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## Research Note

# One minute, intraoperative assessment of the viability of hydatid cysts using a simple reagent strip test

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## Summary

The aim of the current work was to evaluate the possibility of using a rapid and simple reagent strip test to investigate the viability of hydatid cysts intraoperatively, via testing certain biochemical parameters. Thirty eight HCF samples were processed and examined by different methods for determining the viability status. Using the reagent strip test in the current study, the highest significant level of glucose was detected in HCF samples with the highest viability % at pH 7.5 and the lowest significant level of glucose was detected in HCF samples with the lowest viability % at pH 8.5, indicating a likely correlation between glucose concentration and the viability of PSs. On the contrary, protein was not detected in HCF containing viable PSs and was found to be higher in HCF containing non-viable PSs, denoting the possible degenerative processes in such PSs. Haemoglobin was found in trace amounts in all of our samples. In addition, the strip test detected bacterial contamination in 8 samples and biliary leakage in 7 samples. Our results suggest that the simple reagent strip test can assist in providing fast, uncomplicated primary data regarding the viability status of the hydatid cysts. Thus, it may aid the surgeons to make informed decisions for further management and appropriate follow up to minimise the risk of post-operative recurrence.

**Keywords:** Hydatid-viability-strip test

## Introduction

Hydatidosis, or cystic echinococcosis, is one of the most common worldwide zoonotic infections (Lawn *et al.*, 2004). The disease is prevalent in underdeveloped countries, especially in rural areas, where man maintains close contact with various farm animals which may act as intermediate hosts, and dogs as definitive hosts (Radfar & Iranyar, 2004). Hepatic localization is observed in about 75 % of infected cases. Other organs may be affected as primary sites, or as a result of leakage if such serious cystic lesions are not properly managed (Nunnari *et al.*, 2012). Surgical interventions are frequently used to manage this parasitic infection while,

medical treatment by albendazole is typically recommended as an adjunctive treatment to avoid dissemination of scolices to secondary sites (Skuhala *et al.*, 2014). In general, there is a high risk of disease recurrence which is certainly increased if the cyst is not managed properly. One of the most important factors controlling this issue is the viability of the protoscolices (PSs) which by a way or another should be inactivated (Stankovi, *et al.* 2005; Skuhala *et al.*, 2014). Cyst viability and fertility are mainly determined by the presence of free protoscolices in the hydatid fluid and of growing protoscolices attached to the germinal layer (Galindo *et al.*, 2002). In fact, protoscolicidal agents are used frequently in conservative surgical treatment to kill the functioning germinal layer and prevent

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the possible spread of living PSs. Therefore, revealing the extent of viability PSs is crucial for complete success of the therapeutic intervention (Karaoglanoglu *et al.*, 2011).

Viability of the hydatid cysts was previously tested by variable means such as motility or by using special stains; however these methods require time and certain skills. Conversely, research has clarified that metabolic functions of the viable *Echinococcus* metacestode determine the biochemical composition of the hydatid cyst fluid (HCF). The HCF provides the larval stage with the nutrients necessary for metabolism, subsequent survival and growth (Li *et al.*, 1985; Zhao, 1987). These include carbohydrate (Kagan & Agosin, 1968), protein (Carbone & Lorenzetti, 1957), haemoglobin and bilirubin (Li *et al.*, 2013) that are theorised to regulate vital processes within the parasitic stage (Farhan *et al.*, 1959). Accordingly, determination of these leading parameters in the HCF and/or their changeability may implicate an image to the vitality status of this metacestode (Li *et al.*, 2013). For that reason, the aim of the current work was to evaluate the possibility of using a rapid and simple reagent strip test, in a descriptive manner to reflect the degree of viability of hydatid cyst fluid, intraoperative in one minute technique via investigating important biochemical parameters.

## Materials and Methods

The study included 38 patients who were suffering from isolated hepatic hydatid cysts and were admitted to the Department of Surgery, National Hepatology and Tropical Medicine Research institute between April 2014 and April 2016. All the cases were subjected to detailed history and clinical examination, ultrasonography and triphasic computed tomography, serological testing to detect anti-*Echinococcus* antibodies in addition to the routine pre-operative investigations. Cases were exposed to PAIR technique alone or to PAIR followed by deroofing. Additionally, evacuation of the cyst contents was used for large cysts  $\geq 5$  cm in diameter. Albendazole was administered to all patients at a dose of 400 mg; pre-operative twice daily for two weeks up to one to six months post-operative. Pregnant women, patients with liver cysts  $< 30$  mm in diameter, patients treated with ritonavir or phenytoin, and patients with an active malignant disease were not included in this study, as recommended by ethical committee. Aspirated cystic fluids using sterile needles under aseptic conditions were exposed to post and/or intra operative parasitological examination to investigate cyst contents and viability.

### Processing of HCF samples

Each HCF sample was transferred into two sterile tubes. Each tube was labelled for the Patient's name, number and the therapeutic approach by which the sample was obtained. The first tube was centrifuged at  $10,000 \times g$  for 15 min. The supernatant was used for the measurement of the biochemical parameters of the hydatid cyst fluid using the reagent strip test in a sterile environ-

ment. Taking into consideration that centrifugation was essential only for further parasitological examination and not for biochemical testing. In addition, samples were individually examined by variable means including; physiological appearance to detect obvious characteristics and parasitological examination to identify the morphology & viability of the detected protoscolices. The second tube was prepared for culture and sensitivity to test for bacterial contamination of the HCF.

### I-Parasitological examination

According to Soulsby (1982) with modifications, one drop from the resuspended pellets was examined microscopically as wet unstained mounts to detect protoscolices or hooks using 10 x objectives (3 – 5 smears were examined for each sample). The sample was then observed under the 40x objective to examine for motility and amoeboid like peristaltic movements. For staining procedure, Eosin 0.1 % solution was added to an equivalent volume of cyst fluid containing protoscolices (PSs) and one drop was examined microscopically. According to Macpherson and Smyth (1985), dead protoscolices absorb the dye while those that are viable completely or partially exclude the dye (No colour is observed). Viability within 100 protoscolices was recorded and in the case of an insufficient number, more samples were prepared and repeated. Counting was performed until 100 protoscolices were analysed. A calculation was performed to estimate % of viable and non-viable protoscolices.

### II-Physical examination

Physical characteristics were investigated by the following parameters; A- Volume using a volumetric syringe. B- Colour changes; translucent, whitish, greenish or reddish. C- Aspect; clear, slightly turbid or turbid.

### III- Microbiological examination

Culture for aerobic and anaerobic bacteria was performed to detect the presence of bacteria. A standard plate count reflects the number of viable microbes which was used to estimate the growth strength.

### VI-Biochemical investigation using reagent strip test

The reagent strip test, commercially known as urine strip test, was used in the current study to test the viability (Medi-Test Combi 10 © SGL, MACHEREY-NAGEL GmbH & Co.KG made, Germany). The test is a qualitative semi-quantitative method to detect vital biochemical parameters: Blood, Urobilinogen, Bilirubin, Protein, Nitrite, Ketones, Glucose, pH, Specific gravity and finally Leucocyte. The strip was completely immersed in the test tube for 1 – 2 seconds, read immediately and the data reported. The diagnostic parameters were estimated according to each index test using corresponding cut off levels (glucose positive at  $> 50$  mg/dl; protein at  $> 30$  mg/dl; leukocyte esterase at  $> 10$  granulocytes and bilirubin at  $0.85 \mu\text{mol/l}$ ) (Joshi *et al.*, 2013). In case the hydatid cyst fluid was

positive for bilirubin, we proceeded to perform a Fouchet's test in which positive results confirm the presence of bilirubin due to biliary communication.

#### Fouchet's test

The oxidizing action of Fouchet's reagent converts the bile pigment to green biliverdin. Colours range from olive green to emerald green, depending on the concentration of bile pigment present. The tube was filled with hydatid cyst fluid and Ba.chloride with the ratio 1/2. The sample was centrifuged, the supernatant was removed and Fouchet's reagent was then added. The result was qualitative semi-quantitative according to the intensity of the green ring at the bottom of the tube (nil, trace, +, ++, +++).

#### Statistical analysis

All data were analyzed using Statistical Package for the Social Sciences (SPSS) version 16 for Windows (SPSS Inc., Chicago, IL, USA). Data are reported as mean values for quantitative variables and percentages for qualitative variables. To compare between viability and glucose/protein/pH, we used Analysis of variance (ANOVA) followed by pair wise analysis (Bonferroni test). For variables that did not follow a normal Gaussian distribution (not normally distributed), Mann Whitney test was used to compare between viability and bilirubin/leukotriene. P values  $\leq 0.05$  were considered statistically significant.

#### Ethical considerations

The study was conducted according to the institutional ethical and professional guidelines for the management and follow-up of patients for post-operative care. Patients with multiple hydatid cysts or cysts in other organs and cases, who were excluded from this study, were managed according to the institutional protocol in the corresponding department. Informed written consent was provided by each patient before the procedures were performed.

## Results

In the present study, categorization of our cases was done, in one hand according to the individual parameters investigated by the reagent strip test. On the other hand, another classification was done according to the viability tested by eosin exclusion dye, taking into consideration that the total number of samples was 38 samples which included in all categorization. Protoscolices in 38 HCF samples were microscopically examined before and after eosin staining. Ten out of these 38 samples (26.3 %) obtained 0 % viability of PSs after eosin staining, while the PSs in the remaining 28 samples (73.7 %) showed variable degree of viability that ranged from 10 % to 100 %. Despite the fact that this study did not intentionally aim to assess for motility, it was accidentally observed within some PSs (5 out of the 38 samples) within 4 hours during examination. Concerning morphological features (Figs. 1&2), viable, non-motile PSs showed intact tegument, evaginated PSs and

Table 1. Comparison of viability with glucose.

Glucose	Viability Percent		ANOVA	P value
	Mean	SD		
0 (n = 19)	6	2.60		
1 (+) (n = 9)	29.78	5.06	90.06	<0.001*
2 (++) to 4 (++++) (n = 10)	70.70	23.58		

\*Significant p value

# Bonferroni test showed significant difference between the groups

refractile calcareous corpuscles. Non-viable PSs showed different features such as oedema, tegmental destruction or fragmentation and distortion of hooklets (Fig. 3). Many non-viable PSs appear to morphologically resemble viable PSs e.g. normal architecture of calcareous corpuscles and confirmation was attained only after eosin stain. In general, non-viable PSs showed variable morphological features ranging from apparently normal (Figs. 1 D, E & F, Fig. 2 at the periphery) up to extensive necrosis (Fig. 3). Higher positive results which are statistically significant corresponding to glucose level were recorded in the HCF with higher viability in the detected PSs. The high glucose level (2 to 4 pluses) was detected in 10 samples with the highest viabilities as a % (mean =  $70.7 \pm 23.6$ ). Nine samples with a mean viability % of  $29.8 \pm 5.0$ , obtained lower glucose level (+), while no glucose was detected in 19 samples, considered non-viable (mean viability %  $6 \pm 2.6$ ) (Table 1). Bonferroni test showed significant difference between the groups with variable glucose levels, expressing the significance of glucose in the viability status of hydatid cysts. On the contrary, the highest protein levels (+++) were obtained in samples with the lowest viability % ( $15.4 \pm 13.2$ ), while no protein was detected (0) in samples with the highest viability % ( $97.3 \pm 2.5$ ), (+) to (++) with ( $67.2 \pm 14.2$ ). The differences were statistically significant ( $P < 0.001$ ). These results indicate the presence of higher protein levels in HCF containing non-viable PSs and an

Table 2. Comparison of viability with protein.

Protein	Viability Percent		ANOVA	P value
	Mean	SD		
0 (n=3)	97.33	2.51		
1 (+) to 2 (++) (n=15)	67.20	14.61	79.76	<0.001*
3 (+++) (n=20)	15.37	13.16		

\*Significant p value

# Bonferroni test showed significant difference between the groups



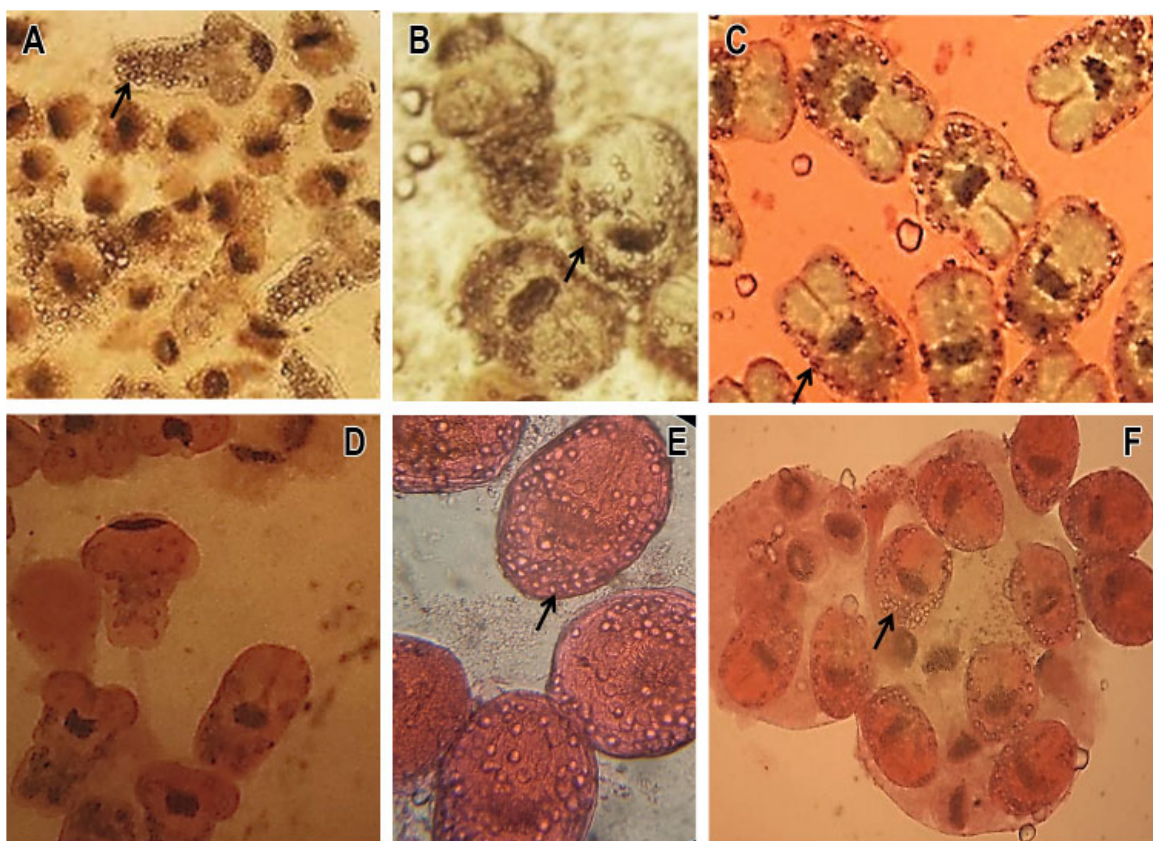


Fig. 1. Viable protoscolices (PSs); A, B (unstained wet mount) & C stained with eosin and appear yellow due to exclusion of the dye. Non-viable PSs (D, E & F) all take eosin stain (pink in colour). Notice the appearance of calcareous corpuscles (arrow) in both viable and non-viable PSs.

absence of protein in HCF containing viable PSs (Table 2). Regarding the pH measured by reagent strip test in this study, 8 cases with the high viability % ( $78.5 \pm 19.2$ ) reported a pH of 7.5, 11 cases with low viability %  $31.6 \pm 6.0$  reported a pH of 8 and the 19 cases with lowest viability % ( $6 \pm 2.6$ ) recorded a pH of 8.5. The differences were statistically significant ( $P < 0.001$ ).

On the other hand, strips displayed positive results at  $>10$  granulocytes/ $\mu$ l in 8 HCF samples with viability %  $9.1 \pm 10.7$ , while in the 30 samples with no bacterial growth, the mean viability % was  $33.9 \pm 31.1$ . The difference was statistically significant ( $P = 0.002$ ). Positive samples were processed for both aerobic and anaerobic cultures. The identified microorganisms were *Escherichia coli*, *Enterococcus* spp. and *Staphylococcus aureus*. Moreover, samples obtained from one patient had sterile HCF in spite of being positive for leukocyte esterase. Bilirubin was detected in 7 samples with reported viability % of  $17.6 \pm 12.7$  (2 of them were reported to have super infection as well), while 31 samples were free of this biochemical parameter and the mean viability % in their samples was  $31.2 \pm 32.0$ , however, the difference was not statistically significant ( $P = 0.42$ ) (Table 3). Therefore, (excluding Bilirubin) our results signify the importance of the previously mentioned biochemical parameters in determining the HCF viability status. Density was the same in all samples in addition to Hb, which was found in trace

amounts in all samples. Nitrite and ketone did not show changes in the reagent strip test, denoting their irrelevance to our samples. Then again, according to the viability evidenced by the parasitological examination, our samples were divided into 4 categories as follow; samples with high viability, in which 90 to 100 % of the protoscolices were viable (only in 3 samples). Medium viability, in which 55 to 85 % of the PSs were viable (5 cases), low viability, in which 25 to 50 % of the PSs were viable (10 cases). Non-viable protoscolices were detected in 20 samples (0 up to maximum 10 % viability). Based on such categorization and the statistically signifi-

Table 3. Comparison of viability with pH.

pH	Viability Percent		ANOVA	P value
	Mean	SD		
7.5 (n= 8)	78.50	19.16	170.37	<0.001*
8 (n=11)	31.55	6.00		
8.5 (n=19)	6.00	2.60		

\*Significant p value

# Bonferroni test showed significant difference between the groups

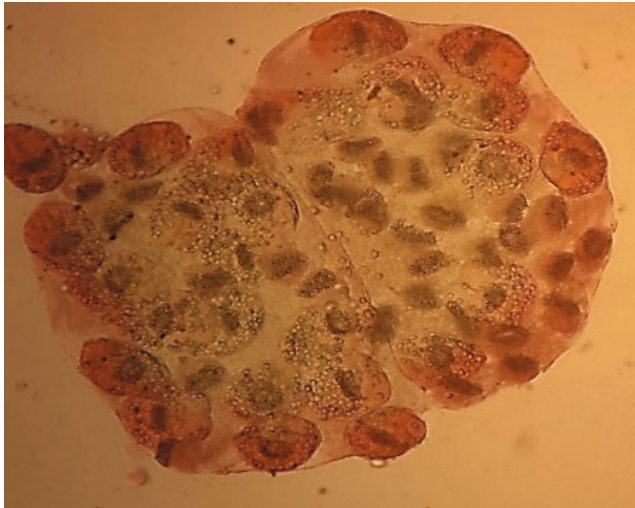


Fig. 2. Some of the protoscolices (PSs) in the centre are viable (exclude the dye) while other PSs are non-viable and take the dye.

cant data of the tested biochemical parameters, Table 4 represents the results of the verified parameters by the reagent strip test in relation to the degree of viability in different samples. Accordingly, there were different possibilities e.g. high glucose, nil protein, pH 7.5; this means very high viability up to 100 % (90 to 100 %). On the contrary, nil glucose and high protein and pH 8.5, this means that the tested HCF sample is not viable. The figures in between reflect degrees of viability that necessitate certain precaution intra and post-operative. In addition, presence or absence of bilirubin or/and leukotriene may indicate on some intra-operative complications.

## Discussion

In the current study glucose was found to be higher in HCF containing viable PSs and not present in HCF containing non-viable PSs. Glycogen has been reported to be present in the germinal

Table 4. Comparison of viability with bilirubin and leukotriene.

		Viability Percent		Mann Whitney test	P value
		Mean	SD		
Bilirubin	Negative (n=31)	31.16	32.01	-0.792	0.42
	Positive (n=7)	17.57	12.71		
Leukotriene	Negative (n=30)	33.87	31.12	- 3.05	0.002*
	Positive (n=8)	9.13	10.72		

\*Significant p value

membrane and a muco-polysaccharide is reported as a major component of both the laminated membrane and the protoscolices (Chemale *et al.*, 2003). Moreover, it is reported that the larval stage of *E. granulosus* conserves greater amounts of glycogen than the adult form of the parasite. This reflects the importance of such a biochemical component for the survival and growth of hydatid cyst (Agosin & Repetto, 1963). The mechanism of glucose uptake from the host has been studied extensively and has been reported to be by active transport, not by simple diffusion (Read, 1967). The previous authors confirmed this mechanism experimentally by changing the glucose concentration in the incubation medium and reported no changes in the glucose uptake via the cyst wall. On the other hand, the same authors reported that pH influenced the rate of glucose uptake; the major uptake peaked at pH 7.5 and the lowest at pH 8.5. This observation supports a link between the recorded pH in our samples and their viability. Wherein, the highest significant level of glucose is detected in HCF samples with the highest viability % at pH 7.5 and the lowest significant level of glucose is detected in HCF samples with the lowest viability % at pH 8.5.

Table 5. Degree of viability of HCF samples in relation to biochemical parameters measured by reagent strip test.

Item of reagent strip	Degree of viability			
	High viability	Medium viability	Low viability	Non-viable
Glucose	++++	++	+	Trace to nil
Protein	Nil or trace	+ to ++	+++	+++
pH	7.5	7.5	8	8.5
Leukotriene	Nil	Nil	±	±
Bilirubin	Nil	Nil	±	±

The protein in HCF, is reported to be composed mainly of albumin, globulin and free amino acids. Most of these proteins are derived from the host proteins (Chen & Yang, 1990; Chemale *et al.*, 2003; Aziz *et al.*, 2011). They cross the cyst wall by both free diffusion and active transport mediated by specific receptors in the germinal layer (Jeffs & Arme, 1988; Huang & Xu, 1994). Some studies hypothesized a functional role for albumen to provide energy to the larvae. Albumin is also suggested to exert functions similar to those in human plasma. These functions include its ability to maintain oncotic pressure, and to act as a transport protein (Chemale *et al.*, 2003; Li *et al.*, 2013). However, the micro-total protein level is equivalent to a very low level of approximately 1 – 2 % in the human serum (Li *et al.*, 1985), hence difficult to be visually identified by a reagent strip test as reported in our study in HCF with viable PSs. In contrast, high protein levels were identified in the HCF with dead PSs. In an attempt to understand this observation, it was mandatory to search for the protein composite of the hydatid cyst structures. Earlier studies recorded that about 9.2 % of the fresh weight of larval protoscolices is a protein which is composed of 94



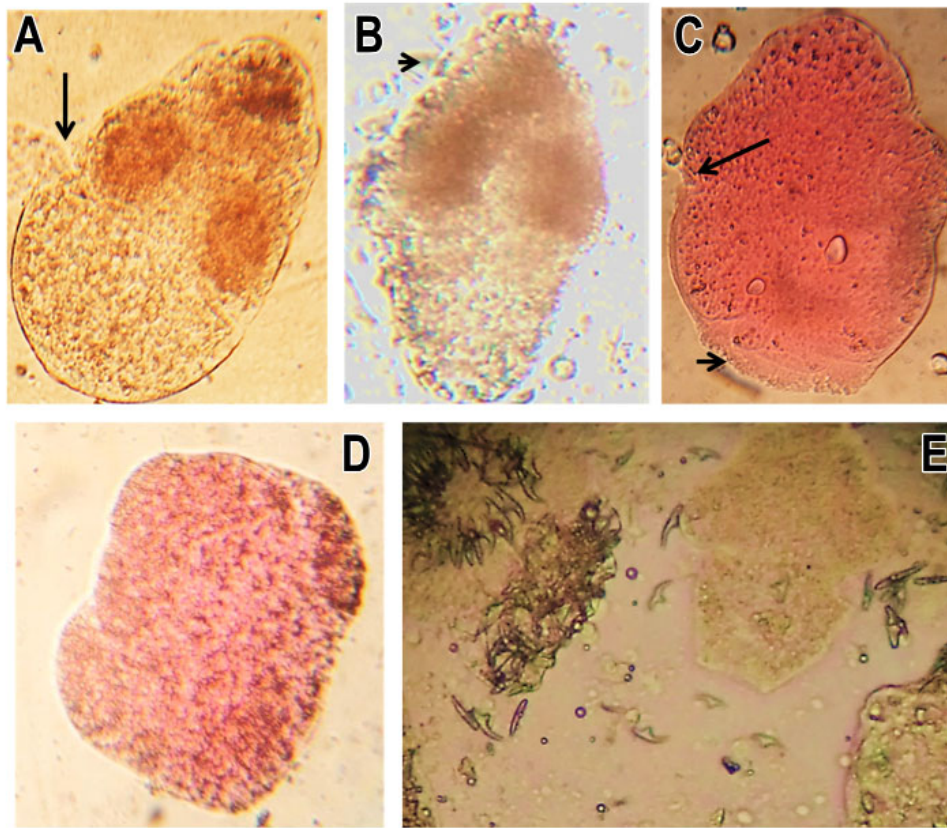


Fig. 3. Swollen disfigured degenerated PSs with absent corpuscles and disturbed hooks & suckers. Arrows in A&C point to disrupted tegument, short arrows in B&C point to the fragmented tegument. Unstained PSs (A&B) & stained one with eosin (all relaxed, evaginated PSs). E: protoscole lost their hooks.

different elements, but albumin and globulins are the major components (Agosin & Repetto, 1965). Laminated and germinal layers are reported to contain proteins as well (Cmelik & Briski, 1953; Krvavica, 1959). Therefore, degenerative processes in non-viable PSs in our samples are perhaps behind the rise in protein level due to its loss into the HCF.

Regarding the alkaline pH recorded in all our HCF samples, when tested by reagent strip test, calcareous corpuscles were suggested to have a chief role behind such alkalinity. These corpuscles are unique structures representing the major component of the protoscoler (Rogan & Richards, 1987). The mineral element composition of corpuscle bodies is calcium, magnesium, phosphorus, sulphur, zinc and molybdenum. The predominant anion is carbonate as seen via X-ray diffraction patterns (Frayha & Haddad, 1980; Smith & Richards, 1991). More recent studies concluded that this high level of  $\text{Ca}^{2+}$  ions controls the pH and prevents acidification of the HCF (Li *et al.*, 2013). Another study reported that the phosphate, within these corpuscles is present in an amorphous and hydrated form, allowing its solubility and mobility for the vital metabolic processes of the larva (Smith & Richards, 1991). Accordingly, these corpuscles are suggested to be major reserve centres for organic compounds and inorganic elements. This enables the hydatid parasite to establish the required intra and ex-

tra cellular concentrations of these chemicals in the cyst fluid and thus maintain alkaline pHs (Li *et al.*, 2013). In our samples, higher significant alkalinity (8.5) was recorded in HCF samples with the lowest viability degree. In samples with degenerated PSs, which probably increase the extracellular concentration of  $\text{Ca}^{2+}$  together with the other minerals resulted in observed biochemical change. While these minerals and particularly  $\text{Ca}^{2+}$  is found in higher concentrations in viable PSs. Thus maintain normal function of the larval *cestoda*. In addition to the aforementioned, glucose uptake stabilizes the pH at 7.5 in HCF with viable PSs.

Concerning haemoglobin, that is found in trace amounts in all our samples, Li *et al.* (2013) reported that  $\beta$ -haemoglobin which is a subtype of haemoglobin is present in the HCF and possibly plays an important role in transporting oxygen and carbon dioxide thus provides necessary energy to the larval stage. This may explain its presence in trace amounts in all samples. On the other hand, in the present work, reagent strips yield positive results of >10 granulocytes in 8 HCF samples denoting infection which certainly affects viability of the larval stage and unfortunately can be a main cause of mortality in cystic hydatidosis (García *et al.*, 2010). The identified microorganisms were *Escherichia coli*, *Enterococcus spp.* and *Staphylococcus aureus*. It is probably the cyst itself that promotes the super-infection by the compression and distortion of the biliary

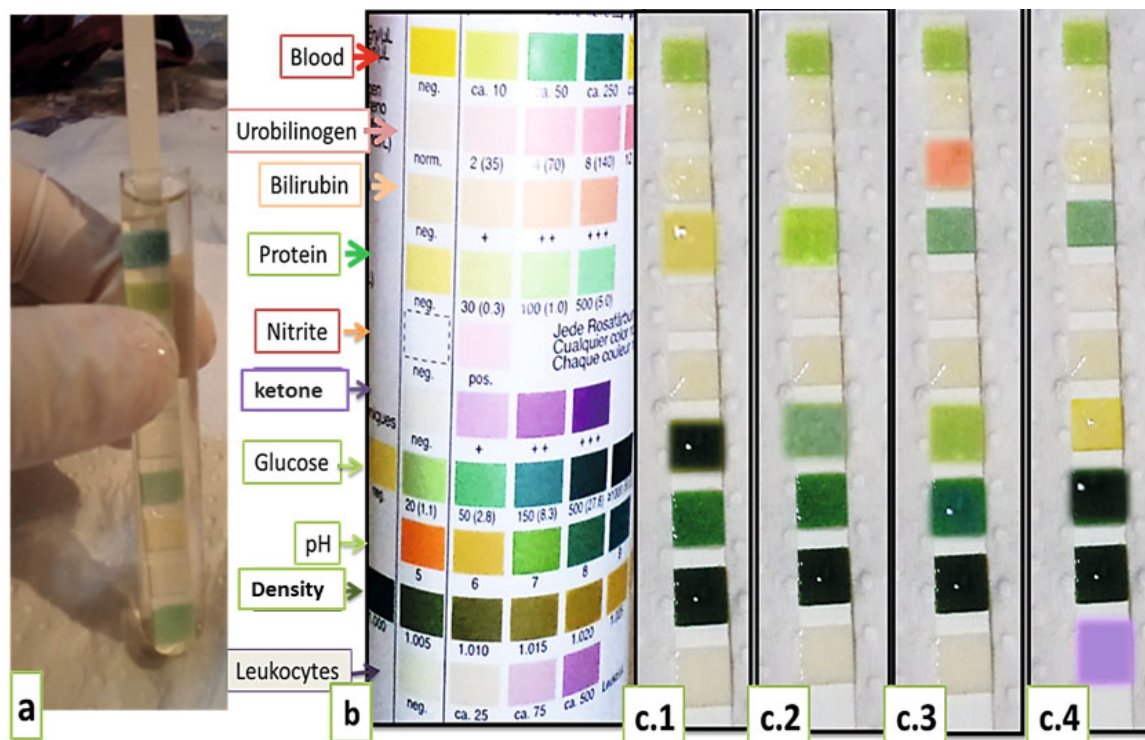


Fig. 4. Reagent strip test used in our work with representative sample (a) shows different parameters measured by the test (b). Samples with different results; c.1-(98% PSs viability) glucose +++, bilirubin nil, protein nil, pH 7.5 and leukocytes nil.c.2-(51% PSs viability) glucose ++, bilirubin nil, protein ++, pH 7.5 and leukocytes nil.c.3-(20 % PSs viability, bile contaminated) glucose +, bilirubin +++, protein +++, pH 8 and leukocytes nil.c.4- (non-viable with bacterial infection) glucose nil, bilirubin nil, protein +++, pH 8.5 and leukocytes +++.

tree (Diaz *et al.*, 1995). This correlates with the results of 2 of our samples, in which both bile and super infection were reported. Bacteraemia arising from a variety of sites is another possible origin of hydatid cyst super-infection in hydatid disease (Chen *et al.*, 2002). Concerning the sample which showed sterile pus in this study, more or less similar observation was previously recorded by Târcoveanu *et al.* (2008) which investigated aspirates from liver abscesses and pus was sterile in 22 patients of their cases. Bile contamination was reported in 7 cases, however, with no significant difference in relation to the viability of the samples. This is just indicating a biliary communication in these cases which is certainly important to be identified or/and confirmed by a simple test. Kayaalp *et al.* (2011) reported that, biliary fistulas are the most common morbidity (up to 26 %) following hydatid liver surgery. Thus, raise the importance of rapid intra-operative verification.

Our results suggest that the use of this simple reagent test which is commercially available with affordable cost and can be applied simply in seconds to clarify the viability status of the parasitic stage, in addition to detection of certain complications as super infection and biliary communication. Hence the simple reagent strip test can assist in providing fast, uncomplicated primary data regarding the viability status of the hydatid cysts. Thus, may help the surgeons in the surgical theatre to take the appropriate decisions, as regard the concentration of scolical agent, dose and duration of

post-operative medical treatment, in addition to the decisiveness steps in the follow up period. Certainly further cohort studies are indeed recommended to evaluate the degree of success, using such approach to minimize morbidity and to avoid recurrence of such serious cystic lesions.

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