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PROTOSCOLICES ANTIGENS FROM *HYDATID CYST* OF HUMAN AND ANIMAL ORIGIN FOR DIAGNOSIS OF CYSTIC ECHINOCOCCOSIS

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Abstract

Hydatid cyst (HC) protoscolices somatic antigens (HCPsS-Ag) derived from the HC extracted from patients, sheep; equine and HC antigen purchased with ready-made diagnostic kits were evaluated in diagnosis of zoonotic hydatidosis. The sensitivity and specificity of these antigens in capturing anti-HC-IgG-antibodies (anti-HC-Abs) were measured by ELISA in infected hosts' sera. Sensitivity of HCPsS-Ag extracted from HC of human origin to diagnose anti-HC-Abs in naturally patients was 90.0%. The sensitivity of HCPsS-Ag extracted from sheep, equine and ready-made diagnostic kits (R.M.K.) were (71.4%; 57.1% & 91.9%) respectively. The median ELISA OD values of positive HC patient samples versus HCPsS-Ag of sheep and equine was significant ($P \leq 0.05$) lower than screened same sera versus HCPsS-Ag of human origin. For agreement analysis, Kappa values did show significant difference between human sera versus HC antigen extracted from human or sheep ($K= 0.385$, $P=0.168$). Receiver Operating Characteristics (ROC) analysis showed accepted human diagnosis by using human or sheep originated antigens. HCPsS-Ag of human origin showed low cross-reaction with *Schistosoma mansoni* and Hepatitis C virus, than those of animal origin.

Keywords: Hydatidosis, Human, Sheep, Equine, Protoscolices, ELISA.

Introduction

The cystic echinococcosis is a silent cyclo-zoonotic disease with risky problems in man and animals worldwide. It is caused by the tapeworm larvae of *Echinococcus granulosus* with dog the main definitive host (Siracusano *et al,* 2012) and asynanthropic transmission cycle between dog and vegetarian animals as sheep and other edible animals as well as man (Swarna and Parija, 2008). In Egypt, hydatidosis endemicity was reported stray dogs (El Shazly *et al,* 2007) in edible animals (Haridy *et al,* 2000; El-Dakhly *et al,* 2019) and man (Ibrahim and Morsy, 2020;El-Sayed *et al,* 2020). The infection is mostly asymptomatic; the symptomatic one shared clinical symptoms with other hepatic diseases and cross serological tests (Fotoohi *et al*, 2013). Thus, imaging methods (radiological and ultrasound) combined with the immunological methods were used to confirm and support clinical signs and symptoms of HC patients (Sunita *et al*, 2011). Also, molecular characterization was used to diagnose hydatdosis in different hosts (Mousa *et al*, 2020). ELISA was one of the commonest used tests as being cheap and easily simple, but sensitivity and accuracy depended on characters and stability of used antigen (Golassa *et al,* 2011; Zhang *et al*, 2012).

Hydatidosis cyst (HC) protoscolicesantigens proved sensitive and specific in diagnosing infection in sheep (75% & 80% respectively) and 62.5% & 66.7% respectively in capturing human anti-HC Abs in sera using ELISA (Rafiei and Craig, 2013). Protoscolices antigen was commonly used instead of hydatid fluid as an antigen in diagnosis hydatidosis (Zhang *et al*, 2003)

The present study aimed to evaluate the freshly fertile HC surgical extracted from a pulmonary infected patient in diagnosing infections in human and animals.

Materials and Methods

The HCPsS-Ag derived from HC extracted from infected patients, sheep and equine

and R.M.K. HC antigen purchased were used in ELISA hydatidosis diagnosis.

Sera from known infected patients, animals and reference positive sera from commercial human ELISA R.M.K, and sera of suspected patients were used. Sera from man and animals infected by other parasites were included as control. Hydatid cysts protoscolices somatic (crude) antigen (HCPsS-Ag) was prepared from fertile HC freshly obtainedthe surgical removal pulmonary human HC. Also, HCs were collected from the lungs of infected slaughtered equine in Giza Zoo Abattoir and infected slaughtered sheep in Cairo Governmental Abattoir. The obtained materials were safely transported to the Research Lab, Department of Parasitology. The protoscolices (Ps) were extracted after aseptic cyst puncture cleaned by washing and sedimentation in pH 7.2 phosphate buffered saline (PBS) and used to prepare the crude antigens (Fotoohi *et al*, 2013). Prepared Ag was dialyzed against 5mM Tris-HCl (pH 7.4) for 48hr at 4°C. The protein contents were estimated by using Bradford method (Bradford, 1976). Antigen was aliquoted into 1ml plastic vials and stored until used at -20°C**.**

Sensitivity and specificity using ELISA: ELISA determined sensitivity and specificity of HCPsS-Ag from human, sheep and equine in diagnosis of anti-HC IgG Abs in sera of corresponding natural infected hosts (Liu *et al*, 2015)with little modifications. Dilutions of the reagents, reference positive and negative samples were optimized for each antigen after checkerboard titration. Optical density (OD) values were measured at 450nm with a full automated Titerteck multiskan ELISA reader. Samples were run in duplicate, associated with 2 positive and 2 negative reference sera in each plate. The commercial Ag of R.M.K. (Acc-Diag-*Echinococcus* IgG ELISA Kit, 8202-35, Diagnostic Automation/Cortez Diagnostics Inc. CA91367, USA) was used in comparison according to the manufacturer's instructions.

Human sera: Sera were collected from outpatients attended 6th October University Hospitals: a- 20 samples from hydatidosis surgically proved patients, b- 20 samples from suspected patients with positive Casoni test and a history of dog contact, which have leukocytosis, eosinophilia and increased enzymes levels but negative sonography, c- 20 sera from active *S. mansoni* patients, d- 10 sera from sero-diagnosed HCV patients, and e- 20 sera from healthy individuals as negative control.

Animal sera: During visits to the slaughterhouse of Giza Zoo and Cairo Governmental Abattoir, identified blood and fecal samples were collected from slaughtered equine and sheep. At postmortem inspection of carcasses, animals with liver, and lung HC as well as other parasites as cysticercosis & fascioliasis were collected in clean labeled containers. These were sera from 20 purely HC sheep, 10 purely *Cysticercus ovis* sheep, 10 purely *Fasciola gigantica* sheep and 10 healthy ones, as well as, 20 purely HC equine, 5 purely sarcocystosis equine, and 10 healthy ones.

Stool samples of all cases were examined using Fluke finder technique (Welch *et al*, 1987) to diagnose large sized eggs. The concentration flotation technique (Soulsby, 1982) was to diagnose other parasites.

Statistical analysis: Cutoff point for positive result was taken as 2x mean of OD of negative control sera (Romero *et al,* 2010). Sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) was evaluated by screening test using different crude antigens against different HC antigens using Chi-square ($\chi 2$) and Kappa agreement tests. The Kappa degree of agreement was measured after Landis and Koch scale (Landis and Koch, 1977), with scores divided into: <0 (no agreement), 0.0-0.20 (slight agreement), 0.21-0.40 (fair agreement), 0.41-0.60 (moderate agreement), 0.61-0.80 (substantial agreement), 0.81-0.99 (almost perfect) and 1 (perfect agreement). Receiver operating characteristics (ROC) analyzed plots of all sensitivity

versus (1-specificity). Area under the curve (AUC) was a parameter indicated the diagnostic test intrinsic accuracy in anti-HC-Abs in infected human and animal sera versus different HCPsS-Ag of different origin. The larger the AUC the more accuracy was diagnostic test. ROC analysis for each screening the true positive rate (TPR) against false positive rate (FPR) was measured compared to gold standard. The gold standard was the screening tests that use the HCPsS-Ag of the same species tested sera. ELISA OD non-parametric data were expressed as median ± IQR (interquartile range). Statistical comparison between the median of the different screening tests was made by independent sample Kruskal-Wallis test and significance values have been adjusted by the *Bonferroni* correction for multiple tests. $P \leq 0.05$ values were considered significant. Allanalyses were done using SPSS version 26 software (SPSS Inc, Chicago, IL, USA).

Ethical approval: This study was assessed and approved by Faculty of Medicine Cairo University Ethics Committee after taking consents from local health authorities and all participants. Animals handling, samples collection and patients' sera were assessed and approved by the institutional review board of the Institutional Animal Care and Use Ethical Committee (CU-IACUC), Cairo University. This study was done from October 2019 to February 2020, Department of Parasitology, Cairo University Faculty of Veterinary Medicine.

Results

Value of HCPsS-Ag from the different origins of anti-HC-IgG Abs in human, and animals natural infected sera using ELISA: Evaluating the HCPsS-Ag sensitivity of the different origins in capturing of anti-HC-IgG Abs in sera showed high sensitivity for each Ag in detection of Abs in naturally infected sera of the correspondinghost. HCPsS-Ag from sheep and equine gave 90% sensitivity in diagnosing infection in naturally infected sheep and equine sera. At the same time, the sensitivity of HCPsS-Ag of human origin diagnosed 87.8% of patients sera,which decreased to 75% versus antigens coated on the plate of R.M.K. Sensitivity of HCPsS-Ag of human origin in diagnosing infection in sheep and equine HC natural infected sera were (50%&45%) respectively. While HCPsS-Ag of sheep and equine origin were detected 62.5% & 50% of anti-HC-Abs in infected patients' sera. Sensitivity of HCPsS-Ag of human origin in diagnosing of infection in suspected patients as compared to HCPsS-Ag of animal origin in suspected patients showed70% & 60% sensitivity by HCPsS-Ag of human and that of R.M.K. comparedto 45% & 40% after screening same patients versus HCPsS-Ag of sheep and equine respectively (Tab. 1).

Statistical analysis of the previous data described in table 2 &3 revealed that marked sensitivity and high absorbance (ELISA OD) value was recorded during testing of each Ag versus infected serum samples obtained from the same host origin. The sensitivity of HCPsS-Ag of human origin versus infected human sera was used as the gold standard for comparing the sensitivity of other antigens. The sensitivity of HCPsS-Ag of sheep and equine origin for diagnosis of anti-HC-Abs in sera of infected patients was 71.4% & 57.1% respectively and 91.9% by using R.M.K. antigens. Also, the 3 antigens gave 100% specificity in diagnosing HC human sera. The HCPsS-Ag of human and equine origin showed 55.6% & 77.8% sensitivity in diagnosing anti-HC-Abs in infected sheep sera and R.M.K. antigen gave 88.9% sensitivity. Anti-HC-Abs in infected equine sera by using HCPsS-Ag of human and she-ep origin, both showed low sensitivity (50% & 72.2%). For analysis agreement, *kappa* values (K= 0.385, p =0.168) did not show significant difference between testing human sera versus HC antigens from human, or sheep (Tab. 2). Sensitivity of HCPsS-Ag extracted from HC of human, sheep, equine and R.M. K. in diagnosing 20 HC suspected patients with positive Casoni test, showed that these antigens detected anti-HC Abs in sera

of 14, 9, 8 & 12 respectively (Tab. 1). Antigens showed significantly moderate *kappa* agreement (K= 0.44-0.519, P =0.008-0.01) with similarity between extracted from sheep (K=0.519 P=0.01) and Ag in R.M.K. (K=0.565, P=0.01).

ELISA OD by different HCPsS-Ag: Cut-off values (2x mean) were 0.0564, 0.4412, 0.4832 & 0.1032 for positive sera by using HCPsS-Ag of human, sheep, equine and R.M.K. origin respectively. Median values of ELISA OD gave significant differences ($p\leq0.05$) between antigens of animal origin instead of that extracted from human one but, without significant difference in values between that using HCPsS-Ag of human HC or using R.M. K. Also no significant difference was between median ELISA OD using HCPsS-Ag of sheep or equine origin in diagnosis of infection (Tab. 3).

ROC analysis of AUC obtained after test ing human HC infected sera versus HCPsS-Ag of human origin as gold standard test showed that sheep and equine HCPsS-Ag gave worthless data in capturing of anti-HC-Abs in patients' sera (0.755 & 0.722 respectively). Ag from sheep or equine gave a bad AUC in suspected patients and Ag of R.M. K. gave (AUC = 0.816) good one (Tab. 4). The HCPsS-Ag from HC of sheep and equine gave cross reactions versus Abs of *F. gigantica, C. ovis, Sarcocystis, S. mansoni* & HCV cases, decreased total specificity to 86.31% for Ag from HC sheep and equine. Ags extracted from human HC and R.M. K. didn't cross reacted with Abs of other human parasites. Also, none of the Ags cross reacted with any Ab controls (Tab. 5).

Table 1: Efficacy of HCPsS-Ag of different origin to detect anti-HC-IgG Abs in man and animals sera.

Tested serum samples		Reaction of HCPsS-Ag originated from			
		human	sheep	Equine	R.M.K.
HC infected human (n=20)	Positive	18(90.0%)	14(70%	10(50%)	17(85%)
	Negative	2(10.0%)	6(30%)	10(50%)	3 (15.%)
HC infected sheep(n= 20)	Positive	10 (50%)	18 (90%)	14 (70%)	16 (80%)
	Negative	10 (50%)	2 (10%)	6 (30%)	4 (20%)
HC infected equine(n= 20)	Positive	9 (45%)	15 (75%)	18 (90%)	8 (40%)
	Negative	11 (55%)	5 (25%)	2 (10%)	12 (60%)
Suspected HC human patients (n= 20)	Positive	14 (70%)	9 (45%)	8 (40%)	12 (60%)
	Negative	6 (30%)	11 (45%)	12 (60%)	8 (40%)

Table 2: Analysis for reaction of HCPsS-Ag of different origin to detect anti-HC- Abs in man and animals sera.

Samples	Gold standard (HCPsS-Ag)	Reaction versus HCPsS-Ag	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	Pearson Chi square (χ^2)	*Kappa* value	*P* value
human HC (n=20)	Human	sheep	71.4	100	75	100	33.3	1.905	0.385	0.168
		equine	57.1	100	62.5	100	25	1.143	0.250	0.285
		R.M. K.	91.9	100	92.5	100	66.6	1.845	0.735	0.003
sheep HC (n= 20)	sheep	human	55.6	100	60	100	20	2.222	0.200	0.136
		equine	77.8	100	80	100	33.3	5.185	0.412	0.023
		R.M. K.	88.9	100	90	100	50.0	1.818	0.615	0.003
equine HC (n= 20)	Equine	human	50	100	55	100	18.2	2.222	0.167	0.178
		sheep	72.2	0	65	86.7	0.0	0.741	(-0.167)	0.389
		R.M. K.	44.4	100	50	100	16.7	1.481	0.138	0.224
Suspected HC patients (n= 20)	Human	sheep	64.3	100	75	100	54.5	7.013	0.519	0.008
		equine	57.1	100	70	100.0	50.0	5.714	0.444	0.017
		R.M. K.	78.6	83.3	80	91.7	62.5	6.706	0.565	0.01

Table 3: Median ELISA OD using HCPsS-Ag of different origin to detect anti-HC-IgG Abs in man and animals sera.

Samples	HCPsS-Ag (human)	HCPsS-Ag (sheep)	HCPsS-Ag (equine)	R.M.K. Ag
HC infected human (n=20)	$0.6295^{Aa} \pm 0.11$	$0.4695^{Bb} \pm 0.50$	$0.3585^{Bb} \pm 0.28$	$0.6000^{Aab} \pm 0.025$
HC infected sheep (n= 20)	$0.2620^{Ab} \pm 0.57$	$0.7030^{Ba} \pm 0.05$	$0.6205^{BCa} \pm 0.35$	$0.6950^{BCb} \pm 0.032$
HC infected equine (n= 20)	$0.305^{Ab} \pm 0.49$	$0.6930^{Ba} \pm 0.34$	$0.7995^{Ba} \pm 0.02$	$0.310^{Aa} \pm 0.505$
Suspected HC humans (n= 20)	$0.4495^{Aab} \pm 0.63$	$0.3330^{Bb} \pm 0.30$	$0.3180^{Bb} \pm 0.27$	$0.4795^{Aa} \pm 0.579$

Data = median ± IQR (interquartile range), Column with different small letters =significant, while row with different capital letters = $p\leq0.05$ (independent sample Kruskal-Wallis test, Significance values adjusted by Bonferroni correction for multiple tests.

Table 4: AUC of ROC of HCPsS-Ag of different origin to detect anti-HC-Abs in man and animal sera

Samples	Gold standard	Screening tests versus	AUC	*Classification*
HC infected human (n=20)	HCPsS-Ag (Human origin)	HCPsS-Ag (Sheep)	0.755	Worthless
		HCPsS-Ag (Equine)	0.722	Worthless
		R.M.K. Ag.	0.830	Good
HC infected sheep (n= 20)	HCPsS-Ag (Sheep origin)	HCPsS-Ag (Human)	0.600	Not good
		HCPsS-Ag (Equine)	0.889	Good
		R.M.K. Ag.	0.914	Excellent
HC infected Equine (n= 20)	HCPsS-Ag (Equine origin)	HCPsS-Ag (Human)	0.361	Not good
		HCPsS-Ag (Sheep)	0.750	Worthless
		R.M.K. Ag.	0.722	Worthless
HC suspected patients (n= 20)	HCPsS-Ag (Human origin)	HCPsS-Ag (Sheep)	0.765	Worthless
		HCPsS-Ag (Equine)	0.756	Worthless
		R.M.K. Ag.	0.816	Good

Null hypothesis: true area = 0.5, AUC: Area under the curve, AUC represents accuracy of screening test.

Table 5: Cross reaction between HCPsS-Ag of different origin and specific antibodies of other parasites.

Serum samples non-infected by HC but infected by other parasites as	No. of +Ve and % of specificity versus HCPsS-Ag originated from			
	human	Sheep	equine	R.M.K.
*S.mansoni*infected patients (n=20)	(3) 85%	(3) 85%	(3) 85%	(2) 90%
Hepatitis C infected (n=10)	(3) 70%	3) (70%)	(3) 70 %	2) 80%
C. ovis infected sheep (n= 10)	(0) 100%	(3) 70%	(2) 80%	(0) 100%
F. gigantica infected sheep (n=10)	(0) 100%	(3) 70%	(3) 70%	(0) 100%
Sarcocystis infected equine (n=5)	(0) 100%	(1) 80%	(2) 60%	(0) 100%
Negative sheep(n=10)	(0) 100%	(0) 100%	(0) 100%	(0) 100%
Negative dquine (n=10)	(0) 100%	(0) 100%	(0) 100%	(0) 100%
Healthy individuals (n=20)	(0) 100%	(0) 100%	(0) 100%	(0) 100%
Mean specificity (n = 95)	(89)93.68%	(82)86.31%	(82)86.31%	(91)95.79%

Discussion

The ELISA proved to be the reliable test in diagnosing hydatidosis (Zhang *et al,* 2012). Bovine or camel HC was used as antigens to measure *E. granulosus*-specific ELISA-IgG antibodies & IgG subclasses in suspected cases (Irabuena *et al*, 2000). But, sheep hydatidosis was routinely used as antigen for different serological techniques or even for manufacturing the R.M.K. used in diagnosing of human infection (Rafiei and Craig, 2013).

Undoubtedly, absence of absolute compatibility between antigens from man and animals led to problems in sensitivity and specificity in capturing of low anti-HC-Abs level in man sera especially in immuno-compromised ones (Brunetti *et al*, 2010; Fotoohi *et al*, 2013). Zhang *et al*. (2003) found that fluid Ag extracted from HC human patient was not valuable antigenic source as it contained proteins such as IgG that cross-reacted with specific immunoglobulins sera in patients. I(n the present study, showed high sensitivity and high OD value for this crude extract in capturing specific anti-HC Abs in infected sera of the corresponding hydatidosis host species. This agreed with Pawlowaski *et al*. (2001) and Sabry and Reda (2008).

The great difference was recorded in sensitivity of HCPsS-Ag of animal origin when used to substitute human origin in HC diagnosis. The HCPsS-Ag extracted from hydatidosis of human origin gave a sensitivity of 87.8% for human diagnosis. This sensitivity decreased to 50.0% & 45% when used to diagnose infection in sheep and equine respectively. Also, the HCPsS-Ag of sheep origin gave a sensitivity of 62.5% when used to diagnose HC infection in patients. This low level of sensitivity showed in-compatibility of antigens extracted from sheep HC to replace that from patients. Rafiei and Graig (2013) reported that protoscolices antigen of sheep gave high sensitivity of 90% when used in ELISA diagnosis of human hydatidosis. The increased sensitivity of HC sheep extracted antigens than that from equine hydatidosis (50%), which agreed with Bauomi *et al*. (2015) and Albadawi *et al.* (2016). The present study found a sensitivity (71.4%) and specificity (100%) with sheep HC-Ag

versus the human one. This sensitivity level didn't occur with hydatidosis equine antigens (57.1%). The sensitivity of HCPsS-Ag of human origin in suspected patients as compared to HCPsS-Ag of animal origin gave sensitivity of 70% & 60% by using human HCPsS-Ag or R.M.K. respectively, and 45% & 40% versus HCPsS-Ag of sheep and equine ones.

The decreased R.M. K sensitivity might be related to some factors, as the supplied Ag was coated on the used plate, without identification of concentration/well, or used kit neither gave the antigen type nor origin.

In the present study, sensitivity of HCPsS-Ag of human origin in diagnosis human sera as the gold standard compared with sensitivity of other antigens, sensitivity of R.M.K. Ag was (91.9%) and (88.9%) versus infected sheep sera (44.4%) versus infected equine serum samples. All tested antigens proved high specificity versus each host ant-HC-Abs in all infected sera of human and animals. This agreed with Fasihi *et al*. (2019).

The high sensitivity of R.M.K. Ag in diagnosing HC patient might be due to the high purification of this antigen compared with other antigens used. The present study recorded low median ELISA OD among positive cases using animal originated HC antigens with significant difference ($P \leq 0.05$) between them and that using human antigen origin. No doubt, HCPsS-Ag sheep or equine origin gave low diagnostic sensitivity in immuno-compromised patients. Also, variation in mean OD values within the infected groups may be related to different sensitivity of each Ag as well as the specific level Abs titer in their sera. Few studies used soluble protoscolices antigen in diagnosing human echinococcosis (Carmena *et al,* 2004), but many proteins of *E. granulosus* as hydatid fluid & protoscolices were immunogenic and used to diagnose specific Abs in dogs and intermediate host as sheep (Carmena *et al,* 2005). Fotoohi *et al*. (2013) reported the suitability of HC Ag of sheep origin to diagnose infection in patients.

The present ROC analyzed sensitivity and specificity simultaneously at different cut-points, which estimated the accuracy using multiple pairs of sensitivity and specificity. ROC analysis plots for all sensitivity versus (1-specificity) showed large AUC indicated intrinsic accuracy of the anti-HC-Abs diagnostic test in infected patients and sick animal sera versus different HCPsS-Ag origin (Landis and Koch, 1977). HCPsS-Ag extracted from sheep or equine HC cross reacted with Abs of other infection as *C. ovis, F. gigantica, Sarcocystis, S. mansoni* and HCV infected cases with different degrees, but all the used Ag did not cross react with any Ab present in controls. Sadjjadi *et al* (2009) reported that serological tests using crude antigens for hydatidosis diagnosis was sensitive, but without accepted specificity. So, difference in sensitivity of HCPsS-Ag from different hosts in capturing of specific anti-HC-Abs was related to the shared amount of specific protein fractions between HCPsS-Ag extracted from different hosts.

The present study proved the high ELISA specificity for using crude extract (HCPsS-Ag). This agreed with El-On *et al.* (1997) who reported that both the purified and crude antigens were comparable sensitivity and specificity for sero- epidemiological studies. But, the present result disagreed with Brun etti *et al*. (2010) who found that hydatidosis serodiagnosis by crude antigens were sensitive, but with low specificity.

Conclusion

Undoubtedly, the echinococcosis/hydatidosis is a worldwide risky problem particularly in the sheep rearing countries.

The suitability of HCPsS-Ag of animal origin to replace human one was affected by animal species. The hydatidosis of sheep origin was the best extract for human than that of equine in ELISA diagnosing zoonotic hydatidosis.

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