ORIGINAL ARTICLE

Proton Nuclear Magnetic Resonance (^{1}H NMR) Metabolomics Study in Serum, Urine, and Cystic Fluid for Differentiating Fertility and Staging of Intra-abdominal Hydatid Cyst in Adults

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Abstract

Background: Cystic echinococcosis (CE) is a parasitic zoonosis caused by the tapeworm *Echinococcus granulosus.* Over the past few years, a lot of research has been done on liver illnesses using metabolomics techniques to identify biomarkers which could identify the diseases in its early stages. The present study was done to explore biomarkers in serum, urine, and cystic fluid which would help in differentiating, staging, and assessing fertility of intra-abdominal hydatid cyst by using proton nuclear magnetic resonance (^{1}H NMR) metabolomics.

Materials and methods: In the study, 28 subjects (16 cases and 12 controls) were enrolled. Staging of hydatid cysts was performed using ultrasonography. In patients complying with case and control definition, blood, urine, and cystic fluid were collected for complete blood count, urine culture, Echinococcus IgG enzyme-linked immunosorbent assay (ELISA), and metabolomic analysis. The 17, 15, and 11 metabolites in serum, urine, and cystic fluid samples were quantified, respectively, to differentiate between case and control group.

Results: In this study, we observed that there was a significant downregulation of succinate metabolite in urine samples of cases, down-regulation of five metabolites (isoleucine, valine, histidine, tyrosine and formate) and upregulation of alanine in cystic fluid of cases.

Conclusion: Current study demonstrates that metabolomics can be used non-invasively for rapid diagnosis of CE. This is one of the very few studies, which used ^{1}H NMR spectroscopy, to analyze the profile of metabolites in serum, urine, and cystic fluid in cases of CE and controls.

Keywords: ^{1}H NMR, Cystic echinococcosis, Hepatic cyst, Hydatid disease, Metabolomics.

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Highlights

- This study explores biomarkers in serum, urine, and cystic fluid using metabolomics, which would help in differentiating intra-abdominal hydatid cysts from other cysts.
- This study determines which metabolites are the most promising diagnostic indicators for hydatid cyst viability or fertility.

Introduction

Cystic echinococcosis (CE) also known as hydatidosis is a parasitic zoonotic illness caused by the tapeworm *Echinococcus granulosus*.[1] The CE typically affects the liver (50–70% cases), lung (20–30% cases), and rarely other organs such as the central nervous system, kidney, and spleen.[2] Majority of hydatic cysts are detected in the liver and are asymptomatic, discovered by chance during regular abdominal ultrasonography (USG) to diagnose other medical conditions.[3] The CE is one of the 17 neglected tropical diseases that the World Health Organization (WHO) has identified for management or eradication by the year 2050.[4]

Sheep, goats, and cattle are domestic intermediate hosts that serve as important disease reservoirs and primary hosts of this zoonotic parasite are domestic dogs (the definitive host).[1] Cystic echinococcosis occurs in humans accidentally consuming *E. granulosus* eggs excreted in the feces of infected dogs, these eggs hatch in the gastrointestinal tract of humans to release oncospheres that travel to the liver via the portal and lymphatic circulation, where they typically settle and mature into larvae.[5]

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Five types of CE (CE1 to CE5) cysts/hydatid cyst stages are described based on abdominal USG findings, as per the WHO categorization system namely: active fertile stage (CE1 and CE2), transitional (CE3) and degenerated, inactive/sterile cysts (CE4 and CE5).[6] Surgery, percutaneous management, a "watch and wait" strategy, and medication therapy are used for treating CE

depending on the stage of disease.[7] The active cysts (CE1, CE2, and CE3) are treated by surgery and percutaneous management while, inactive cysts are monitored regularly by a "watch and wait" strategy.[8,9]

Most of the time, imaging techniques yield enough information to diagnose CE, however, even with these advanced techniques of radiological imaging, diagnosis of early stage CE continues to be challenging.[10] When imaging results are not characteristic, serological testing may be used, however, these tests are unable to differentiate between active and inactive CE stages.[11]

Over the past few years, a lot of research has been done on liver illnesses using metabolomics techniques to identify biomarkers which could identify the diseases in its early stages.[12] However, the metabolomics approach based on proton nuclear magnetic resonance (^{1}H NMR) and its use for early diagnosis of echinococcosis has not been extensively studied.

With this background, the present study was done with an aim to explore biomarkers in serum, urine, and cystic fluid which would help in differentiating, staging, and assessing fertility of intra-abdominal hydatid cyst by using ^{1}H NMR metabolomics in order to (1) Determine detailed metabolite profiles of intra-abdominal hydatid cyst fluid samples (if patient is operated), serum and urine from cases as compared to controls. (2) To correlate these profiles with hydatid cyst activity/viability as diagnosed by the established imaging, light microscopy, and histopathological techniques. (3) To determine which metabolites are the most promising diagnostic indicators for hydatid cyst viability or fertility.

Materials and Methods

The present observational, case–control study was conducted at Dr Ram Manohar Lohia Institute of Medical Sciences and Centre for BioMedical Research, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow over 3 years (December 2014 to December 2017).

In the study, 28 subjects were enrolled out of which there were 16 cases with evidence of intra-abdominal hydatid cyst (Hepatic cyst in 15 patients and splenic cyst in 1 patient) and 12 were controls (without evidence of intra-abdominal hydatid cyst) diagnosed with Liver abscess, hemangioma, etc.

Staging of hydatid cysts was performed using USG and/or computed tomography (CT) and/or magnetic resonance imaging (MRI). Echinococcosis was classified according to its viability into active fertile (CE1 & CE2), transitional (CE3); and degenerated, inactive and infertile/sterile cysts (CE4 & CE5).

In patients complying with case and control definition, blood was collected in a plain/EDTA (ethylenediaminetetraacetic acid) vial, urine in a sterile container, and 2–5 mL of cyst fluid in a sterile tube (if the patient had undergone abdominal surgery). The following tests were performed on these samples:

- Blood in EDTA vial was sent for complete blood counts. A serum sample was sent to look for elevated Liver enzymes.
- If the patient underwent abdominal surgery, 1–2 mL of cyst fluid was sent for light microscopy – hydatid cyst fluid samples categorized as fertile if the protocolizes were present and sterile if these were absent.
- Echinococcus Ig G enzyme-linked immunosorbent assay (ELISA) was performed on serum samples of patients with suspected intra-abdominal hydatid cysts (DRG Instruments GmbH, Marburg, Germany).
- Metabolomic analysis: 1 mL of the cystic fluid (if operated), serum and urine flash frozen in liquid nitrogen/–80°C after collection were sent for ^{1}H NMR experiments at Centre for Bio-Medical Research, SGPGIMS, Lucknow.
- Urine was cultured for bacterial pathogens. Culture-positive urine samples from cases/controls were excluded from metabolomics analysis.

Nuclear Magnetic Resonance (NMR) Experiments and Resonance Assignments

The NMR experiments were performed using a Bruker (Billerica, Massachusetts, United States) Avance III 800 MHz FT-NMR spectrometer equipped with a 5 mm Triple resonance ^{1}H/^{13}C/^{15}N TCI cryoprobe with a Z-shielded gradient, operating at a proton frequency of 800.26 MHz. The ^{1}H NMR spectra were acquired with water pre saturation at 300 K. In case of glucose, the α-anomeric proton resonance was quantified with a factor of 36/100 to get the weight of total serum glucose, as α- and β-glucose are present in solution in a ratio of 36:64 under physiological conditions.

Statistical Analysis

Data comprising ^{1}H NMR spectra recorded for serum and urine samples were phased, baseline corrected, aligned, and then subjected to statistical analysis. As per the analysis, 17, 15, and 11 metabolites in serum, urine, and cystic fluid samples were identified and quantified, respectively. Using Statistical Package for Social Sciences (SPSS) Version 22.0, we performed a univariate nonparametric *t*-test to differentiate between case and control group. The values obtained along with Mean ± SD calculations depicted the elevation and depletion of the quantified metabolites. A p-value < 0.05 was taken to indicate statistical significance.

Results

In both the cases and control groups, patients were aged from 18 to 65 years, showing no significant difference. In case group, proportion of females was higher (87.5%) as compared to controls (33.3%) but this difference was not significant. Abdominal pain was the most common clinical feature whereas none of the patients in either of two groups had cholangitis. Statistically, no significant difference between two groups was observed with respect to any of the clinical features. The demographic and clinical profile of cases and controls is shown in Table 1.

On comparing the laboratory findings between the cases and control groups, we observed that leukocytosis was seen significantly more (p-value = 0.0238) in the control group. The hematological and other laboratory findings of cases and control group are shown in Table 2. Histopathological and radiological examination of cyst was done in 11 cases; 7 had fertile hydatid cysts while in 4 cases hydatid cysts were sterile. The radiological staging of the cyst is shown in Table 3. Echinococcus IgG ELISA positivity was seen in 93.75% of cases as compared to none of the controls.

On metabolomic analysis using the NMR spectrometer 17 metabolites in serum, samples of cases and controls were identified and quantified, respectively. However, none of the metabolites in serum were found to be statistically significant to differentiate between case and control groups. The mean values of serum metabolites analyzed are shown in Table 4.

Table 1: Comparison of demographic and clinical profile of cases vs controls

Serial No.	Characteristic	Cases		Controls	
		No.	%	No.	%
1.	Age (Years)				
	18–30	6	37.5	5	41.66
	30–45	5	31.25	5	41.66
	45–55	3	18.75	1	8.33
	55–65	1	6.25	0	0
	>65	1	6.25	1	8.33
2.	Gender				
	Male	2	12.5	8	66.6
	Female	14	87.5	4	33.3
3.	Abdominal pain	13	81.25	12	100
4.	Abdominal lump	6	37.5	2	16.66
5.	Cholangitis	0	0	0	0
6.	Dyspepsia	1	6.25	0	0
7.	Weight loss	1	6.25	2	16.66
8.	Vomiting	2	12.5	3	25.0
9.	Low grade fever	6	37.5	8	66.66
10.	Enlarged liver	2	12.5	3	25.0
11.	Prior antibiotic/anthelmintic use	0	0	0	0

Table 2: Hematological and laboratory findings

Serial No.	Characteristic	Cases (n = 16)		Controls (n = 12)		p-value
		No.	%	No.	%	
1.	Anemia	10	62.5	6	50	0.508
2.	TLC (Total leukocyte count)					
	Leukopenia	0	0.0	0	0.0	–
	Leukocytosis	1	6.25	5	41.6	0.0238
3.	Eosinophilia	3	18.75	2	16.66	0.8867
4.	SGPT					
	Increased	6	50	8	66.66	0.1266
5.	Fertility/viability status of hydatid cyst on basis of light microscopy, histopathological examination and radiological investigations					
	Fertile	7	43.75	NA	NA	NA
	Sterile	4	25	NA	NA	
	Total	11	68.75	NA	NA	
6.	Echinococcus IgG ELISA positivity	15**	93.75	0	0	

**In one case, Echinococcus IgG ELISA was negative, which was diagnosed as splenic hydatid cyst; NA, not applicable

On metabolomic analysis using the NMR spectrometer 15 metabolites in urine, samples of cases and controls were identified and quantified, respectively, and downregulation of succinate was found to be statistically significant (p-value = 0.03) to differentiate among case and control groups. The mean values of urine metabolites analyzed are shown in Table 5.

On metabolomic analysis using the NMR spectrometer, 11 metabolites in cystic fluid samples of cases and controls were identified and quantified, down-regulation of 5 metabolites (isoleucine, valine, histidine, tyrosine and formate) and upregulation of alanine in cystic fluid of cases were found to be statistically significant to differentiate among case and control group. The mean values of cystic fluid metabolites analyzed are shown in Table 6.

In this study to determine which metabolites were the most promising diagnostic indicators for hydatid cyst viability or fertility, we compared the levels of different metabolites in serum, urine, and cystic fluid samples of cases with fertile and sterile cysts as shown in Table 7. Only upregulation of citrate in the urine of cases was found to be statistically significant in differentiating between sterile cysts and fertile cysts.

Discussion

The results of the current study demonstrated that metabolomics can be used non-invasively for rapid diagnosis of CE. This is one of the very few studies, which used ^{1}H NMR spectroscopy, to analyze the profile of metabolites in serum, urine, and cystic fluid in cases of CE and controls.

In this study, we found that there was a significant downregulation of succinate metabolite in urine samples of cases. However, in the study by Lin et al. urine samples from the CE patients were characterized by significant upregulation of glutamate, histidine, and acetate.[13] Also, none of the metabolites in serum was found to be statistically significant to differentiate between the cases and controls, however, in the study by Lin et al., a significant elevation of glutamate, histidine, and lactate was seen in serum samples of CE cases while Bai et al. in their study reported a significant decrease of glutamate in serum samples of CE cases.[13,14]

In this study, we observed that there was a significant downregulation of five metabolites (isoleucine, valine, histidine, tyrosine, and formate) and upregulation of alanine in cystic fluid of CE cases. Similar observations were also reported by Lin et al. in their study, where they found a higher concentration of alanine in cystic fluid (the most abundant amino acid) and a lower concentration of isoleucine, histidine, and tyrosine similarly, Chen et al. reported a higher concentration of alanine in their study.[13]

In this study to determine which metabolites were the most promising diagnostic indicators for hydatid cyst viability or fertility we found that only upregulation of citrate in urine was found to be significant in differentiating fertile cyst from sterile cyst. However,

Table 3: Showing radiological characteristics of the cases

Serial No.	Radiological stage	Observation	No. of cases
1.	CE1	Smooth, round or oval, well-defined cystic lesions with thin walls. Having a maximum size of 15 cm.	2
2.	CE2	A cyst presents as unilocular cysts with tiny daughter vesicles organized peripherally. It is considered that the existence of a daughter cyst is pathognomonic for hydatid cysts.	5
3.	CE3	The cyst presents as a main maternal cyst with several larger minor cysts often termed as daughter cysts, which may acquire shapes that resembling wheels, rosettes, or honeycomb structure.	None

(Contd...)

Table 3: *(Contd...)*

Serial No.	*Radiological stage*	*Observation*	*No. of cases*
4.	CE4	A cyst presents as a cystic lesion including floating membranes or serpentine bands that symbolize the ruptured or detached membranes.	3
5.	CE5	A dead cyst with a thick calcified wall is seen. The cysts might be partially or completely calcified, depending on the degree of calcification.	1
			Total = 11

Table 4: Mean ± SD values of serum metabolites among case and control group

Serial No.	*Metabolites*	*Case (Mean ± SD)*	*Control (Mean ± SD)*	*p-values*
1.	Isoleucine	0.69 ± 0.36	0.90 ± 0.60	0.26
2.	Valine	2.38 ± 0.94	2.45 ± 1.26	0.86
3.	Alanine	4.64 ± 1.46	3.97 ± 0.89	0.17
4.	Acetate	1.17 ± 2.97	0.24 ± 0.13	0.29
5.	Acetone	0.05 ± 0.04	0.06 ± 0.05	0.63
6.	Succinate	0.26 ± 0.28	0.28 ± 0.26	0.86
7.	Glutamine	6.24 ± 1.75	6.98 ± 6.70	0.67
8.	Citrate	0.98 ± 0.30	0.99 ± 0.43	0.93
9.	Glycine	2.51 ± 1.20	2.26 ± 0.99	0.55
10.	Lactate	30.38 ± 19.91	39.41 ± 23.21	0.28
11.	Threonine	0.62 ± 0.65	0.56 ± 0.66	0.81
12.	β-glucose	42.18 ± 29.17	47.86 ± 26.68	0.60
13.	α-glucose	31.05 ± 19.41	36.79 ± 17.79	0.43
14.	Tyrosine	0.51 ± 0.24	0.62 ± 0.24	0.26
15.	Histidine	0.98 ± 0.42	1.01 ± 0.81	0.91
16.	Phenylalanine	0.77 ± 1.17	1.83 ± 2.95	0.20
17.	Formate	0.26 ± 0.34	0.13 ± 0.09	0.21

Table 5: Mean ± SD values of urine metabolites among case and control group

Serial No.	*Metabolites*	*Case (Mean ± SD)*	*Control (Mean ± SD)*	*p-values*
1.	Alanine	0.31 ± 0.31	0.39 ± 0.54	0.65
2.	Acetate	0.08 ± 0.08	0.15 ± 0.20	0.27
3.	Acetone	0.009	0.004	0.58
4.	Succinate	**0.08 ± 0.06**	**0.17 ± 0.13**	**0.03**
5.	Citrate	3.80 ± 2.86	4.27 ± 6.13	0.80
6.	Dimethylamine	0.18 ± 0.16	0.17 ± 0.18	0.88
7.	Trimethylamine	0.02 ± 0.02	0.04 ± 0.03	0.29
8.	Glycerophosphocholine	0.23 ± 0.45	0.13 ± 0.10	0.48
9.	Trimethylamine-N-oxide	0.29 ± 0.25	0.47 ± 0.72	0.41
10.	Glycine	1.26 ± 1.28	1.32 ± 1.60	0.91
11.	Urea	10.58 ± 6.12	12.51 ± 6.77	0.46
12.	Histidine	1.23 ± 1.10	1.32 ± 1.53	0.86
13.	Phenylalanine	3.63 ± 7.24	4.78 ± 4.79	0.65
14.	Hippurate	2.68 ± 2.71	3.05 ± 3.08	0.75
15.	Formate	0.08 ± 0.06	0.20 ± 0.27	0.0951

Statistically highly significant value $p < 0.05$

Ciftci et al. in their study reported an increase in 3-phosphoglycerate in the plasma of patients with inactive cyst,[15] while Hosch et al. in their study reported that active (fertile) cyst fluid was characterized by significantly higher levels of malate, succinate, acetate, and lower levels of lactate compared to inactive/sterile cyst.[16]

Table 6: Mean ± SD values of cystic fluid metabolites among case and control group

Serial No.	*Metabolites*	*Case (Mean ± SD)*	*Control (Mean ± SD)*	*p-values*
1.	Isoleucine	**1.17 ± 1.15**	**14.51 ± 10.68**	**<0.0001**
2.	Valine	**6.60 ± 6.50**	**34.67 ± 39.11**	**0.0087**
3.	Alanine	**13.54 ± 14.95**	**3.56 ± 5.94**	**0.038**
4.	Acetate	2.51 ± 2.91	5.98 ± 6.80	0.0776
5.	Succinate	0.65 ± 0.66	1.25 ± 1.44	0.1512
6.	Citrate	5.68 ± 10.07	6.55 ± 0.12	0.7682
7.	Glycine	9.09 ± 12.61	15.79 ± 12.39	0.1729
8.	Lactate	15.76 ± 8.63	28.46 ± 23.10	0.053
9.	Histidine	**1.73 ± 1.67**	**11.51 ± 10.90**	**0.0015**
10.	Tyrosine	**1.44 ± 1.42**	**7.88 ± 2.77**	***p* < 0.0001**
11.	Formate	**0.14 ± 0.02**	**0.65 ± 0.70**	***p* = 0.0069**

Statistically highly significant value $p < 0.05$

Table 7: Mean ± SD values of urine metabolites among fertile and sterile cyst

Serial No.	*Metabolites*	*Fertile (Mean ± SD)*	*Sterile (Mean ± SD)*	*p-values*
1.	Alanine	0.38 ± 0.36	0.31 ± 0.32	0.72
2.	Acetate	0.05 ± 0.04	0.86 ± 1.19	0.12
3.	Acetone	0.02 ± 0.007	1.54 ± 2.44	0.15
4.	Succinate	0.11 ± 0.06	0.11 ± 0.07	0.85
5.	Citrate	3.85 ± 1.89	1.08 ± 1.32	**0.02**
6.	Dimethylamine	0.20 ± 0.13	0.13 ± 0.12	0.41
7.	Trimethylamine	0.01	3.08 ± 4.29	0.11
8.	Glycerophosphocholine	0.37 ± 0.69	0.43 ± 0.45	0.86
9.	Trimethylamine-N-oxide	0.42 ± 0.28	1.26 ± 1.48	0.20
10.	Glycine	1.50 ± 1.23	7.75 ± 10.27	0.17
11.	Urea	13.60 ± 6.28	6.12 ± 6.19	0.08
12.	Histidine	1.91 ± 1.29	31.99 ± 55.50	0.21
13.	Phenylalanine	2.37 ± 1.71	23.16 ± 37.25	0.20
14.	Hippurate	3.41 ± 2.95	1.02 ± 1.07	0.12
15.	Formate	0.13 ± 0.06	0.31 ± 0.44	0.33

Metabolomics has been extensively used to find novel biomarkers and study metabolic pathways in several liver diseases. The use of NMR-based metabolomics approach has certain characteristic features such as its high repeatability, quick detection time, and its ability to quantify several metabolites in one measurement, making it a useful tool for early diagnosis of CE.[17] The early diagnosis of CE cases can be done with use of both metabolomics and imaging techniques. One such example is the

MRI technique based on Chemical Exchange Saturation Transfer (CEST) which allows the detection of specific metabolites within the tissues like amino acids non-invasively.[18]

This study had some limitations firstly, the sample size of the study was small (16 cases and 12 controls), and a greater number of participants is required to improve the accuracy and reliability of the current results. Secondly, metabolite levels in humans are influenced by a variety of factors including age, gender, microbiota of the gut, comorbidities, and hormonal levels and in this study, we did not control the patients' dietary habits and were unable to evaluate the hormonal levels in cases and controls.

Conclusion

In this study, we used the ^{1}H NMR-based metabolomics approach to evaluate the different metabolite levels in CE cases and controls. This study shows that evaluation of metabolite levels can be used to differentiate CE cases from other causes of hepatic cyst. The metabolites that distinguished between CE cases and control was succinate in urine and 6 metabolites namely: isoleucine, valine, histidine, tyrosine, formate and alanine in cystic fluid in serum samples, statistical analysis was inconclusive as none of the metabolites were found to be statistically significant to differentiate the groups. While comparing the fertile and infertile cases, citrate in urine was found to be significant in differentiating fertile cysts from sterile cysts.

Ethics Approval

Ethical clearance was duly obtained from the Institute Ethics Committee vide letter no. IEC/3381/RMLIMS/2014 dated November 24, 2014, before starting the study.

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