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ORIGINAL ARTICLE

GI tract Mycobiome in Chronic Hepatitis C Virus Infection, a Case Control Study

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ABSTRACT

Background: *Human microbiota including intestinal fungi (mycobiota) plays an important role in health and disease. Mycobiome is a term refers to the genetic composition of fungal microbiota. The relation of intestinal mycobiota to chronic viral hepatitis and its progression has not yet been totally explored.* ***Objectives:*** *This study was designed to compare GI fungal composition and fungal genetic load (mycobiome load) in patients with chronic hepatitis C virus (HCV) infection including liver cirrhosis and healthy subjects.* ***Methodology:*** *Stool samples were collected from patients with varying degrees of chronic HCV infection. Sabouraud dextrose agar was used for culture detection of fungi. Real-time PCR was done for estimation of mycobiome load in patients with chronic HCV infection, HCV cirrhosis and in healthy control.* ***Results:*** *This study enrolled 53 patients with chronic HCV infection including 27 patients with chronic HCV hepatitis and 26 patients with HCV liver cirrhosis. Fifty-five subjects were considered as healthy controls. There is a significant increase in mycobiome load in patients with chronic HCV infection including patients with cirrhosis more than the control group (P value <0.05). By culture method, Candida species were obtained with more frequent isolation in HCV patients. There was an increase in the non albicans Candida in chronic HCV cirrhosis group more than albicans Candida.* ***Conclusion:*** *HCV patients are more predisposed to candidal colonization than normal subjects. The load of enteric mycobiome is increased in chronic HCV patients. The load of GI mycobiome may increase with the progress of HCV infection.*

INTRODUCTION

Viral hepatitis is a worldwide health problem. About 2 million people die every year from viral hepatitis. HCV infection represents a major concern in Egypt. According to WHO, Egypt is the country with the highest HCV prevalence. It is estimated to be around 7% [1]. Major percentage of patients with HCV infections are prone to develop serious complications as liver cirrhosis which may predispose to hepatocellular carcinoma [2].

Microbiota refers to complex microbial communities including bacteria, fungi, viruses and archaea that are normally inhabit different parts of human body[3]. Microbiota is present in GIT, Skin, respiratory tract, oral cavity, female reproductive system and even in sterile body areas[4-7]. However, GIT is still representing the main site of microbota[7]. Gastrointestinal (GI) microbiota is composed of about 10^{14} cells [8]. Gut microbiota regarding load, composition and diversity are proved to play a role in both health and many diseases[9-11]. Microbiome is a term that describes the genetic content of bacteria, fungi, and viruses microbiota. Gut microbiome refers to collection of genetic material of different organisms present in human GIT. It is estimated to be more than 3 million genes which is 150 larger than the whole human genome. So, gut microbiota is now proved to be a human organ [12-13]. The term mycobiome describes the genetic composition of fungal microbiota [12, 14].

The relation between gut bacterial microbiota and autoimmune hepatitis, alcoholic hepatitis, and chronic HBV has been discussed in many studies [15-17]. The association of intestinal fungal communities and chronic HBV are investigated in some studies [18]. However, to our knowledge the relation of human mycobiome load and chronic HCV infection and cirrhosis has not been investigated.

The current study, aimed at giving insight to GI fungal composition in cases of chronic HCV infections, cirrhosis and healthy subjects using culture dependant methods. In addition, real time PCR was used to compare the fungal genetic load (mycobiome load) in these patients and healthy controls.

METHODOLOGY

Study design

This study is a case control study which enrolled 53 patients with HCV infection and 55 healthy subjects as controls during the period extending from April 2015 to

January 2017 in the department of internal medicine, Mansoura University hospitals, Egypt. The study protocol was approved by institutional review board in faculty of medicine, Mansoura University. Written consents were obtained from patients and controls sharing in the present study. The patients group includes 27 patients with chronic HCV hepatitis and 26 patients with HCV liver cirrhosis. The case and control groups were matched in age, sex and body mass index table (1). Diagnosis of Hepatitis C viral infection was based on serological detection of antibody against HCV by ELISA. Reverse transcriptase real time PCR (rt-PCR) was used for detection of the virus RNA load in patients group. HCV RNA load was classified into mild, moderate and severe.

The diagnosis of liver cirrhosis was based on fibroscan and liver function tests. Absence of HCV infection in the control group was diagnosed by ELISA for HCV antibody. Liver function tests were done for both groups table (1). Stages of liver fibrosis in patients group were identified by fibroscan. HCV virus load estimated by real time rt-PCR was classified into mild, moderate and sever. Patients with less than 10^5 copy number/ ml (mild infection), 10^5 -10^6 copy number/ ml (moderate infection), and more than 10^6 copy number/ ml (sever infection) table (2).

Patients with intestinal dysmotitilty, peptic ulcer, cancers, and with other types of hepatitis including other viral hepatitis were excluded. The patients and control groups were not receiving any antimicrobial agents including antibiotics, antifungal and antiviral drugs in the previous 3 month before the study.

Table 1: Demographic characteristics and liver function tests of patients and control groups

	Chronic HCV (Total =27)	***HCV cirrhosis (Total =26)***	***Healthy control (Total =55)***
Age (years)	(33-65)	(37-69)	(26-56)
(mean±SD)	52.6 ± 6.7	56.8 ± 10.6	50.1 ± 10.2
Sex			
Male (No%)	12 (44.4)	14 (53.8)	31 (56.4)
Female (No%)	15 (55.6)	12 (46.2)	24 (43.6)
Body mass index (mean ± SD)	31.8 ± 4	31.3 ± 5	34.9 ± 3.8
ALT (mean±SD)	41 ±17	28 ± 14.3	21.9 ± 4.8
AST (mean±SD)	45.8 ± 19	35.6 ±8.2	30 ± 3.6
Serum Bilrubin (Total) (mean ± SD)	1.2 ± 0.5	1.6 ± 0.6	0.9 ± 0.07
Serum Bilrubin (Direct) (mean ± SD)	0.4 ± 0.4	0.6 ± 0.3	0.2 ± 0.02
Albumin (mean ± SD)	4.1 ±0.5	3.4 ± 0.6	4.3 ± 0.2
Prothrombin time (mean ± SD) sec	13 ± 0.2	15 ± 0.7	12.9 ± 0.2
Serum alpha feto protein	4 ± 2.6	18 ± 8.8	2.4 ± 0.8

Table 2: HCV load in relation to liver fibrosis stages in the patients group

HCV RNA load by real-time rt PCR (copy number /ml)	***Stage 1 No% (Total = 13)***	***Stage 2 No% (Total=14)***	***Stage 3 No% (Total=20)***	***Stage 4 No% (Total=6)***
<10^5	1 (7.7)	2 (14.3)	5 (25)	0
Moderate (10^5-10^6)	3 (23.1)	4 (28.6)	3 (15)	3 (50)
>10^6	9 (69.2)	8 (57.1)	12 (60)	3 (50)

Preparation of stool samples

Stool samples were collected from patients and controls in sterile containers. Each sample was divided into two parts each was 500 mg weight; for culture detection of fungi and real-time PCR. The portion for molecular analysis was homogenized in 0.1 M sodium phosphate buffer (pH 6.5) and stored until processing.

Culture:

The first part of the sample was used for cultivation of fungal component of microbiota. The samples were inoculated in duplicate on SDA supplemented with chloramphenicol (40 mg/mL) and kanamycin (50 mg/mL). The samples were then cultured for 7 days at 25 °C and 37 °C. Species identification of yeast was performed by morphology, germ tube test and API 20 C (Bio-Merieux Company, France) [19-20].

DNA extraction:

DNA extraction was performed using the QIAampDNA Stool Mini Kit (QIAGEN Company, Germany) following the manufacturer's instructions [21].

Real-time quantitative PCR

Fungal genetic load (mycobiome load) in stool samples was determined by quantitative real-time PCR using ABI-Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). SYBR-Green

PCR 2X master mix was used for quantification of total fungal load in 96- well plates. The reaction was done in total volume of 25 µl with 10µl of DNA sample. Each reaction was performed twice. Amplification was performed according to the following reaction conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 64°C for 45 seconds, 72°C for 30 seconds with a final extension step of 5 minutes at 72°C [22]. Melting curves were analyzed to confirm the identity and fidelity of amplification products [23]. The following primers specific for 18S rRNA gene were used: 5'ATTGGAGGGCAAGTCTGGTG 3' and 5'CCGATCCCTAGTCGGCATAG 3'[24]. The threshold cycle for each sample was determined.

Data were calculated using the 2-{2(-Delta DeltaC[T])} method [22, 24].

Statistical analysis

Descriptive data were presented in the form of number, parentages, and mean ± standard deviation. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS, version 17.00; Chicago, IL, USA). Chi-square test ($\chi 2$) was used for analysis of categorical data. Kolmogorov-Smirnov test was used to assess normality of continuous data. Independent sample t-test was used to compare continuous data of two groups. ANOVA test was used to compare continous data between more than two groups.

Values of P<0.05 were considered to be significant. The results of quantitative real time PCR for mycobiome load were converted to the average logarithms ($\log_{10}$) of target DNA fragment copy number in one gram feces. The averaged $\log_{10}$ of mycobiome load was expressed as mean ± SD and used for statistical analysis.

RESULTS

Study population:

This study was conducted in the department of internal medicine, Mansoura University hospitals, Egypt in the period extending from April 2015 to January 2017. Fifty three HCV patients (26 patients with HCV liver cirrhosis and 27 patients with chronic HCV liver infection) and 55 healthy subjects were enrolled. The mean age of the chronic HCV group was 52.6 ± 6.7, cirrhosis group 56.8 ± 10.6 and the control subject was 50.1 ± 10.2. The demographic characters and the liver function test expressed as mean ± SD are presented in table (1). Stages of liver fibrosis were identified by fibroscan. Patients of stage 3 liver fibrosis were the major sector of the patient group (20/53) (37.7%). HCV RNA load estimated by reverse transcriptase real time PCR and the stages of liver fibrosis in the patient group are shown in table (2). Patients were classified according to the viral RNA load into mild, moderate, and sever infection table (2). Stool samples were collected from patients and controls. Each sample was divided into two parts for culture of fungal microbiota and for molecular estimation of mycobiome load.

Composition of fungal microbiota by culture dependant method:

By culture dependant method, *Candida* species were the only detected fungal microbiota. Rate of *Candida* isolation by culture method from HCV chronic infected patients was 66.7% (18/27), 73.1% (19/26) from cirrhosis patient and 43.6% (24/55) from the controls. On comparing the two HCV patients groups and control group, there is a statistically significant difference in colonization rate of the HCV infected patients and the controls (P value < **0.01**). The albicans *Candida* was the most commonly isolated species in both patient and control groups. The presence of non albicans *Candida* was most common in HCV cirrhosis without significant difference (P=0.8). Different composition of *Candida* species was observed in control, chronic HCV and in HCV cirrhotic patients (table 3). The most numerous non albican species in controls was *C. tropicalis*. The most commonly isolated non albicans species in chronic HCV patients and cirrhosis were *C. tropicalis* and *C. parapsilosis* respectively.

Mycobiome quantification by real-time PCR

In the current study quantitative real-time PCR was used for estimation of mycobiome load in patients and control groups. The $\log_{10}$ mycobiome load was significantly higher in the two patients groups compared to the control P value < **0.01** (table 4). Comparing $\log_{10}$ of mycobiome load among liver fibrosis groups, significant difference was found P value < **0.01** (table 5).

Table 3: *Candida* species isolated by culture dependant method from stool sample of the studied groups

Fungus species by culture method	*HCV infected patients No% (Total = 27)*	*HCV cirrhosis No% (Total = 26)*	*Healthy control No% (Total = 55)*	*P value*
C. albicans	**11 (40.7)[a]**	**10 (38.5)[b]**	**15 (27.3)**	0.8[a]
Non albicans Candida	**7 (29.6)**	**9 (34.6)**	**9 (16.4)**	
C. tropicalis	3(11.1)	2 (7.7)	4 (7.3)	
C. glabrata	1 (7.4)	2 (3.8)	2 (3.6)	
C. krusei	1 (3.7)	1 (7.7)	0	
C. parapsilosis	2 (7.4)	4 (15.4)	3 (5.5)	
Total number of isolated *Candida* species	**18 (66.7)**	**19 (73.1)**	**24 (43.6)**	**<0.01[b]**

[a] Statistically insignificant difference in distribution of *Candida* albicans/ non albicans between the three groups

[b] Statistically significant difference in *Candida* colonization between chronic HCV infected patients and the control group.

Table 4: Log_{10} mycobiome load in different groups (mean ± SD)

	HCV infected patients	*HCV cirrhosis*	*Healthy control*	*P value*
Log_{10} mycobiome load	5.8 ± 0. 24	5.9 ± 0.18	5.4 ± 0.26	**<0.01[a]**

[a] **Statistically significant**

Table 5: Correlation of liver fibrosis stages with log_{10} mycobiome load

	Liver fibrosis stage 1 Number (13)	*Liver fibrosis stage 2 Number (14)*	*Liver fibrosis stage 3 Number (20)*	*Liver fibrosis stage 4 Number (6)*	*P value*
Log_{10} mycobiome load Mean ± SD	5.56 ± 0. 22	5.58 ± 0.18	5.84 ± 0.14	6.12 ±0.07	**<0.01[a]**

[a] **Statistically significant**

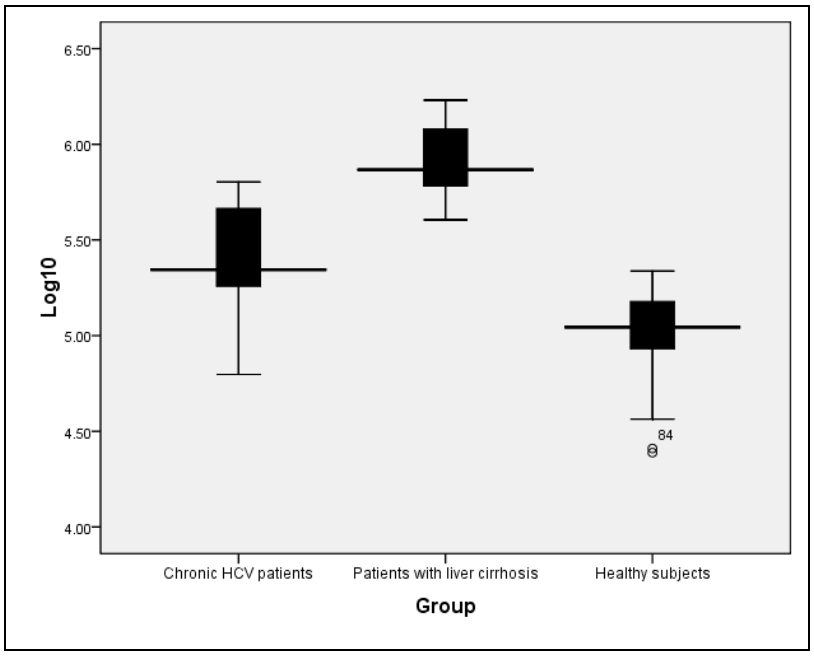

Fig. 1: Log_{10} of the mycobiome load in control and patients groups

On studying the possible relation between HCV RNA load and log_{10} mycobiome loads, independent sample t test was performed table (6). No significant relation was found (P value= 0.8).

Table 6: Correlation of HCV RNA load with log_{10} mycobime load by real-time PCR

HCV viral load with real time PCR		**Log 10 mycobiome load (mean)**	**P value**
Mild	8	6.1 ±3.3	0.8[a]
Moderate	13	6.4±5.7	
Sever	32	5.5±3.6	

[a] Statistically insignificant

DISCUSSION

Fungi are among the most environmentally common and divergent eukaryotes [25]. More than 600 fungal species are conveyed to cause human disease and are related to a variety of diseases [26].

Many studies have pointed to the importance of the mycobiome in gastrointestinal health. In 2008, *Stephan Ott* in Christian-Albrechts-University and the University Hospital Schleswig-Holstein in Kiel, Germany, and colleagues found that the fecal fungal community in patients with inflammatory bowel disease (IBD) was substantially different from that of healthy controls [27].

Little is known about the gut mycobiota of HCV patients. We sought to explore the gut mycobiota composition and load in HCV patients with no other underlying disease, in comparison with healthy controls from the same geographical area (having similar diet and lifestyle).

Although, culture is the golden standard for the detection of fungal species, PCR techniques based on the detection of the small subunit ribosomal RNA (18S rRNA), had reemerged and are able to provide the quantitative information about uncultivable fungi which compromise the largest part of GI mycobiota. This method is of higher sensitivity than traditional culture methods [26, 28].

This study enrolled 53 patients with chronic HCV infection including 27 patients with chronic HCV hepatitis and 26 patients with HCV liver cirrhosis. Fifty-five subjects were considered as healthy controls. We compared the composition and load of intestinal fungal microbiota using culture method and quantitative real-time PCR amongst the three groups. In the current study, SYBR Green real-time PCR was used for quantitation of whole mycobiome load in fecal samples.

Our culture findings revealed only very limited fungal species could be detected with culture method. These intestinal fungi include *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis* in both patients and control groups.

The highest rate of *Candida* colonization was detected in cirrhotic patients followed by chronic HCV infected patients (73.1% and 66.7%). *Candida* species were isolated only from 43.6% of the healthy subjects.

This agrees with *Khatib et al*. [29] who reported fecal colonization with multiple species in healthy subjects. Colonization with multiple *Candida* species was detected in 69.8% of hepatitis patients. Also, this result agrees with other previous studies [30-31]. *Candida* species represent important category of gut flora. The rate of *Candida* colonization in healthy subjects is ranging from 20% to about 75%. Comparing patients and healthy persons, significant difference was observed regarding *Candida* colonization between HCV infected patients and healthy control (P value < 0.01). Healthy persons and chronic HCV patients are more likely to harbor *Candia* albicans, while cirrhotic patients are more likely to harbor non albicans *Candida*. No statistically significant difference was detected in the distribution of *C. albicans* among the three groups (P value: 0.8).

However, this comparison is based on rate of culture dependant isolation of fecal fungi which is of low sensitivity. In addition, in the present study the data is insufficient to decide species persistence in the gastrointestinal tract. Whether the isolated yeasts are permanent or temporary residents is unclear. We recommend further studies employing sequencing for more accurate identification of different fungal species and to take more than one specimen from both patients and healthy persons at different times.

In our study, there is a significant increase in mycobiome load in patients with chronic HCV infection including patients with cirrhosis more than the control group (P value <0.01).

Our study shows a higher load of fungal mycobiome in patients with hepatitis C cirrhosis than in patients with chronic hepatitis C, and the latter was higher than the healthy persons

This agrees with *Chen et al* study [18], which shows a higher load of fungal species in patients with hepatitis B cirrhosis than in patients with chronic hepatitis B, and the latter was higher than that in HBV carriers and healthy volunteers. Also, *Chen et al* study shows a little alteration in fecal fungal diversity between HBV carriers and healthy volunteers.

Perhaps the most significant influence of HCV on the gut microbiota is related to the pathophysiological alterations of the liver, eventually interfering with its digestive functions. For example, HCV infection leads to low bile production, subsequently leading to fungal and bacterial overgrowth and changes in gut microenvironment and microbial community [32].

Up to our knowledge no other studies were done to show the relation between intestinal fungal species load in relation to HCV.

Our results indicate that the mycobiome load significantly correlated with the disease progression of patients with different degrees of chronic HCV infection. This finding may be explained by the disturbed intestinal microecology which results in increased intestinal microbial population including bacterial and fungal flora. Patients with HCV chronic hepatitis and liver cirrhosis are more prone to this altered intestinal microbiota [33-34].

Many studies illustrate that during hepatic affection, there is a decrease in intestinal motility. Also, damage of the bowel barrier occurs, leading to increased permeability and fungus translocation and consequently fungus infection. The use of PCR could have a benefit in management of fungus infection [35-36].

Our study has some limitation. First, we did not study the correlation of intestinal fungal coloniazation and mycobiome load with the development of infections in patients with different stages of HCV hepatic affection and cirrhosis. Second, our study did not include HCV patients who developed hepatocellular carcinoma and the possible role of mycobiota in development of hepatocellular carcinoma. Further studies are recommended to investigate these important topics.

CONCLUSION

HCV cirrhotic patients are more predisposed to high candidal colonization than normal subjects. The load of enteric mycobiome is significantly increased in

HCV liver cirrhosis. The load of GI mycobiome may increase with the progress of HCV infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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