

Autoimmunity in human CE: Correlative with the fertility status of the CE cyst

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Summary

Cystic echinococcosis is speculated to exert several immune-evasion strategies involving autoimmune-phenomena. We evaluated the hypothesized that the prevalence of autoantibodies increases in the sera of CE patients that may evidence the association between the parasite and autoimmune diseases. Sera from 63 subjects at distinct types of CE cyst fertility were investigated for antinuclear antibodies (ANA), and anti-CCP antibodies. Plasma levels and cellular production of IL-17A cytokine were specifically defined as being assumed to prime for autoimmunity. Healthy-controls were age and gender-matched to test sera. ANA expressions inside the surgically removed metacestode and adventitial layer were also assayed. Out of 63 patients, 35 % had fertile highly viable cysts (group-1), 41 % had fertile low viable cysts (group-2) and 24 % had non-fertile cysts (group-3). A four-fold increase in ANA sera-levels was detected in group-1 compared with their controls (p -value 0.001) while anti-CCP levels were of insignificant differences. In group-2 and group-3, no significant differences were detected between ANA and anti-CCP sera-levels in CE patients and their controls. IL-17A sera-levels in group-1 and group-2 were significantly higher than their healthy-controls while being of insignificant differences in group-3, p -value= 0.300. No association was detected between sera-levels of IL-17A and ANA as well as anti-CCP antibodies. Interestingly, relative IL-17A cellular expression associated positive ANA deposition in the parasite cells and adventitial layer. Collectively, based on the parasite fertility, IL-17A and ANA seemed to be involved in the host immune defenses against CE. There is no association between CE and anti-CCP antibodies.

Keywords: CE; ANA; IL-17A; anti-CCP antibodies

Introduction

Echinococcosis is one of the 17 neglected tropical and zoonotic diseases (NTDs) documented by the World Health Organization (WHO). In endemic regions, prevalence rates in humans exceed 50 per 100,000 people/ year. In some regions in Central Asia, China, Argentina, Peru, and East Africa incidence rate may in-

crease up to 5 – 10 %. In hyperendemic areas of South America prevalence of echinococcosis in the livestock in slaughterhouses, diverges from 20 % up to 95 %. This parasitic infection ensues also extensively in Australia, particularly in eastern regions, where it cycles chiefly in a wildlife pattern (Craig *et al.*, 2007; Brunetti *et al.*, 2010; Barnes *et al.*, 2012; Thompson *et al.*, 2014; WHO, 2020). According to WHO in 2018, increased numbers of infected

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people reported in a multicenter study revealed the real burden of this neglected parasitic disease in some European Regions and Turkey. In Egypt, a recent study determined the wide diversity of this parasitic disease in slaughterhouses (El Dakhly *et al.*, 2019). Therefore, it becomes necessary for the World Health Organization (WHO) to prioritize efforts for human cystic echinococcosis (CE) (WHO, 2018; WHO, 2020).

It worthy to mention that the establishment of CE disease conserves infection of the intermediate host (herbivorous animal) or the end-host (man) by ingesting the infective stage of the parasite (eggs) that disperse in the dogs' feces; consequently, oncospheres would release and approach the portal blood circulation to lodge in the hepatic parenchyma in about 60 % of the cases. Later, each oncospheres undergoes multiple insidious morphological alterations that involve disintegration of its hooklets, vesiculation, and multiplication of its cellular territories, to be recreated into a cyst surrounded by a germinal layer that frequently produces the characteristic CE cyst fluid. Accordingly, in many years it can attain a larger size. The inner germinal layer reproduces asexually forming inside buds of protoscoleces (PSC) and brood capsules (asexual-reproduction) while depositing to the outside the laminated layer for its protection (Bortoletti *et al.*, 1973; Galindo *et al.*, 2002; Atmaca *et al.*, 2019).

Probably in the intermediate host cystic echinococcosis exists in two variable forms: fertile CE cyst, in which PSC is attached to the germinative layer and liberated into the CE cyst fluid. Also, the viability of the fertile CE cyst, that is, the proportion of live PSC, fluctuates between 100 % and 2,8 % (Dueger *et al.*, 2001; Lahmar *et al.*, 2009; Salem *et al.*, 2011; Lahmar *et al.*, 2013; Zait *et al.*, 2013). Contrarily, non-fertile CE cysts have no PSC neither connected to the germinal layer nor detached free in the CE cyst fluid, and thus are incapable of continuing the parasite life cycle; and the reason beyond CE cysts infertility remains unclear (Rogan *et al.*, 2006; Irshadullah *et al.*, 2011).

However, the innate immunity against the developing oncospheres tempts to defeat the infection via recruitments of eosinophils and macrophages. Consequently, in few weeks, lymphocytic T cells become stimulated and incorporated in the inflammatory response that starts preliminary with the activation of T helper (Th)-1 cells to eliminate the parasite; however, dynamic alterations in the T cells retain the predominance of the Th-2 and T regulatory (Treg) cells (Gottstein *et al.*, 1991; Vatankhah *et al.*, 2018; Abo Aziza *et al.*, 2020; Jiménez *et al.*, 2020). Interestingly, the immunomodulatory mechanisms supervened by the host-derived IL-10 and TGF- β would be invested by the parasite to house itself silently at the expense of the surrounding liver microenvironment (Hanilou *et al.*, 2008; Siracusano *et al.*, 2008). Therefore, the clinical presentation of CE disease remains asymptomatic or presents with non-specific symptoms to be undetectable for a long time until complications ensue (Sahin *et al.*, 2006; Saidi, 2006; Frider *et al.*, 2010; Amit *et al.*, 2015).

Recently, IL-17, a signature interleukin produced chiefly by Th17

cells, has been defined in the progression of liver inflammation (Hammerich *et al.*, 2011) and gastric cancers (Zhong *et al.*, 2015). Besides, in a human study, the expression level of IL-17 appeared to affect the progression of cystic echinococcosis (Tursun *et al.*, 2010). However, the factual role of IL-17 in CE remains not well understood especially if this interleukin had been implicated in several autoimmune (Furue & Kadono, 2019; Nalbandian *et al.*, 2019) and infectious diseases (Iwakura *et al.*, 2008).

In this framework and on the account of previous studies, CE in humans seemed to be related to autoimmune diseases (Girelli *et al.*, 1993; De Rosa *et al.*, 2001; Tekaya *et al.*, 2009). Review articles published by Zhang *et al.* (2003), Zhang *et al.* (2012), and Grubor *et al.* (2017) proposed several mechanisms exerted by the parasite to avoid the host-protective immunity that involves antigen mimicry.

In the current study, serum samples for CE were used to define autoimmune populations implied in the diagnosis of autoimmune and rheumatic diseases; anti-nuclear antibody (ANA) and anti-cyclic citrullinated peptide (anti-CCP) antibodies.

ANAs are a subset of autoantibodies that attack the *nuclear* macromolecules including DNA, histones, centromeres, cyclin A, and small nuclear ribonucleoproteins; that share chiefly in the pathogenesis of SLE (Wu *et al.*, 2006). ANAs were found to be elicited as a part of the anti-parasite immune response including cystic echinococcosis (CE), plasmodium infections, and schistosomiasis (Hsieh *et al.*, 2008; Romani, 2008; Aslan *et al.*, 2009; Brahimi *et al.*, 2011; Wang *et al.*, 2018; Rivera-Correa & Rodriguez, 2019). However, the real function of ANAs in echinococcosis still not clear. Anti-CCP, citrullination is a physiologic process that occurs during cellular death by the diminution of arginine amino acid that exists in certain human proteins; a process that requires the presence of the peptidyl arginine-deiminase enzyme (PAD). Expression of PAD occurs normally in the leucocytes during the inflammatory processes. However, the release of PAD in the bloodstream may citrullinate extracellular proteins. Production of anti-cyclic citrullinated peptide antibodies appeared to be critical for the development of rheumatoid arthritis (RA) (Schellekens *et al.*, 2000; Lee & Schur, 2003; Zendman *et al.*, 2006; Ortancil, 2011).

In the present work, based on the fertility rate of the metacestode we determined the serum levels of IL-17A and auto-immune populations (ANA and anti-CCP antibodies) in CE patients comparing with healthy controls. Localizations of the inflammatory cytokine IL-17A and ANA in the metacestode and adventitial layer were also investigated.

Material and Methods

The specimens were obtained from the National Hepatology and Tropical Medicine Research Institute and Nasser Institute for Research and Treatment in the period from January 2018 up to May 2020. The 63 patients were confirmed for CE in respect to clinical, radiological, and serological findings; and underwent either

surgical removal or PAIR technique in association with medical treatment. All these patients were with no known disease at the time of sample recruitment or preceding anti-CE therapy in earlier times. Small CE cysts were considered still developing and have not initiated producing PSC. Control subjects were selected on account of being age and gender harmonized to the assessed sera and of no known illness meanwhile obtaining the blood samples. Sample processing regarding the standardized procedures was performed in the Pathology Department, Faculty of Medicine, Cairo University, Egypt. Readings and titrations of the target auto-immune immunoglobulins were performed in Theodor Bilharz Research Institute (TBRI), Immunology and Therapeutic Evaluation Department, Cairo, Egypt.

In a descriptive-analytic cross-sectional study, the influence of CE infection on autoimmune antibody populations in the serum of the patients and in situ in surgical CE cysts specimens was evaluated besides the possible role of IL-17A. The study involved 63 patients diagnosed with CE and 63 age and sex-matched healthy subjects were used as controls in the current study.

Quantification of autoimmune antibodies-sera levels (ELISA):

Using pre-operative serum samples the autoimmune populations; Antinuclear antibody (ANA) and Cyclic citrullinated peptides (Anti-CCP) were quantified using enzyme-linked immunosorbent assays (ELISA) as recommended in the manufacturers' instructions (Biocompare, Inc. company, Biorbyt, # orb339622, South San Francisco, United States for ANA, MyBioSource, # MBS703683, Creative Diagnostics, # DEIA1227, the USA for Anti-CCP, and RayBiotech #ELH-IL17-1, Norcross, Georgia for IL17A). Optical density was measured by switching the plate reader at 450nm for ANAs, anti-CCP, and anti-IL-17A. IL-17A detection level was 80 pg/ml. In further absolute quantification of the ANAs (using lab-to-lab service), titer less than 1:80 was considered negative, $\leq 1:160$ was a cut-off, and $\geq 1:320$ was positive meanwhile in anti-CCP titers more than 20 EU/mL were regarded as being positive.

Characterization and classification of Cystic Echinococcosis

HCF obtained by PAIR and cystectomy was evacuated into sterilized test tubes such that the shreds of the germinative layers, brood capsules, and protoscoleces were allowed to precipitate. The parasite's products were then washed three times in NaCl sterile solution at 0.9 %. Thereafter, the liquid was filtered using sterilized gauze (with wide porous mesh) to eliminate large fragments. The obtained filtrate was poured into sterile test tubes left to precipitate at room temperature for 5 min (Khammari, 2019). Obtained samples were classified based on their fertility rate, a process that involved estimation of the average number of protoscoleces and brood capsules/ml, and the viability rates in percentage where viability below 85 % were to be considered low.

The supernatant was removed and 100 μ L of the protoscoleces' sediment was obtained for counting; where 20 μ L was smeared and the parasites (protoscoleces and brood capsules) were enu-

merated and logged as the parasite number per each 100 μ L sediment. The process was repeated 2 – 3 times to assure the average number (Shahnazi *et al.*, 2013; Hajhossein *et al.*, 2015). After the average protoscoleces counting, one drop from the sediments of the CE sand (protoscoleces; brood capsules, and shreds of the germinal layer) was inspected microscopically as wet unstained mounts to determine the existence of protoscoleces or hooks. Protoscoleces were examined for amoeboid-like movements, the activity of the flame cells, and integral morphology by light microscopy. Dye-exclusion test was performed using eosin 0.1 % solution as a vital stain where eosin was added to the same volume of cyst fluid holding CE sand. Due to loss of membrane integrity, the dye is absorbed by dead protoscoleces while viable ones remained unstained (Hanks and Wallace, 1958; Ducci *et al.*, 2002). Thereafter viability was calculated in percentage. Moreover, viability was assured using the method of the urine strip test, where the highest glucose and the lowest protein measurements signified high viability and vice versa with those specimens of low viability (El Saftawy *et al.*, 2017).

Processing of the metacestode: Cyst wall specimens from both fertile and non-fertile cysts were formalin-fixed, paraffin-embedded, and subjected to the ensuing staining processes:

Hematoxylin and Eosin stain (H&E) (Kiernan, 2001): H&E stain was used to demonstrate the considerable details of the metacestodes. All slides were examined for the germinal and laminated layers, attached protoscoleces, and the adventitial layer where photomicrographs were captured using Olympus Microscopy, CX-23.

In-situ biomarkers immunoassay:

(1) Immunohistochemical Staining for IL-17A (El Aal *et al.*, 2015): tissue cut sections were dewaxed and the intrinsic peroxidases were blocked with Hydrogen-Peroxide Block stock for 10 – 15 min. to hinder the nonspecific background staining. Tissue sections were then washed 2 – 3 times with PBS and incubated with ScyTek Pro-Block solution for 20 – 30 min to block proteins. Tissue sections of both fertile and non-fertile cysts were then incubated with Anti-IL-17A antibody, polyclonal antibody (Abcam, catalog number ab214588) (1:200) for 30 minutes at 4°C. After washing in PBS, tissue sections were incubated with Ultra-Vision Detection System, a labeled streptavidin-biotin immune-enzymatic antigen detection kit, (TP-015-HD, Thermo-Fisher Scientific, USA) for 10 min. at 25°C. Finally, tissue cut sections were embedded in DAB solution for 15 – 25 min. to be finally counter-stained with hematoxylin. Control slides were performed where the step of immune staining with the anti-IL-17A antibody was omitted.

Estimation of IL-17A immune-reactive cells: the intensity of the immunohistochemical stain was performed by Image J software

Images by being transformed to the format, 8-bit gray-scale. Two definite assessments were made in the current study: a) the digitized immune-stained tissue images were segmented into IL-17A immune reactive areas versus the non-immune reactive areas and b) the quantification of the staining intensity within IL-17A immune positive areas where the threshold of positive pixels was set above the background intensity (Smorodinova *et al.*, 2015; Smorodinova *et al.*, 2017). Positive signals were automatically selected to designate counts of the positive signals per mm².

(2) Direct immunofluorescence staining:

Tissue sections from both fertile and non-fertile CE cysts were subjected to the staining procedure. Besides, the study held a process to convert hydatid sand into cut sections that involved embedding the samples of informal aldehyde in a test tube, then the sediment was obtained and allowed to be settled and wrapped in gauze. Thereafter it was applied in tissue cassettes to be embedded in melted paraffin wax. Cut sections were consistently prepared using microtome to be ready for staining.

Anti-nuclear antibody (ANA), FITC:

ANAs IgG, one of the evaluated autoimmune populations in the patients' sera, was tested for its existence in the stable immune-complexes in the metacestodes' structures using direct immunofluorescence staining (Ensink *et al.*, 2015, Wang *et al.*, 2016) with IgG fluorescein isothiocyanate (FITC) Conjugate w/ Evans Blue (#1410b327) kits. Reference pattern of ANA: a- homogenous i.e. full nuclear staining which is pathognomonic for any autoimmune disease; b- rods and rings pattern: which is thought to be a drug-induced pattern in hepatitis-C; c-speckled: coarse and fine speckles throughout the nucleus; c-nucleolar: inspection of the immune-stain around the nucleolus in the inside of the nucleus; d- centromere: as the immune staining is visualized along the chromosomes (Calise *et al.*, 2015; Chan *et al.*, 2016, www.ANAPatterns.org).

Positive tissue control

Hoechst dye stock is a membrane-permeable fluorescent stain that labels the nuclear DNA by binding to adenine (A)-thymine (T)-rich regions in the minor groove emitting blue fluorescence. Therefore, it was used as a control fluorescent stain to inspect the live and fixed cells in each independent group. In the present work, the Invitrogen™ Hoechst 33342 (catalog no. H3570, Thermo Fisher Scientific, USA) was utilized in a dilution, 1:2,000 in PBS (Crissman and Hiron, 1994; Stinton *et al.*, 2013; Chazotte, 2011; Crowley *et al.*, 2016).

Controls were exposed to similar conditions of tissue processing and staining procedures were similar for both ANA immune-stain and Hoechst dye. Fixed cells and tissues were incubated in the stain for 5 – 10 minutes at room temperature. Samples were then washed properly with PBS to be ready for reading under the con-

ventional fluorescent microscopy at 490 nm wavelength for FITC and 461 nm for Hoechst dye.

Notably, the whole procedure was kept away from the light and was guided by the manufacturer's protocol. The images were measured for optical intensities using Image J software, NIH, USA; by being segmented and exposed to thresholding creating binary- images; i.e. black and white pixels (Ensink *et al.*, 2015). Specimens of fertile and non-fertile cysts were assessed in 100 images (20× objective) with means ± S.D. values per mm².

Negative tissue control

Healthy Guinea liver was supplied from Pathology Department, Faculty of Veterinary Medicine, Cairo University and manipulated to establish negative (healthy) tissue control (Swindle *et al.*, 1988; Dyson *et al.*, 2012) when becomes stable in ANA staining.

Statistical methods:

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data were summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. For comparison of paired measurements within each patient, the non-parametric Wilcoxon signed-rank test was used (Chan, 2003a). Correlations between quantitative variables were done using the Spearman correlation coefficient (Chan, 2003b). *p* -values less than 0.05 were considered as statistically significant

Ethical Approval and/or Informed Consent

In the current study, all measures regarding human contributors complied with the ethical standards established by the National Research Committee and the 1964 Helsinki Declaration 1964 (on 1/10/2014 in Kasr Al-Ainy School of Medicine, Cairo University, Egypt) and its successive regulations and analogous ethical standards. Objectives and dealings of the study were clarified to all participants. Informed consent was obtained from all participants in the current study. Patients of any age who suffered of cancers in addition to pregnant women and children younger than five years old were all excluded.

Results

Characterization of cystic echinococcosis (CE):

CE cyst fluid was collected from 48 male patients (76 %) and 15 female patients (24 %) of mean age 50 yrs ± 5 old. Morphohistological analysis of the fertile CE cysts revealed variable PSC viability, germinal layer with PSC attached (Fig.1), thick laminated layers, and adventitial layer composed mainly of fibroblasts and collagen fibers (Figs.1F and G). Additionally, histological sections of fertile

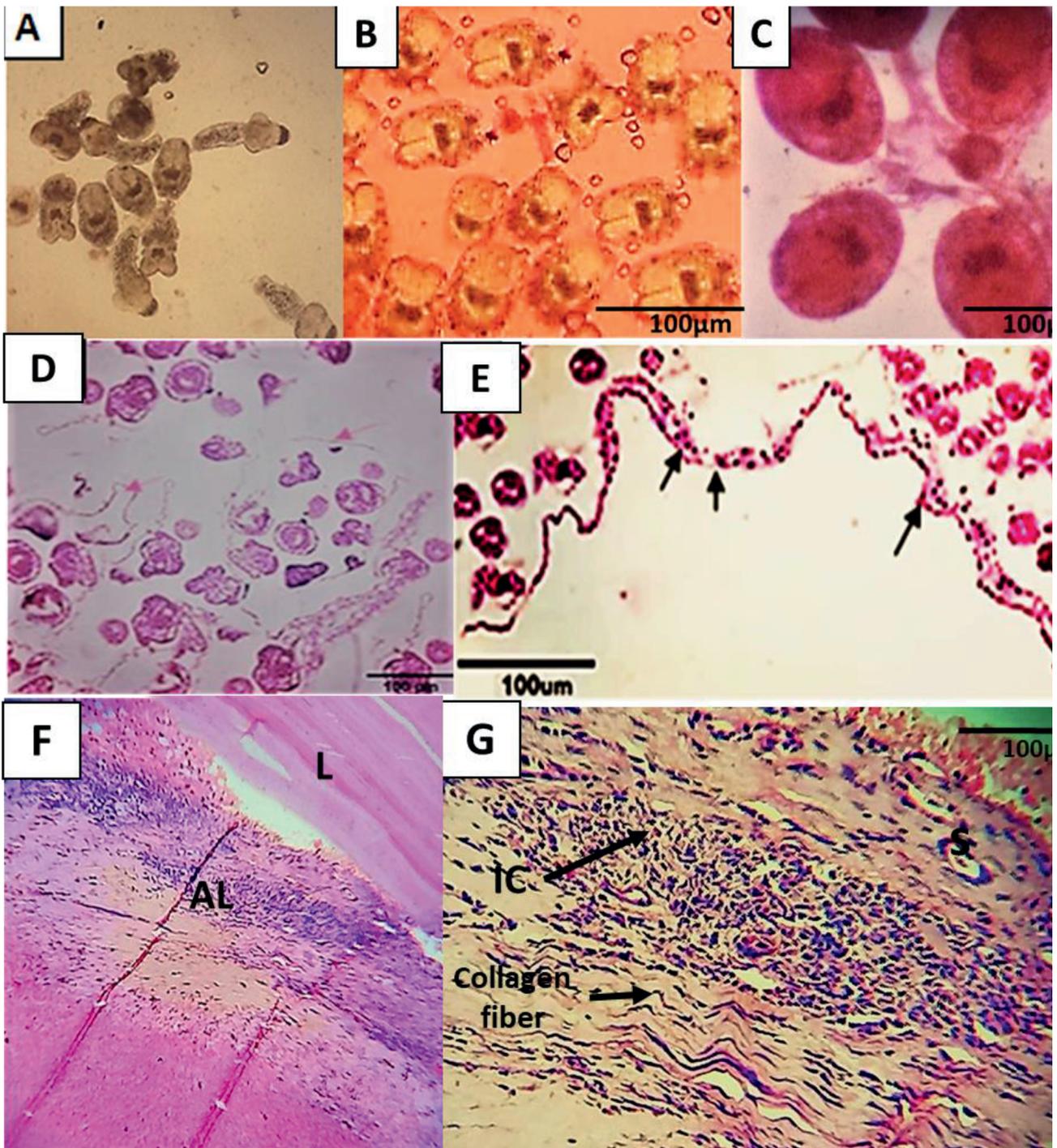


Fig. 1. Show the structures of the fertile hydatid cysts removed from liver tissue.

(A) Light microscopy of free evaginated hydatid protoscoleces in wet mount. (B,C) Determination of parasite viability using 0.1 % eosin dye-exclusion test showing viable and dead protoscoleces respectively. Note stalks are still joining protoscoleces to the germinal layer (red asterisk) in (C). (D) Developed brood capsules encompass protoscoleces. (E) Growing protoscoleces still attached to a piece of germinal layer from a fertile cystic echinococcosis are observed; note the cells with nuclei bigger than parasite cells within the germinal layer (arrows). (F) Laminated (L) layer adjacent to the adventitial layer (AL). (G) Adventitial layer encompasses mixed inflammatory cells (IC) with sinusoids (S) and collagen element. Scale bare, 50 μ m (A);100 μ m (B-G).

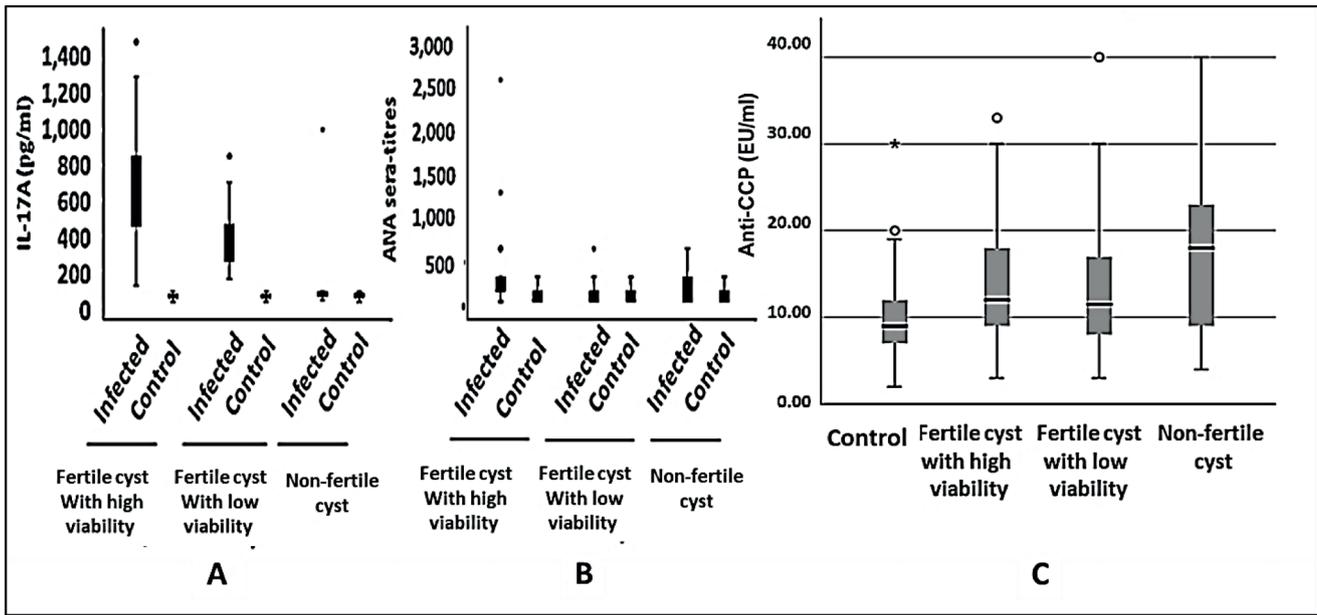


Fig. 2. Plasma concentrations of IL-17A (A), ANA-sera titres (B) and anti-CCP serological values (C) in cystic echinococcosis (CE) patients using ELISA test.

Patients were assembled according to the fertility state of their infection, that is, fertile with high viability, fertile with low viability, or non-fertile cystic *echinococcosis*. Comparisons of the paired measurements within each group were performed using the non-parametric Wilcoxon signed rank test. All data are presented as means \pm SD in the three independent groups.

CE cysts, showed that among the germinal layer cells, several cells have bigger nuclei than the small pignotic nuclei of the germinal layer of the *parasite*, signifying a mammalian origin rather than CE tissue (Hidalgo *et al.*, 2018) as shown in Figure 1 E. It is worth noting that this feature was not found in every non-fertile CE cyst specimen.

From 63 patients, 22 (35 %) had fertile cysts, with protoscoleces viability higher than 85 %, group-1 (plate 1), and 26 patients (41 %) with fertile cysts of low protoscoleces viability, group-2 meanwhile 15 (24 %) patients had non-fertile cysts with no protoscoleces budding from the germinal layer nor detached in the CE fluid, group-3. Grouping of study participants in association with some variables concern fertility of CE cysts is shown in Table 1. The results verified the relationship between cyst fertility and the type of the cyst as well as the parasite mean counts that were higher in group-1 and group-2 compared with group-3 (p -value < 0.05).

Sera- levels of proinflammatory IL-17A cytokine and autoimmune family members (ANA and anti-CCP) in CE patients and disease-free controls

To evaluate the inflammatory profile of IL-17A in CE patients, the

percentages of IL-17A cytokine among the three groups were assessed in serum as shown in Figure 2A. The proportions of IL-17A was significantly higher in group-1 and group-2 than those in the control group (mean 696.59 ± 349.87 vs 52.95 ± 15.73 , p -value < 0.001 and mean 370.77 ± 172.51 vs 51.19 ± 15.53 , p -value < 0.001 , respectively) while being of insignificant differences in group-3 with their controls p -value = 0.300.

The distribution of autoimmune antibody populations according to the three groups and their controls was shown in Figures 2B and C. In group-1, patients have almost a four-fold increase in ANA than their controls (405.45 ± 589.99 vs. 120.00 ± 92.38), this difference is statistically significant (p -value = 0.001). The high standard deviation (SD) in the group-1 indicates that the quantitative data extend over a wide range of values. Conversely, the anti-cyclic citrullinated peptide (anti-CCP) antibodies have a mean value (14.14 ± 7.71) similar to disease-free subjects (11.32 ± 6.52), p -value = 0.19.

Fertile with low viability and non-fertile CE cysts have overall higher ANA mean values (158.46 ± 171.76 and 45.12 ± 8.09), and anti-CCP (218.67 ± 206.67 and 74.47 ± 14.59), these alterations although, are not statistically significant (p -value > 0.05) when

Table 1. Distribution of patients into three groups based on fertility rate of hydatid cysts in the current study.

No of group, n=63	Type of cysts		Mean value \pm SD of protoscoleces	Mean value \pm SD of brood capsules
	Solitary cysts	Multivesicular cysts		
Group-1, n=22	13(59.0%)	13(59%)	1020.45 \pm 479.22	18.82 \pm 9.28
Group-2, n=26	18(70%)	8(30%)	498.08 \pm 232.16	18.65 \pm 8.78
Group-3, n=15	14(93%)	1(7%)	0	0

Table 2. Mean±S.D. plasma concentrations of ANA and anti-CCP antibodies in each of the three groups of cystic echinococcosis patients versus their infection- free controls

Category of patients	Autoimmune-population	Mean±SD of diseased group	Mean±SD of control group	p -value
Group-1	ANA	405.45±589.99	120.00±92.38	0.001
	anti-CCP	14.14±7.71	11.32±6.52	0.109
Group- 2	ANA	158.46±171.76	27.12 ±88.31	0.063
	anti-CCP	45.12 ±8.09	10.58 ±6.31	0.291
Group- 3	ANA	218.67 ± 206.67	37.73 ±23.72	0.084
	anti-CCP	74.47 ± 14.59	12.60 ± 9.14	0.209

compared with their disease-free controls. The complete mean values of the auto-immune population measurements are available in Table 2.

Overall, ANA sera-levels were significantly lower in males than in females. Another finding, anti-CCP, did not express gender dimorphism in CE patients or healthy participants. Age was insignificantly associated with IL-17A levels or the autoantibodies array.

Correlation of interleukin-17A with the production of autoimmune-populations

Correlation between IL-17A level and ANA and anti-CCP antibody concentration levels was evaluated in the three groups of CE patients (n = 63). Spearman *rho*-values and *p*-values are displayed in the Table 3. No correlation was detected between IL-17A levels and ANA as well as anti-CCP antibodies (*p*-value > 0.05).

Localization of IL-17A in immunohistochemical stained CE tissues and its correlation with ANA-immunofluorescence assay

In both fertile and non-fertile cysts, palisading IL-17A positive mononuclear cells were marginal to the laminated layer tracked with accumulated lymphocytes. The main distributions of IL-17A in fertile CE cysts with high viability versus non-fertile cysts were shown in Figure 3. Throughout CE, a trend of constantly growing values was perceived in the whole fertile samples being more in those with high viability *p*-value ≤ 0.05. The immune-reactivity of IL-17A remained a low occasional expression of IL-17A in non-fertile cysts. The mean densities of IL-17A within ten defined and scored regions in the adventitial layer in the three groups are given in Figure 5.

Reactivities of CE for ANAs polyclonal IgG, FITC, and Hoechst dye

Immunofluorescence assay was performed on the prepared cut sections of the metacestode (processed walls and CE sand) and the flanking adventitial layer. ANAs were labeled with polyclonal antibodies FITC (green). Nuclei of the metacestodes' structures and adventitial layer were stained with Hoechst dye (blue) as positive tissue control. Images are median optical sections obtained by confocal fluorescence microscopy. Inserted images are indicating the distribution of ANAs (Fig. 4 I – 1st row of images).

Threshold-based segmentation of the adventitial layer and parasite structures in ANA-fluorescent images

Distributions of the immune complexes' deposits were predominantly noted in the fertile cysts (Sato *et al.*, 1995) being more prevalent in those with high viability as shown in Figure 4 I – 2nd row of images. In sterile cysts, there were low surface fluorescent intensities for the ANAs. The mean quantitative data of ANA in all three groups were of *p*-value ≤0.001.

Characterization of blue-green fluorescence emission ratios in the three independent groups

ANA stain (tagged with green immunofluorescence) had an intensity higher than or equal to the assumed threshold in a typical image of the same group stack of the metacestode's structure and the adventitial layer with the blue-immunofluorescent tagged Hoechst stain (Fig. 4 II). We found in their intensities statistically significant variances in group-1 and group-3 (*p*-value = 0.001 and 0.012, respectively) or subtle differences in group-2 (*p*-value= 0.638).

Table 3. Spearman coefficient of IL-17A plasma levels with autoimmune populations.

Patient groups	Interleukin IL-17A	ANA autoimmune population sera titres		Anti-CCP autoimmune population sera titres	
		Spearman <i>rho</i> -value	<i>p</i> value	Spearman <i>rho</i> -value	<i>p</i> value
Group-1		-0.004	0.984	-0.139	0.538
Group-2		0.021	0.919	0.177	0.388
Group-3		0.257	0.355	0.460	0.084

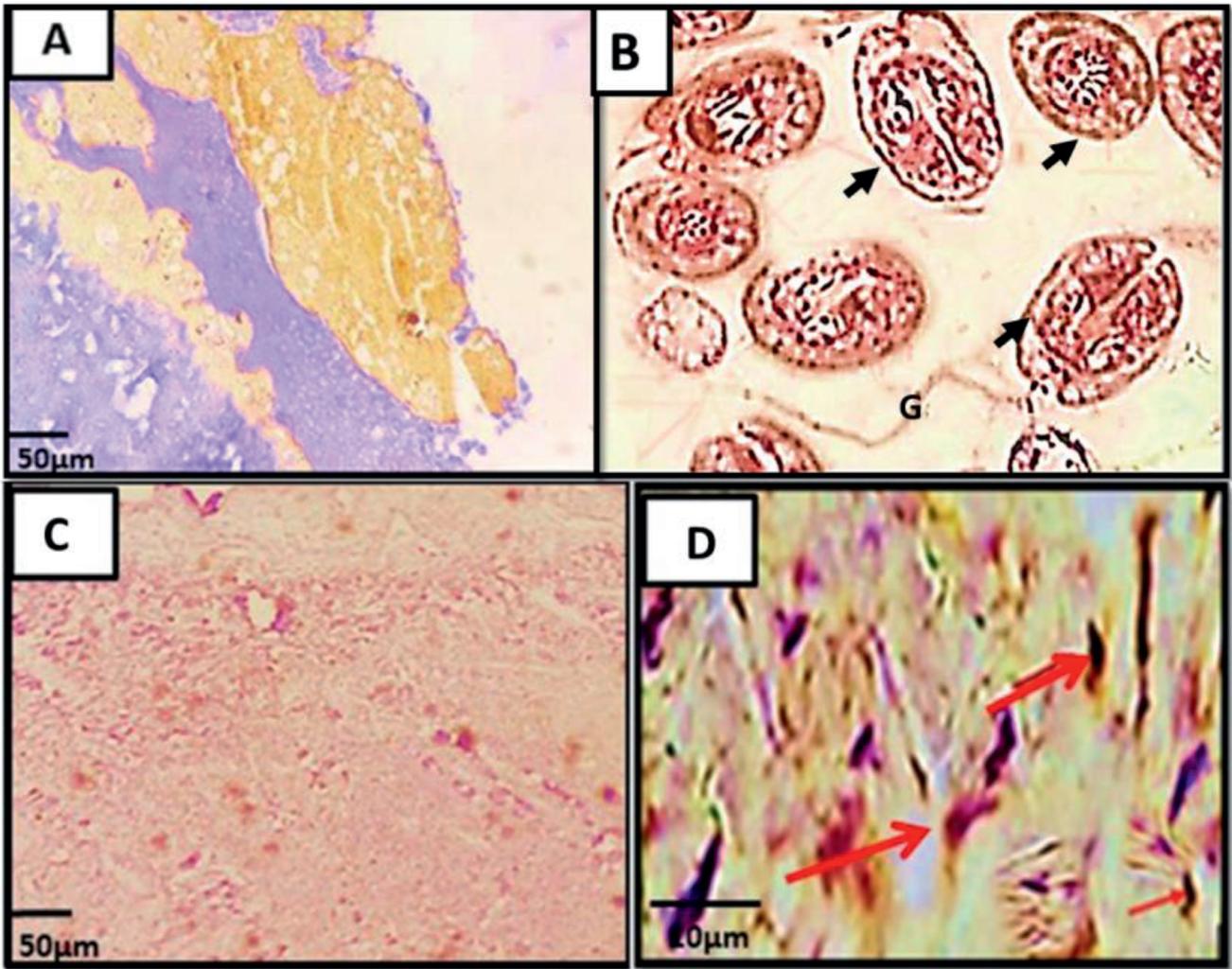


Fig. 3. Photomicrography of IHC-stained hydatid slides for IL-17A obtained using Olympus, CX-23, USA microscopy. Fertile hydatid cyst with high viability: (A) frequent IL-17A immune reactive cells infiltrates marginal to AL. (B) immune reactive cells among the cellular territories of the parasite's protoscoleces along the cuticle. Note the structural composition of a protoscolex (black arrows) still attached to germinative membrane (G). Non-fertile hydatid cysts: (C,D) showing low immune-positive cell infiltration within fibrous elements.

Characterization of the negative tissue control

Healthy Guinea liver exhibited a lower ANA- green fluorescence emission than the stained metacestodes structures or their adventitial layer in the three groups, p -value < 0.001.

The correlation coefficient between IL-17A and ANAs in situ production

ANAs immune positivity had a similar distribution to IL-17A positive immune cells, with a higher value in the structures of cysts with a high fertility rate. The IL-17A was also found to have a statistically significant correlation with ANA regarding the grade of CE cyst fertility (p -value < 0.05), (Fig. 6).

Limitation of the study

3 patients refused to accomplish the study and their results were

omitted. Also, 4 patients had serological titer 160 and their tests were repeated after 2 weeks in regards to the manufacture instructions to confirm the diagnosis in addition to the radiological findings.

Discussion

Satisfactory CE treatment entails an acquaintance of certain features related to the viability of the parasite (Odev *et al.*, 2000; Pauluzzi *et al.*, 1966). Protoscoleces viability was relatively high in the viable solitary and multivesicular cysts, while almost absent viability was detected in solitary cysts. There are few studies concerning protoscoleces viability and CE cyst fertility, however, the results obtained in the current study were similar to a prior study published by Lahmar *et al.* (2012) in Tunisia. Manterola *et al.* (2006)

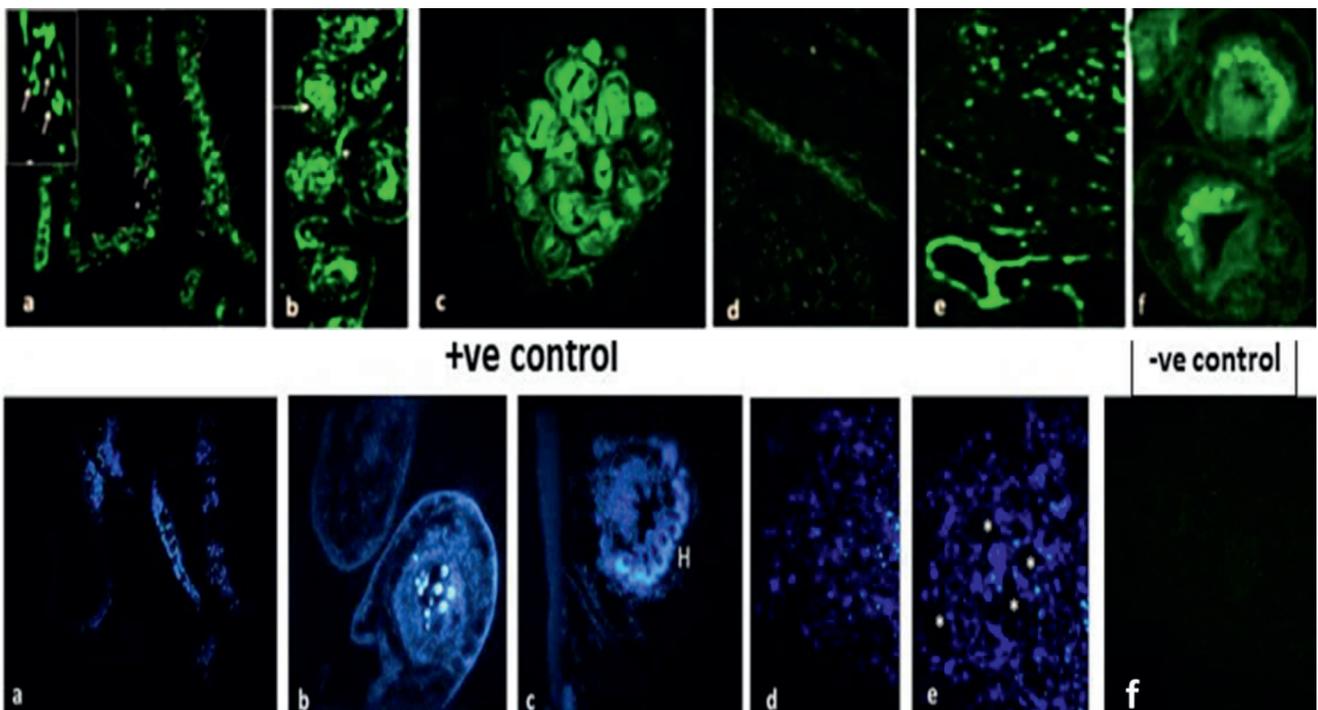


Fig. 4. Immunofluorescence photomicrographs of hydatid cysts.

I-(a-g) the deposits of immune complexes showed a predominant dispersal in the cellular territories of the germinal layer. (a) The rod and ring distribution pattern of ANA. (b) Protoscoleces (P) attached with stalks (s) to the germinal layer (g). (c) Protoscoleces bounded by germinal layer, brood capsules (b). (d) Protoscoleces with chief distribution of immunecomplexes in the hooklet region (h) and moderate to faint existence in the suckers (sk.) and outer rim of the protoscolix. (e) Mononuclear immune cells infiltrating adventitial layer in homogenous ANA pattern. (f) Sinusoidal endothelium showing positive immune reactivity with apparent defenestration (white arrows). II (a-e) Representative images of positive controls in group-1 (fertile cyst with high viability stained with Hoechst dye showing total nuclear counts). In II (f), the negative control, healthy Guinea pig's liver stained for ANA. Scale bar in pixels.

employed the association between fertility and some variables including type, location, and the diameter of CE cysts as well as the incidence of biliary communications.

High fertility and viability of the cysts evidences the high metabolic activity of the parasite and thus the production of antigenic secretory/excretory by-products being available to host immunity to be phagocytosed and introduced to T helper cells by antigen-presenting cells (Virginio *et al.*, 2012).

Histological analysis revealed the infiltration of mixed inflammatory cells in the adventitial layer similar to a prior study published by Barnes *et al.* (2011). However, Moudgil *et al.* (2019) conveyed the existence of mononuclear infiltrations in the lung tissues adjacent to cystic echinococcosis. Meng *et al.* (2012) assumed the recruitment of the inflammatory cells to be related to apoptosis in the hepatocytes adjacent to the metacestode.

The higher percentage of IL-17A in the inflammatory milieu observed in CE patients with fertile highly viable cysts than in patients with non-fertile cysts suggests the implication of the cytokine in host immune responses against the helminthic infection, following prior-studies (Mezioug & Touil-Boukoffa, 2012; Pan *et al.* 2017). These data indicate a possible relationship between CE cyst fertility and cytokine production in patients' serum and the possible use of IL-17A in the immunosurveillance of CE disease. Labsi *et*

al. (2018) reported that in vivo treatment with IL-17A attenuated the development of cystic echinococcosis and the associated liver fibrogenesis in an experimental model. Such findings drive the attention if IL-17A may have a possible role in the progression of cystic echinococcosis towards non-fertility?

However, the somatic antigens of the protoscoleces were suggested to be related to the regulation of IL-17A production in B cells and CD-4⁺T cells. Pan *et al.* (2017) evidenced that in vitro cultivation of B cells with the excretory/secretory products released by the protoscoleces exhibited lower percentages of Th-17 and B-17 cells and higher percentages of B-10 cells.

Interestingly, it has been assumed that the balance between B-17 cells and B-10 is similar to that between Th-17 and T-reg cells; however, during chronic infection, there are higher percentages of T-reg cells and B-10 contributing to the immune evasion and pathogenesis of the metacestode (Tuxun *et al.*, 2012; Pan *et al.*, 2013; Tan *et al.*, 2013; Pang *et al.*, 2014; Pan *et al.*, 2017).

Similarly, using animal models Wang *et al.* (2014) and Kim *et al.* (2019) postulated the possible therapeutic effects of CE cystic fluid on airway inflammation due to allergy by different strategies including down-regulation of IL-17A.

ANA is the easiest and most frequently assessed biomarkers for autoimmune diseases at a population level (Wandstrat *et al.*,

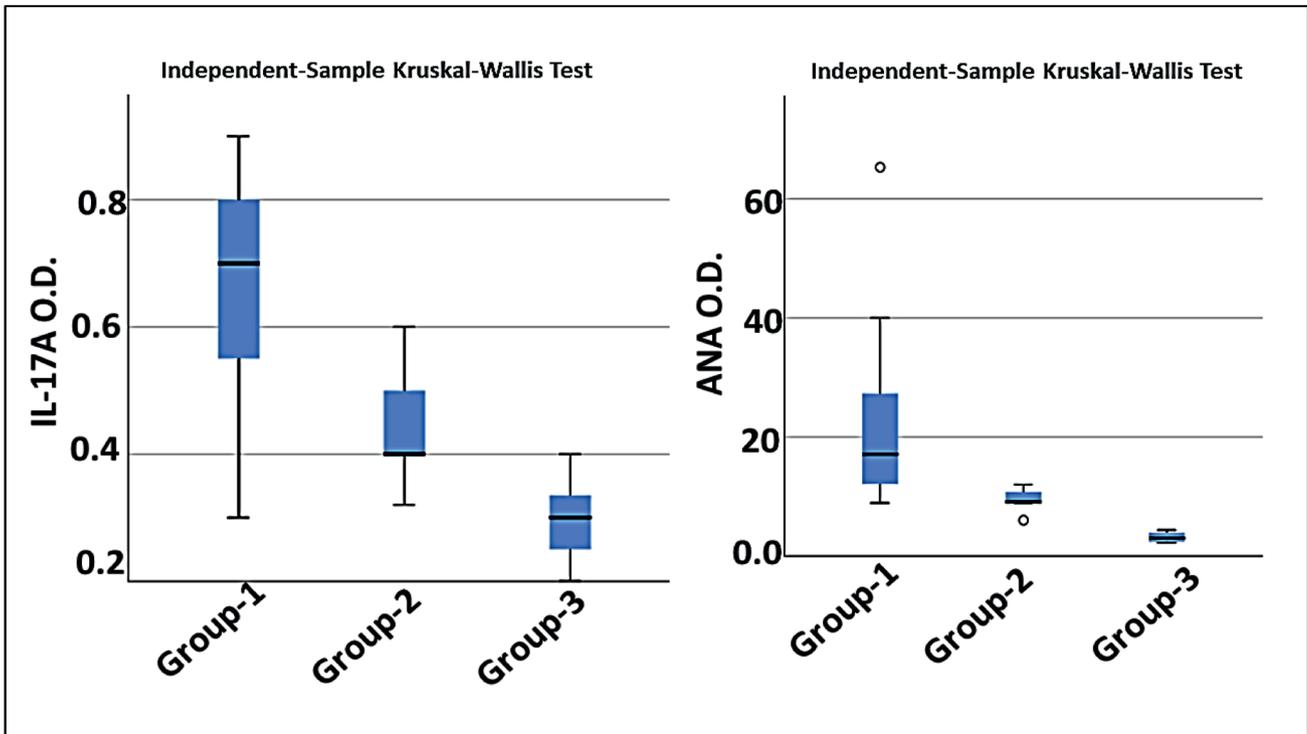


Fig. 5. Immunohistochemistry study of the hepatic hydatid cyst adventitial layer (AL) for interleukin factor IL-17A and ANA immunofluorescence in the three independent groups of hydatidosis patients. The level of significance was adjusted by the non-parametric Kruskal-Wallis and Mann-Whitney tests. The significant differences between the examined groups are indicated ($P < 0.01$).

2006). Estimating the prevalence of ANA in the CE seemed to be critical to understanding the possible parasitic relationship.

Our findings of high ANA seropositivity in fertile CE cysts with high viability is puzzling; and similar to our results Aslan *et al.* (2010) detected significantly high levels of ANAs and other autoantibodies in the sera of patients with CE. One prospect is that ANA binds to nuclear antigens that are charged molecules, chiefly with histones and DNA (Tan *et al.*, 1989). As a consequence, seropositivity shows a robust response and their interpretation necessitates caution, especially in clinical settings of this parasitic disease (Copple *et al.*, 2007; Meroni & Schur, 2010).

Another explanation for the wide frequency of ANA expression (wide standard deviation) among patients with fertile and highly viable CE cysts might be related to intrinsic immunological disorders. Perhaps as a species-specific criterion, humans are more liable to autoimmunity, with ANA response a shot in the arm in several autoimmune diseases (Pisetsky, 2011; Rai & Wakeland, 2011). In animal-based experiments, the production of ANA can occur in absence of other systemic lupus erythematosus-related manifestations, reflecting that enhancement of some immune cells' activities seemed to be dependable on the actions of specific genes (Richard & Gilkeson, 2018).

Rai and Wakeland (2011) postulated that the human genome may contain several polymorphisms to increase the species qualifica-

tion to defeat infections or to help in wound healing. Also, Oates *et al.* (2003) suggested that the selective and deep pressure created by certain infections, that may involve for instance nitric oxide production on a molecular level, that associates diseases such as rheumatoid arthritis and systemic lupus erythematosus as well as resistance against malaria.

However, several studies illustrated that ANA can be existing in healthy individuals (Grygiel-Górniak *et al.*, 2018) as well as in patients with non-rheumatic diseases such as hepatitis-C (Hsieh *et al.*, 2008), cancers (Mohammed & Abdelhafiz, 2015), and thyroid diseases (Inamo & Harada, 1997). In prior studies ANA had been postulated to be of low diagnostic value and that ANA-seropositive individuals in the general population shouldn't necessarily associate autoimmune disease or even develop the disease in the future. In this regard, monitoring of ANA sera-levels is speculated to be only mandatory in cases of clinical symptoms for further rheumatologic diagnosis (Grygiel-Górniak *et al.*, 2018; Karamelic *et al.*, 2007; Pisetsky & Lipsky, 2019).

The finding of being higher in females simulates several earlier studies (Watanabe *et al.*, 2004; Hayashi *et al.*, 2008; Satoh *et al.*, 2012). However, Li *et al.*, (2011) postulated the increased risk for autoimmunity in ANA seropositivity, especially in females.

Another notable finding was the minor changes in ANA levels in individuals with fertile CE and low viability and those with non-fer-

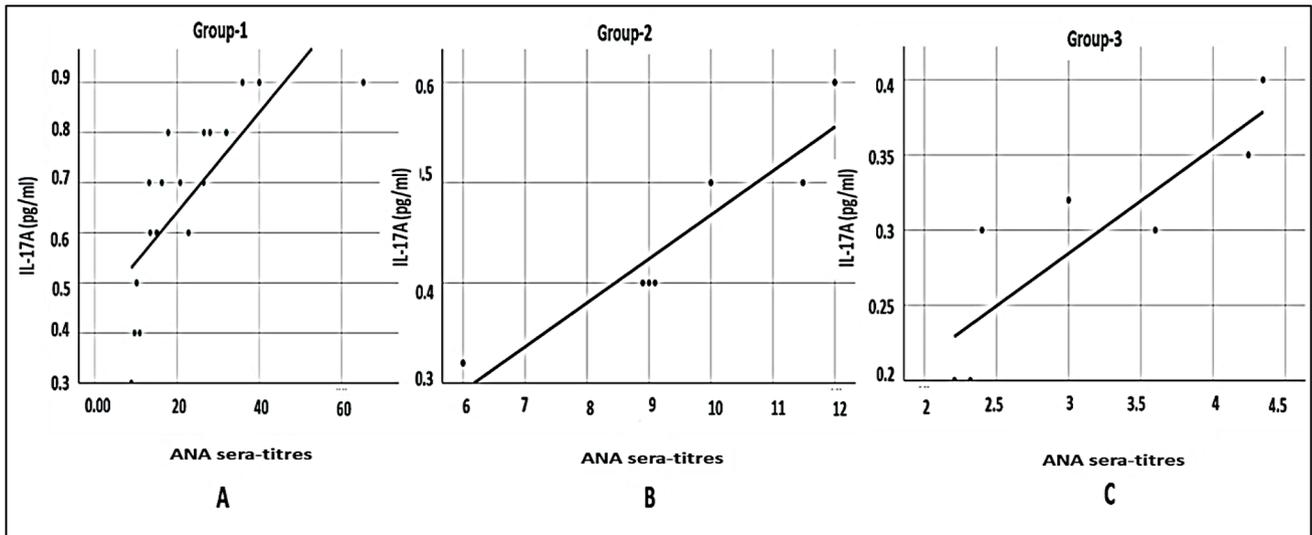


Fig. 6. Correlation between IL-17A immunohistochemical profile and ANA fluorescent intensities in the three independent groups of hydatidosis patients: IL-17A showed positive correlation with ANA in the three hydatid cyst groups. Open symbols and lines represent mean intensities of the tested specimens of each group.

tile cysts that were of insignificant differences compared with disease-free subjects that seemed to be related to the low expression of the parasite antigens.

We investigated serum samples from all patients and their controls to test for the presence of any disease-specific effects as anti-CCP antibodies are recently overlooked as a serologic marker more sensitive and specific than rheumatoid factor (El Sawi *et al.*, 2011). Among the 63 serum samples assayed, positive titers were of insignificant differences with healthy subjects. These findings propose that there is no definite relationship between CE and the level of autoantibodies. In agreement with our results; Colebrook and Lightowers (1995), concluded the lack of association between CE and autoantibodies to a wide-scale of self-antigens.

Interestingly, several case reports documented reactive-arthritis secondary to the infestation of cystic echinococcosis at a distant site in absence of a broad-spectrum of autoantibodies including ANA and anti-CCP antibodies or evidence of other auto-immune disorders, a matter that seems to be related to the potential intense anti-helminthic immunological response (Buskila *et al.*, 1992; Tekaya *et al.*, 2009; Alim *et al.*, 2017). Sánchez *et al.* (1983) reported the existence of special anti-CE antibodies, complement deposits, and vasculitic changes in synovial vessels in patients with a prior diagnosis for Reiter's syndrome. However, Al-Sakee (2011) postulated the significant increase of anti-phospholipid and anti-ardiolipin levels in CE patients and suggested a substantial association of these autoreactive antibodies to cystic echinococcosis. T cells are assumed to be the key mononuclear inflammatory cells in CE that to be differentiated into T-helper cells encompassing the proinflammatory Th1 cells, the anti-inflammatory Th2 cells, immunomodulatory Treg cells, and recently Th-17 cells (Gottstein *et al.*, 1991; Vatankhah *et al.*, 2018; Jiménez *et al.*, 2020; Abo Aziza *et al.*, 2020). In concern to our work, the immuno-reactive expression

of IL-17A was mainly of the mononuclear inflammatory infiltrates that seemed to mirror Th-17 cells possess a definite share in the lymphocytic compartment of the adventitial layer. However, Tan *et al.* (2013) and Ma *et al.* (2014) described neutrophils and Kupffer cells to be involved in the production of IL-17A. Prior studies assumed that the protective role of IL-17A against CE involves the attraction of the macrophages and neutrophils to the site of metacystode to act consecutively with their panel of cytokines (interferon- γ (INF- γ) and IL-6) (Hetta & EIKady, 2017).

Similar to our results in the non-fertile cysts, Monin and Gaffen (2017) reported IL-17A in the laminated membrane within the extracellular traps of the neutrophils along with the enzymes released from their cytoplasmic granules and nuclear histones. Nakajima *et al.* (2014) and Verissimo *et al.* (2019) assumed IL-17A expression to be directly related to the inflammation intensity via inducing Th-2 cells. According to Diaz-Godinez and Carrero (2019), this immunological setting contributes to pathology development rather than being curative.

The dynamics of IL-17A were reported to occur in a time-dependent manner (Hattori *et al.*, 2015). Hai *et al.* (2019) hypothesized that IL-17A alters the immune response milieu towards an effective pro-fibrotic process that encompasses the release of TGF- β 1 from Kupffer cells and HSCs to share in vascular and architectural changes in the liver cells.

IL-17A production was affected significantly by the parasite viability and fertility; in accordance to Das and Khader (2017). Amusingly, it has been suggested that TGF- β 1 released by the IL-17A primed-kupffer cells not only contribute to the parasite immune evasion and chronicity but also facilitates the expansion and differentiation of IL-17A producing cells (Tian *et al.*, 2020).

The current study introduces the first representative estimates of the incidence of ANA in the parasite cells in human CE. In dead,

the definite burden of the cytotoxic ANA auto-immune reactions in different populations was mainly allocated in the highly cellular structures of the parasite and the adventitial layer in a viability-dependent manner.

In the metacestode, the homogeneous nuclear pattern of ANAs seemed to signify the metabolically active parasite cells encompassing protoscolecocytes, germinal layer, and brood capsule (Galindo *et al.*, 2008). Castedo *et al.* (2004) speculated that DNA proliferation can be typified by ANA homogenous pattern where ANAs in the metaphase stage bind to the dense chromatin plate and the interphase stage is found in the outer rim of the nuclei.

In the germinal cells rod and/or ring patterns of ANAs, according to previous descriptions of the ANA fluorescent pattern, are mostly supposed to attack the peri-nuclear cytoplasm of the parasite cells that to our knowledge, had no apparent explanation. In prior studies, it was reported in the perinuclear cytoplasm, against myeloperoxidase enzyme, and considered as a distinctive trait to viral hepatitis-C therapy (Feldrihan *et al.*, 2019).

Infiltration of IL-17A significantly related to ANAs-immune-complex production similar to prior results published by Hetta and EIKady, 2017, Hetta *et al.* (2017), and Zhao *et al.* (2011) who supposed that IL-17A multi-facets involve priming for autoimmune diseases. In this accordance, Nalbandian *et al.* (2009) presumed the direct influence of IL-17A to stimulate the production of auto-antibodies against self-antigens including DNA. Moreover, Miletić *et al.* (2012) defined IL-17A in the inflammatory reactions due to autoimmunity.

Conclusion

Our study examined the serum levels of IL-17A and autoimmune populations (ANA and anti-CCP antibodies) and evaluated and compared their relevance following CE cyst fertility and simultaneously, for the first time, we investigated the localization of IL-17A and ANA in the metacestode and adventitial layer. The present work demonstrated that IL-17A serum levels were significantly increased in CE patients following the fertility of CE. ANA despite being of a four-fold increase in fertile cysts with high viability there was no correlation between IL-17A cytokine and ANA and anti-CCP antibodies serum levels along with the study. There is a lack of association between CE and anti-CCP antibodies. Relative IL-17A expressions in the adventitial layer and among parasites' cells were significantly increased and were associated with elevated ANA intensities being higher in fertile cysts with highly viable protoscolecocytes compared with non-fertile cysts. The present work suggests that, in addition to IL-17A, ANA also seems to participate in immune responses against CE infection in a fertility and viability-dependent manner.

Conflicts of Interest

The authors declare that there are no conflicts of interests

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