers in non-fertile CE were confined with inflammatory cells with palisading aggregates of lymphocytes. Significant deposition of fibroblasts and fibrous strands was noted, but for fertile CE. Quantification was built on image gridding (black color) by Image J software and lymphocytes were manually selected (asterisk with numbers) within the boundaries of squares. Results were expressed as number of intra-adventitial lymphocytes/20 high-power fields. Ratios of mean values (+/- SD) of cell counts of fertile and non-fertile CE to mean pixel area (+/-SD) adventitial layer cut-sections were of significant differences (P-value ≤0.05) and higher in non-fertile cysts.

Evaluation of intra-adventitial immuno-hist-

ochemicical analysis showed all CE samples had immune cell infiltrates in the adventitial layer but, the extent and presence of each one differed. Mean± SD of lymphocytic cells quantitative data of CD-8^+ (cytotoxic T cells), CD-4^+(T helper cells), and GATA-3 (Th2) figured out cellular patterns of the local T-cell infiltrates in the adventitial layer. There was a local increase in the expression of the GATA-3 marker to CD-8^+ & CD-4^+ markers in all CE. GATA-3 high expression levels were visually observed by intensely stained brown areas evidenced in tissue sections.

Twenty-five % of fertile cysts had high infiltration with CD-8^+. But, non-fertile CE evidenced more than 65% of the samples with high immune-positive cells. CD-4^+ cells follow a similar pattern, with high scores in 55% of the non-fertile samples.

Details were given in figures (1, 2, 3, 4, &5).

Discussion

In the present study, two types of CE occurred either fertile cyst as its inner germinative layer able to produce protoscoleces to complete the life-cycle, or non-fertile ones without protoscolex, non-infective final host, which agreed with El Saftawy *et al*. (2017).

In the present histological study, all the CE showed the parasite in complex form with all gradually and temporary morphology due to several host-parasite interactions, which agreed with Khadidja *et al.* (2014). The adventitial layer may be restrictive to the CE cyst growth rate, but protected the hydatid cysts from the host immune reactions (Barnes *et al*, 2011). Thus, in the present study the adventitial layer was thicker in the fertile cysts with high viable protoscolex compared to others. Moreover, the non-fertile CE possessed thinner adventitial layer features, as reported by Hidalgo *et al*. (2019) on an animal model. However, Al SeÂ (2012) reported that the fibrous and necrotic layer was significantly affected by difference of host species due to dissimilar defense mechanisms and cellular effects.

The present study, showed greater thickness of germinative membrane in the fertile cysts with highly viable protoscolex suggesting great parasite activity that elucidated the high rapid growth rate in hosts, which agreed with Barnes *et al*. (2011). In sheep, ultrastructural aspects of protoscolex growth and formation from buds in proliferating germinative layer of fertile CE increased cell size and secretion of extracellular material, giving rise to hooklet structure of protoscolex accompanied with increase in size of the involved cells (Galindo *et al*, 2002). In the present study, non-fertile CE had a thin or scarce germinative layer unable to produce protoscolex. This agreed with Paredes *et al*. (2007), who reported apoptosis as a CE infertility mechanism. Laminated layer and protoscoleces production is the germinative layer main metabolic activity (Thompson, 2017).

In the present study, fertile CE with viable high protoscolex had thick laminated layer (660µm+/-55.2), but non-fertile one showed a thin laminated layer, sometimes <160µm. Bortoletti and Ferretti (1978) reported a direct relation between the laminated layer thickness and cyst development. In human CE laminated layer was a widely crucial component of host-parasite relationship, in shielding metacestode from being attack by host immune cells (Díaz *et al*, 2009), in role of parasite glycoproteins and carbohydrates (Lin *et al*, 2013) and in regulating host's immunity (Dai *et al*, 2011). Carbohydrates residues in the laminated layer elucidated to interact with the Kupffer cells receptors, expressed only in liver macrophages, cells identified and contributed to tolerogenic antigen present that distinctive of this organ (Hsu *et al*, 2013). These features appeared to be interrelated to the parasite survival inside their hosts.

In the current study, the adventitial layer is composed of palisading foamy macrophages, lymphoid follicles, and multinucleated giant cells. The adventitial layer was described as a fibrous capsule outwardly comprised of a pack of fibrous elements and an impressive arra-

ngement of immune cells in form of a layer of palisading macrophages and monocytes, occasional polymorphonuclear cells, an eosinophil-containing layer, a significant aggregated T and B-lymphocytes (Vismarra *et al*, 2015). Barnes *et al*. (2011) reported that in sheep adventitial layer protected cysts from host immune reactions. Díaz *et al.* (2018) added that pigs and cattle form a typical granulomatous reaction with epithelioid macrophages and multinucleated giant cells around the CE and an outer mononuclear cell infiltrate.

In the present study, lymphocytes were the predominant inflammatory cells in adventitial layer of liver CE, but presence and magnitude of inflammation varied. Fertile CE had low adventitial layer inflammation scores with relatively high numbers of lymphocytes and fibroblast without high infiltrated eosinophils. Non-fertile CE had the entire samples with high adventitial layer inflammation scores. Jiménez *et al.* (2020) showed that the overall distributive pattern of inflammatory cells was great in the non-fertile cysts than fertile ones. Sakamoto and Cabrera (2003) reported that eosinophil-mediated destruction of parasite laminated layer was involutedly regressive.

The present study showed that in most cases of non-fertile cysts, CD-8^+ cells were predominant in pericystic adventitia, and a relatively similar number of CD-4^+ cells. Hernández *et al*. (1999) reported that peripheral lymphocyte subpopulations in hydatidosis symptomatic ones had significantly lower CD-3^+ & CD-8^+ lymphocytes levels than controls. Sakamoto and Cabrera (2003) reported that in the adventitial layer around regressive and involution CE, there were infiltrating lymphocytes mostly of CD-4^+ cells, and CD-8^+ cells predominated in pericystic adventitia in cases with progressive CE, assuming that subpopulations of lymphocytes infiltrating the liver CE were usually imitative to the cells in nearby draining lymph nodes.

In the current study, GATA-3 was involved in patients against fertile and non-fertile cysts. Pang *et al*. (2014) reported that mRNA levels of GATA-3 were significantly increased in patients before therapy compared with controls. Also, the expression of GATA-3 and IL-4 genes (Th2 related cytokine) significantly increased in several therapeutic trials in murine models (Parastouei *et al*, 2020). Dorosti *et al*. (2016) reported that in cattle rate of IL-4 gene expression in the hepatic CE fibrous capsule was 9.84 times more than control, and interleukin played a crucial role in host-parasite interactions. Vatankhah *et al*. (2015) found that in chronic hydatidosis T cell-mediated immunity was impaired as compared to other types of chronic inflammatory reactions, suggesting an immune-regulatory role for the parasite.

Conclusion

The Egyptian hydatidosis was well documented in many governorates. Interaction between patient and CE differed with fertile than sterile. Fertile CE stimulated patient's immune mechanism than sterile one. Local host immunity in the adventitial layer of fertile CE disrupted the CE sustainability and progressed into its infertility later on. So, fertile CE stimulated local immune mechanism of body than sterile one, which produced anti-CE-antibodies and infection prognosis.

Conflicts of interest: Authors declared that they have neither conflicts of interests nor received fund

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Explanation of figures

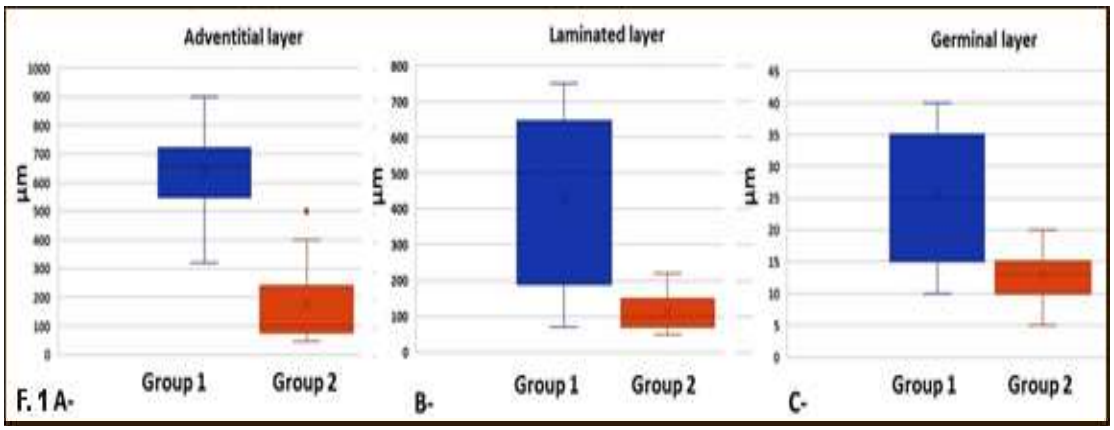
Fig. 1: Stock columns showed median, minimum and maximum values of adventitial, laminated, and germinated layers thickness (µm) according to cyst fertility. Quantitative mean +/- SD of each group. G1: n= 15, G2: n = 10. Measurements significantly higher in G1 (fertile cysts) when compared with G2, non-fertile cysts ($P < 0.05$).

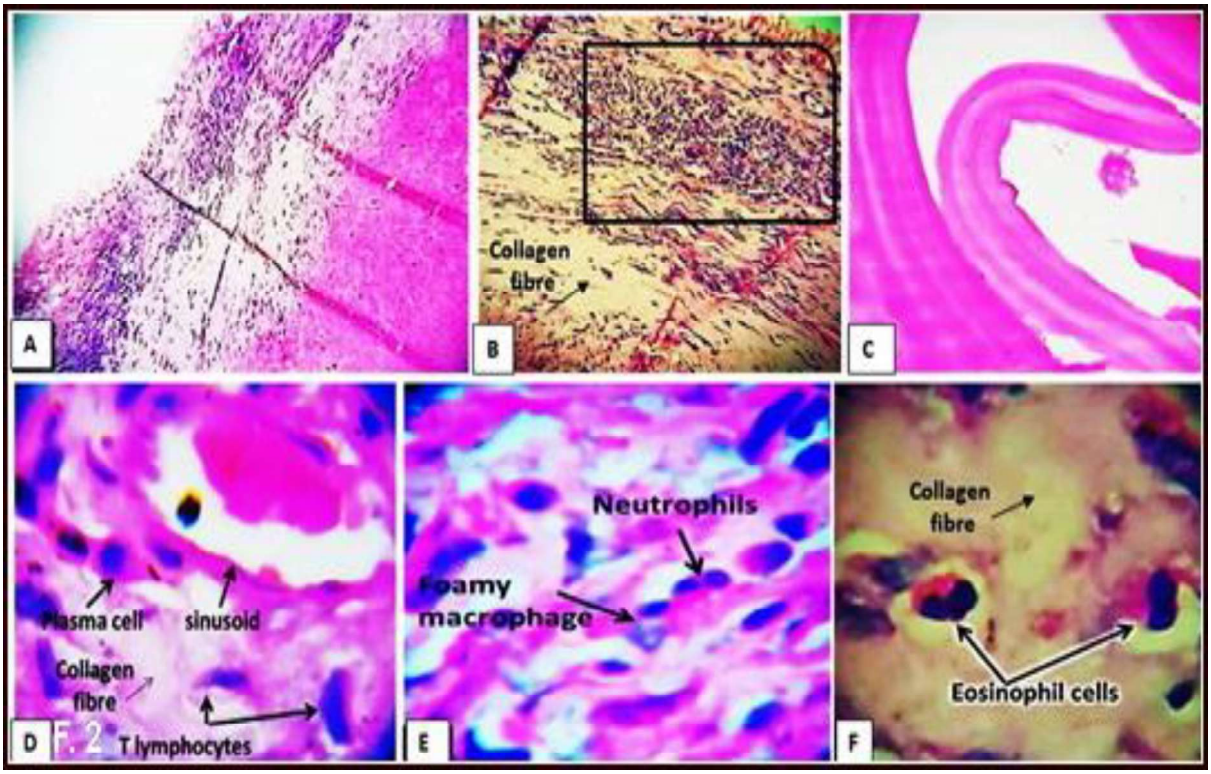
Fig. 2: Histological stain analysis: (A,B) adventitial layer (X40 & X100 respectively) from non-fertile cysts showed aggregates of inflammatory cells, boxed area in B, (C) disorganized laminated layer (X100) as a control, (D) lymphocytic aggregation (H & E, X1000), (E) neutrophils and macrophages (X1000), (F) eosinophils (X1000).

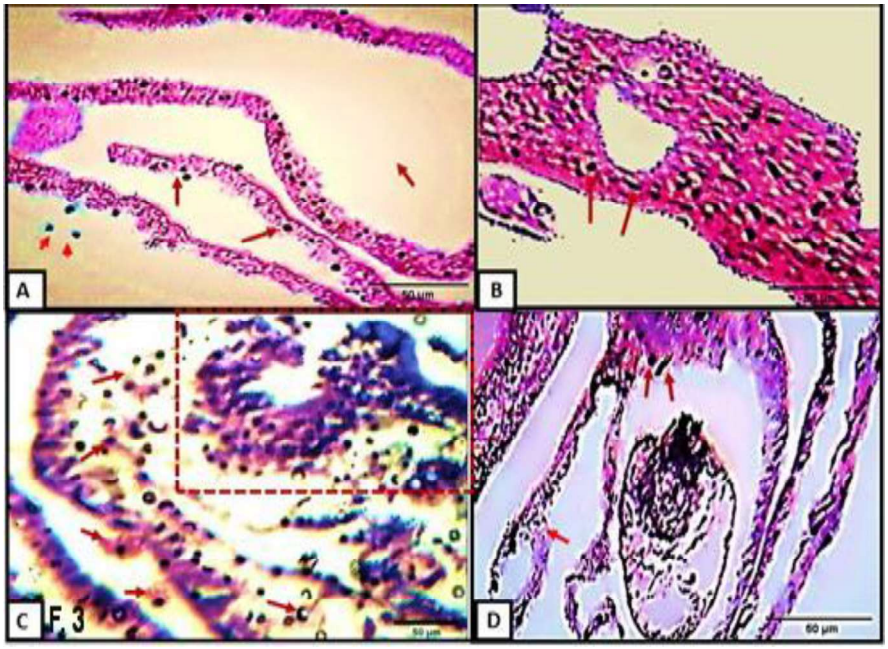
Fig. 3: Germinated layer histological sections: (A, B) stained with H&E showed multi-nuclear patterns within germinated layer of fertile CE, (C) stained with Masson's Trichrome stain showed an area of germinated layer infiltrated with mononuclear cells (boxed area), (D) protoscolex in fertile CE enclosed by germinated layers, 50µm.

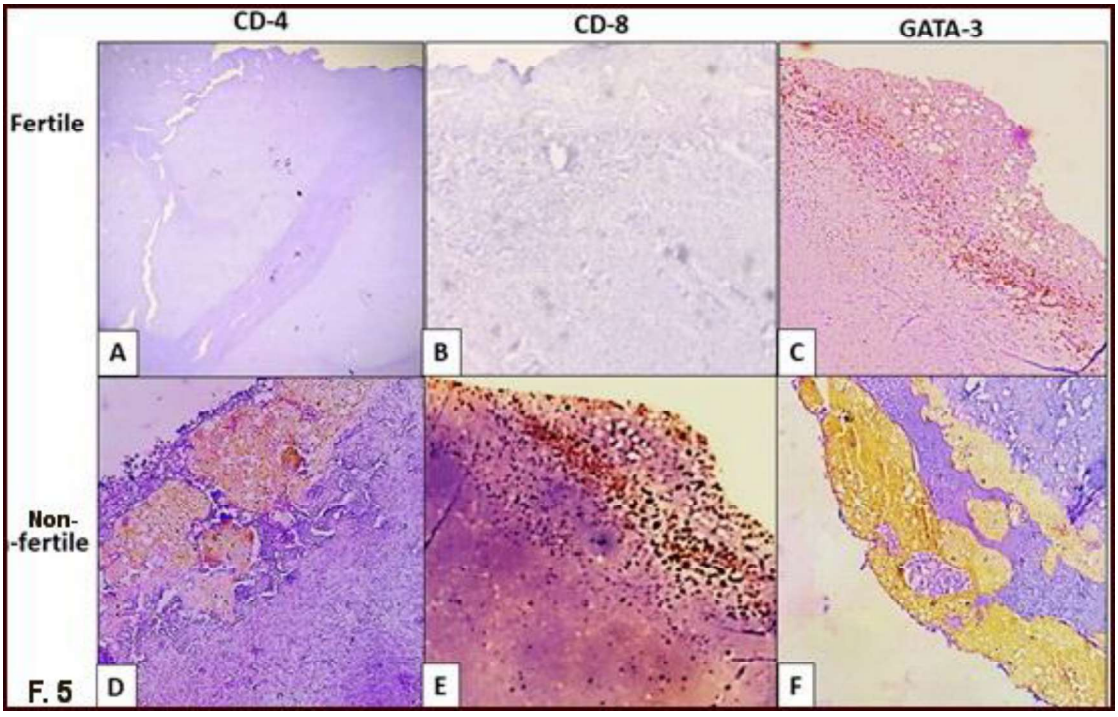
Fig. 4: Intra-adventitial lymphocyte counts in CE biopsies: (A) Region of adventitial layer in fertile CE cyst (G1), with discrete lymphocytes and (B) showed sheets of cells in non-fertile cysts (G2). (C, D) images in grids from A & B respectively with plotted cell counts.

Fig. 5: Intra-adventitial immune-histochemical analysis of groups of human CE by low power: anti-CD-8^{+} (A, D), anti-CD-4^{+} (B, E), and GATA-3 (C, F).






A
B
C
D
F. 3


CD-4
CD-8
GATA-3
Fertile
Non-
-fertile
A
B
C
D
E
F
F. 5