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The Association of Chronic Hepatitis C with Respiratory Microbiota Disturbance on the Basis of Decreased *Haemophilus* Spp. Colonization

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Background: *Haemophilus* species are the most common microbiota in humans. The aim of this paper was to investigate *Haemophilus* spp., mainly *H. parainfluenzae* prevalence, in the upper respiratory tract of chronic hepatitis C (CHC-positive) patients with or without therapy using pegylated interferon alfa and ribavirin.

Material/Methods: We collected 462 samples from 54 healthy people and 100 CHC-positive patients at various stages: before (group A), during (group B), and after (group C) antiviral therapy. Identification of bacterial isolates including biotypes and antimicrobials susceptibility was accomplished by means of standard microbiological methods.

Results: In 70.4% of healthy people (control group) and in 27.0% of CHC-positive patients, the presence of haemophili, mainly *H. parainfluenzae* was observed, and those differences were statistically significant ($p<0.0001$). Statistically significant differences in *Haemophilus* spp. colonization were also observed among healthy people and CHC-positive patients from group A ($p=0.0012$) and from B or C groups ($p<0.0001$). Resistance to ampicillin in beta-lactamase-positive isolates and multidrug resistance (MDR) of *H. parainfluenzae* was detected mainly in group A.

Conclusions: The obtained data suggest that chronic hepatitis C, together with antiviral therapy, may influence the respiratory tract microbiota composition as found using haemophili, mainly *H. parainfluenzae*.

MeSH Keywords: Haemophilus • Hepatitis C • Microbiota

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Background

Chronic hepatitis C (CHC) infection is a contagious disease determined by interaction between the hepatitis C virus (HCV) and the host's immune system. It can cause serious health problem or deterioration in quality of life [1–3]. Natural microbiota of the mucosal surfaces of the body, primarily in the gut and the upper respiratory tract, play a role in preventing the establishment of potential pathogens, and it is very important for the proper functioning of the human body, including its role as a defense mechanism [4–7]. Additionally, it can be regarded as a reservoir of opportunistic pathogens. *Haemophilus parainfluenzae*, a common microorganism in the respiratory tract (mainly the oropharynx), is increasingly recognized as commensal bacteria and sporadically as an opportunistic pathogen causing local or systemic diseases, e.g., respiratory tract infections [8], endocarditis [9,10], bacteremia [11], sepsis [12], and septic arthritis [13], meningitis [14], peritonitis [15,16], epiglottitis, and other infections [17–20], especially in patients with a impaired immunity.

The microbiota colonizing the mucosal surfaces of the upper respiratory tract play a crucial role in preventing the establishment of potential pathogens in the early priming of the reaction patterns of the host immune system [7,21]. The most recent analyses of the natural microbiota composition and the consequences of its changes were performed in various groups of people. In CHC-positive patients, it has been little-studied and only in terms of the pathogen's prevalence or the intestinal flora. The aim of our study was to determine if the nature of the disease, i.e., chronic hepatitis C, together with an antiviral therapy using pegylated interferon alfa (peginterferon-alfa) and ribavirin, has an effect on the rate of overall colonization of the upper respiratory tract by haemophili, including by *H. parainfluenzae*, as the respiratory microbiota.

Material and Methods

We included 154 persons aged 30–75 years, in that 100 patients aged 30–65 years (average 48.1) with chronic hepatitis C (CHC-positive group) who were admitted to the Department of Infectious Diseases of Medical University of Lublin. Among all CHC-positive patients, 3 groups were studied: group A – before (n=46), B – during (n=31), and C – after (n=23) antiviral therapy with a combination of peginterferon alfa and ribavirin. Within 30 days before being admitted to hospital, no patients took any antimicrobial agents or drugs influencing the immunological system, nor had they undergone any blood transfusions or suffered from an allergic disease. Additionally, the included patients had no coexistent chronic diseases of the respiratory tract. None of the patients had cirrhosis of the liver. The control (CHC-negative group) group comprised 54 healthy volunteers aged 35–75 years (average age, 44.6 years). The age distribution was similar in both groups. The percentage of people smoking cigarettes was small and similar for CHC-positive patients and healthy volunteers (about 10% and 8%, respectively). The Ethics Committee of Medical University of Lublin approved the study protocol (KE-0254/75/2011).

A of total of 462 specimens from the mucous membrane of the throat and both nostrils (3 samples from 1 patient) were collected. The specimens were taken by means of sterile cotton swabs and they were immediately placed onto a selective medium for haemophili (*Haemophilus* chocolate agar, HAEM, bioMérieux, France) and for *Candida* spp. yeasts (Sabouraud agar with chloramphenicol, Biocorp, Poland). The presence of bacteria or yeasts in the upper airways in at least 1 sample was considered as colonization. HAEM media inoculated with haemophili were incubated in an atmosphere with increased CO_2 concentration (appropriate for microaerophilic bacteria) for 48 h at 35±2°C. For the initial identification of isolated haemophili, the growth morphology on HAEM agar and requirements for hemin (X factor) and nicotinamide adenine dinucleotide (V factor) on TSA (Tripticasein Soy LAB-AGAR, Biocorp, Poland) medium with diagnostic Oxoid discs DD3 (X factor), DD4 (V factor), DD5 (both X and V factors) were determined. Biochemical identification of isolates growing only in the presence of V factor was carried out using the API NH microtest (bioMérieux, France). Biotyping of *H. parainfluenzae* isolates was performed according to the classification of Kilian [22] on the basis of indole production and urease or ornithine decarboxylase activity (I–VIII biotypes). Sabouraud agar with chloramphenicol inoculated with yeasts was incubated in the aerobic atmosphere for 48 h at 35±2°C. *Candida* spp. isolates were identified using the biochemical microtest API 20 C AUX (bioMérieux, France). The ability of *Candida* strains to produce hyphae, pseudohyphae, or chlamydospores was also evaluated.

Antibiotic sensitivities of 36 *H. parainfluenzae* and 6 *Haemophilus* spp. isolates from CHC-positive patients were established by the disc diffusion method using Haemophilus Test Medium (HTM, Oxoid) according to the Clinical Laboratory Standards Institute (CLSI) recommendation for *Haemophilus* species [23]. Direct colony suspensions standardized to 0.5 McFarland standard (~10^8 CFU, colony-forming units/ml) were prepared using the colonies from an overnight HAEM agar incubation at 35°C in atmosphere with about 5% CO_2. *H. influenzae* ATCC 10211 was used to verify the growth promotion properties of HTM. We tested ampicillin (AM, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg), ampicillin-sulbactam (SAM, 20 µg), cefuroxime (CXM, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), imipenem (IPM, 10 µg), aztreonam (ATM, 30 µg), azithromycin (AZM, 15 µg), tetracycline (TE, 30 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg), and ciprofloxacin (CIP, 5 µg) discs (BD BBL). Multidrug-resistant haemophili

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Table 1. The frequency and statistical analysis of nasopharyngeal colonization by *Haemophilus* spp. in healthy people (control group) and CHC-positive patients divided into three groups: A – before, B – during, and C – after the antiviral therapy with peginterferon-alfa and ribavirin.

People	Healthy people n=54 (%)	CHC-positive patients		
		Group A n=46 (%)	Group B n=31 (%)	Group C n=23 (%)
Uncolonized by haemophili	16 (29.6)	29 (63.0)	24 (77.4)	20 (86.96)
Colonized by *Haemophilus parainfluenzae*	31 (57.4)	15 (32.6)	7 (22.6)	3 (13.04)
p value	Referent	0.016	0.003	0.0004
RR (95%CI)		0.6 (0.4–0.9)	0.4 (0.2–0.8)	0.2 (0.1–0.7)

CHC – chronic hepatitis C.

isolates were defined as having resistance to at least 3 different classes of antimicrobials. β-lactamase production was screened using *Pen* test (API NH, bioMerieux) and nitrocefin as the chromogenic cephalosporin method (Cefinase disks, BD BBL). MIC (minimal inhibitory concentration) for ampicillin was determined by E-test strip (AB Biodisc) and interpreted according to the CLSI recommendation [23].

Data processing and analysis were performed using StatSoft, Inc. STATISTICA 2010 for Windows. Contingency table analysis for comparing proportions was conducted by Fisher's exact test. The relative risk (RR) and its 95% confidence intervals (CI) were calculated. Statistical significance was set at $p<0.05$.

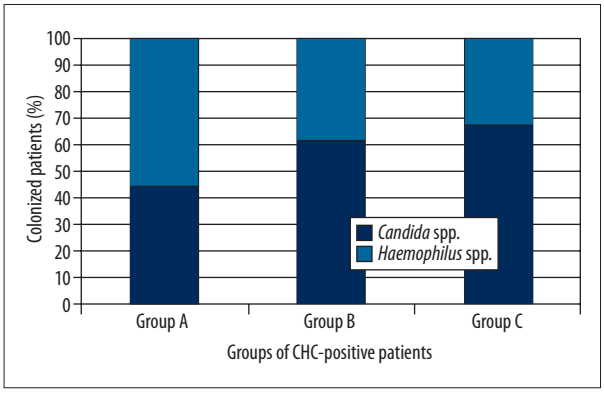

Figure 1. Prevalence of *Haemophilus* spp. *vs. Candida* spp. colonization in CHC-positive patients before (group A), during (group B), and after (group C) antiviral therapy.

Results

Differences were found between healthy people (control group) and CHC-positive patients (total and A – before, B – during, or C – after antiviral therapy group) in overall *Haemophilus* spp. or in the *H. parainfluenzae* colonization. Among all healthy people, 38/54 (70.4%) were colonized by haemophili, including 31/54 (57.4%) colonized by *H. parainfluenzae*. In contrast, among all CHC-positive patients 27/100 (27.0%) were colonized by haemophili, including 25/100 (25.0%) colonized by *H. parainfluenzae*. The differences among the number of healthy people and all CHC-positive patients uncolonized and colonized by *Haemophilus* spp. or by *H. parainfluenzae* were statistically significant ($p<0.0001$, RR=0.4, 95%CI=0.3–0.6). According to Table 1, similar differences were demonstrated in the case of colonization by *Haemophilus* spp. or by *H. parainfluenzae* between the referent group of healthy people and individual groups of CHC-positive patients: group A –before ($p=0.016$), group B – during ($p=0.003$) and after ($p=0.0004$) the antiviral therapy. It was found that in comparison to group A of CHC-positive patients the antiviral therapy reduced the number of patients colonized by different haemophili species; approximately 2.4 times in group B, and 5.7 times in group C. Similar differences as compared to those in group A also occurred in the case of *H. parainfluenzae* (approximately 2.14 times in group B, and 5.0 times in group C). There were no differences in the occurrence of haemophili colonization in men and women ($p>0.05$).

Among all CHC-positive patients 30/100 (30.0%) were colonized by *Candida* spp., including 28/100 (28.0%) colonized by *C. albicans*. Prior to the antiviral therapy (group A) 13/30 (43.3%) patients were colonized by *Candida* spp. A decreased number of patients colonized by yeasts was noticed both in groups B and C from 11/30 (36.7%) to 6/30 (20%), respectively, but these differences were not statistically significant ($p>0.05$). *C. albicans* was the predominant species in all 3 groups of patients – it was isolated from 12/30 (40%) patients of group A, 11/30 (36.7%) patients of group B and 5/30 (16.7%) patients of group C. Non-*albicans Candida* spp. were rarely found: in 3/30 (10%) patients of group A (1 case, *C. lusitaniae*; 1 case, *C. glabrata* and *C. albicans*; 1 case, *C. glabrata* together with *C. tropicalis* and *C. albicans*), in 2/30 (6.7%) patients of group B

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Table 2. Distribution of *Haemophilus* spp. isolates cultured from the upper respiratory tract of healthy people and CHC-positive patients before (group A), during (group B) and after (group C) the antiviral therapy.

People group		No. (%) of haemophili isolates (n=104)	
		***Haemophilus parainfluenzae* (n=83)**	**Other *Haemophilus* spp. (n=21)**
Healthy people		46 (55.4)	15 (71.4)
CHC-positive patients group	A	22 (26.5)	3 (14.3)
	B	11 (13.3)	1 (4.8)
	C	4 (4.8)	2 (9.5)

CHC – chronic hepatitis C.

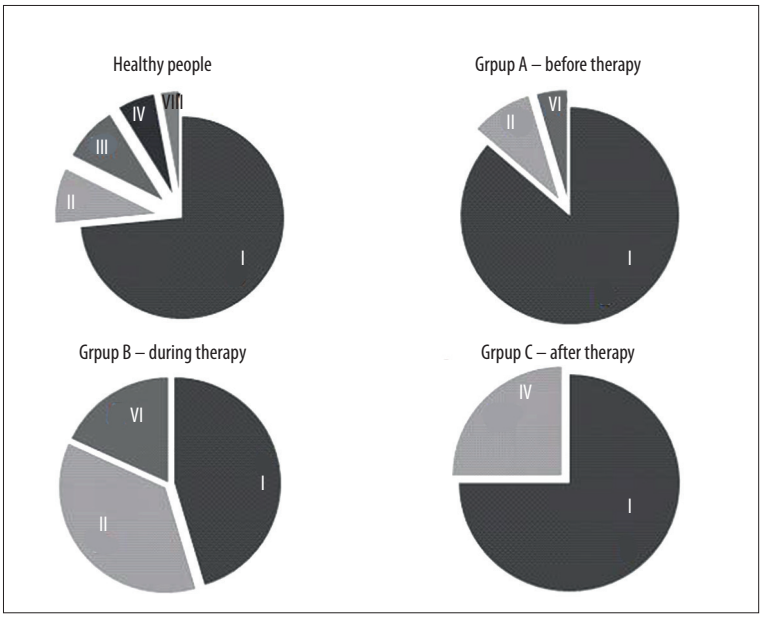

Figure 2. Biotypes of *Haemophilus parainfluenzae* isolates from the upper respiratory tract of healthy people and patients with chronic hepatitis C (CHC-positive) divided into 3 groups: A – before, B – during, and C – after the antiviral therapy with peginterferon-alfa and ribavirin.

(2 cases, *C. glabrata* and *C. albicans*), and in 1/30 (3.3%) patients of group C (1 case, *C. tropicalis*).

A difference (p>0.05) in the percentage of CHC-positive patients colonized by *Haemophilus* spp. *vs. Candida* spp. was shown within groups A, B and C (Figure 1). Before the antiviral therapy (group A) the number of patients colonized by bacteria *vs.* yeasts was 17/30 (56.7%) *vs.* 13/30 (43.3%). It was found that in comparison to group A the antiviral therapy reduced the number of patients colonized by *Haemophilus* spp. *vs. Candida* spp. in group B – 7/18 (38.9%) *vs.* 11/18 (61.1%), and C – 3/9 (33.3%) *vs.* 6/9 (66.7%).

Totally, 43/104 (41.35%) isolates of haemophili were selected in the group of CHC-positive patients (Table 2). It was shown that 37/43 (86.04%) isolates of *H. parainfluenzae* were selected. Additionally, from the CHC-positive patients the majority of *H. parainfluenzae* isolates were selected before the antiviral therapy (group A), while a decrease of haemophili isolation was observed also during (group B) or after (group C) the antiviral therapy. Only 6/43 (13.95%) of the other *Haemophilus* spp. isolates were selected from CHC-positive patients. However, from 1 patient 2 or 3 isolates representing different phenotypes (in group A – from 6 patients, in B – from 4 patients, and in C – from 2 patients) of *H. parainfluenzae* were selected. These *H. parainfluenzae* phenotypes differed in their growth morphology and biochemical properties or susceptibility to antimicrobial agents. *H. influenzae* was not isolated at all.

It was shown that 61/104 (58.7%) isolates of haemophili were selected in the group of healthy people (Table 2). Despite the different rates of total isolation of *Haemophilus* spp. in healthy people and CHC-positive patients, *H. parainfluenzae* was the most frequently identified (46/61, 75.4%) species.

On the basis of indole production, urease activity and ornithine decarboxylase activity *H. parainfluenzae* isolates were assigned to various biotypes. According to Figure 2, there were differences in *H. parainfluenzae* biotypes distribution among the 3 tested groups of CHC-positive patients. In group

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Table 3. Prevalence of antibiotic resistance in *Haemophilus parainfluenzae* and other *Haemophilus* spp. isolated from the throat of CHC-positive patients before (group A), during (group B) and after (group C) the antiviral therapy.

Isolates	CHC-positive patient group	No. (%) of isolates				
		Am	Ctx	Caz	Te	Sxt
Haemophilus parainfluenzae (n=36)	A	3 (8.3)	–	2 (5.6)	3 (8.3)	3 (8.3)
	B	–	1 (2.8)	–	–	2 (5.6)
	C	1 (2.8)	–	2 (5.6)	1 (2.8)	1 (2.8)
Other *Haemophilus* spp. (n=6)	A	–	–	1 (16.7)	2 (33.3)	–
	B	–	–	–	–	–
	C	–	–	–	–	–

CHC – chronic hepatitis C; Am – ampicillin; Ctx – cefotaxime; Caz – ceftazidime; Te – tetracycline; Sxt – trimethoprim/sulfamethoxazole; (–) – not found.

A (n=22 isolates) it was the following: I – 19 (86.4%), II – 2 (9.1%), VI – 1 (4.6%); in group B (n=11 isolates): I – 5 (45.5%), II – 4 (36.4%), IV – 2 (18.2%); in group C (n=4 isolates): I – 3 (75%), IV – 1 (25%) biotype. Multiple isolates recovered from the same patient were often of the same biotype. The most frequently isolated was biotype I (27 isolates), comprising 19 (70.4%), 5 (18.5%) and 3 (11.1%) in groups A, B, and C, respectively. Typical reactions of this biotype include ornithine decarboxylase production but no production of indole or urease. The predominant numerical profile shown in about 74% of *H. parainfluenzae* and included in biotype I was 7360: in group A there were 17 (89.5%), and in group B there were 3 (60%) isolates, while in group C 3 (75%) the isolates were with the numerical profile of 7362. Despite the differences in the prevalence of *H. parainfluenzae* biotypes in CHC-positive patients, the most frequently isolated was biotype I (mainly with numerical profile of 7360).

It was assessed that among 42 haemophili isolates cultured from CHC-positive patients, 28 (66.7%) were susceptible to all tested antimicrobials, while 14 (33.3%) were resistant to at least 1 to 4 antibiotics – 12 (28.6%) isolates of *H. parainfluenzae* and 2 (4.8%) isolates *Haemophilus* spp. (Table 3). Resistance to ampicillin was observed in 4 beta-lactamase positive isolates of *H. parainfluenzae* (MIC=6–256 mg/l), 2 isolates had MIC=1.5 mg/l (intermediate) and 30 isolates of this species were sensitive to ampicillin with MIC ranging from 0.094 to 1 mg/l. In groups B and C less resistant strains were isolated. Multidrug resistance (MDR) was detected in only 2 (4.8%) *H. parainfluenzae* isolates – 1 isolate was resistant to ampicillin, tetracycline and trimethoprim/sulfamethoxazole, and the other isolate – to ampicillin, ceftazidime, tetracycline, and trimethoprim/sulfamethoxazole. None of the tested isolates was resistant to amoxicillin/clavulanic acid, ampicillin/sulbactam, cefuroxime, imipenem, aztreonam, azithromycin or ciprofloxacin.

Discussion

According to our results, *Haemophilus* species isolation from the upper respiratory tract mucosa was decreased markedly and in a statistically significant way from CHC-positive patients both without and at different stages of the antiviral therapy using pegylated interferon alpha and ribavirin. To our knowledge, this study demonstrates for the first time the association of HCV infection and exposure to the antiviral therapy with the presence of respiratory microbiota. It was shown that both the disease itself and the course of the antiviral therapy have an effect on the disturbance of the respiratory tract mucosa colonization by *Haemophilus* spp., mainly *H. parainfluenzae*. It was also found that the standard treatment of patients with chronic hepatitis C and microbiota changes identified on the basis of haemophili had a partial effect on the occurrence of *Candida* spp., mainly *C. albicans*.

Chronic diseases and the scheme of the therapy may result in a reduced quality of life and disturbances of nonspecific defense mechanism important for the health (e.g., natural intestinal microbiota composition or extrahepatic syndromes) [5–7,24–26]. CHC-positive patients have an increased risk of later developing mild or acute infections [1–3,27–29]. Loguercio et al. [30] clearly suggest that manipulating the composition of the intestinal microbiota by probiotic in patients with various types of liver diseases may be important for the liver condition and as an adjunctive therapy in some types of this group of diseases. A combined peginterferon and ribavirin therapy may result both in improved antiviral efficacy and a number of undesirable effects like fatigue, influenza-like symptoms, hematologic abnormalities, and neuropsychiatric symptoms [31–35].

Few studies demonstrated that changes of the microbiota composition and the possible disturbance of its functioning in CHC-positive patients or scheme of the therapy may have a negative effect on health conditions [25,26,29,36]. Although the

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microflora of the upper respiratory tract also plays a significant role in people's health, we did not find any data in the literature on the effects of hepatitis C and the antiviral therapy on the presence of bacteria on the respiratory mucosa and the possible consequences of bacterial microbiota disturbance. However, it can be possible that the composition of the respiratory tract microbiota may also play an important role for patients with chronic hepatitis C as protection against opportunistic diseases (e.g., candidiasis) or it may improve the general condition.

As was shown during our studies, decreased haemophili colonization in the group of CHC-positive patients during and especially after the antiviral therapy with pegylated interferon alfa and ribavirin was partially associated with a slight increase in *Candida* species colonization of the respiratory mucosa. According to Biernasiuk et al. [37], the antiviral therapy had no effect on the prevalence of *Candida* species in the upper respiratory tract of patients with chronic hepatitis C with or without the standard antiviral therapy. Nagao et al. [38] also found no correlation between, e.g., HCV genotype, interferon type, dose or administration schedule, or effect of interferon therapy with *Candida* prevalence. However, these authors found that the presence of oral mucosal lesions was the most significant factor associated with *Candida* spp. colonization. It should also be noted that candidiases were diagnosed statistically more frequently in immunocompromised patients with alteration in the microbiota composition [39]. In our opinion, it may be associated with a microbiota disturbance.

Haemophilus species, especially *H. parainfluenzae*, as the bacteria with low pathogenicity common in the human oral cavity and pharynx microbiota, colonizing them asymptomatically and only occasionally in susceptible people, especially in young children or in immunocompromised patients, takes part in opportunistic infections [8,10,12,40]. Gilley and Orihuela [41] demonstrated on the basis of asymptomatic colonization of the nasopharynx by *Streptococcus pneumoniae* forming a biofilm that this state is the major protective form by which the bacteria interact with the host without invasion but with more resistance to desiccation. According to literature [42–44], *H. parainfluenzae* can live in a biofilm form both on abiotic and eukaryotic cells and probably it lives in a biofilm form as avirulent and protective microbiota during the nasopharyngeal colonization in healthy people. In our opinion, reduction of haemophili presence in CHC-positive patients especially during the antiviral therapy can affect the health condition and may suggest increased susceptibility to a variety of external factors partially associated with a destruction of the protective biofilm.

According to our data, in case of CHC-positive patients some differentiation between the selected *H. parainfluenzae* biotypes existed depending of the group of patients; biotype I was found to be the predominant strain isolated from the respiratory tract independent of the patient's condition or the antiviral therapy but the number of biotypes was decreased. Several reports described the high frequency of oropharyngeal isolation of different biotypes of *Haemophilus* species [45–47]. According to Martel et al. [45], biotypes I, II, and III of *H. parainfluenzae* were more prevalent in the oropharynx, while biotypes I and II were more prevalent in the anogenital area. Most authors agree that the other biotypes of *H. parainfluenzae* were isolated less frequently. According to Kosikowska et al. [48], chronic hepatitis C may have an impact on the microbiota of the host organism but there were small differences in the occurrence of *H. parainfluenzae* biotypes within the upper airways in CHC-positive and CHC-negative groups; biotype I, showing the activity of ornithine decarboxylase but with no ability to split urea and to produce indole during L-tryptophane metabolism, predominated in both groups. It is probable that the isolates with the activity of both enzymes mentioned above possess an advantage of colonizing the upper airways; the activity of beta-galactosidase and ornithine decarboxylase may promote the growth of *H. parainfluenzae* and may also contribute to increased adherence of the isolates.

As was shown in our study, 4 ampicillin-resistant beta-lactamase positive isolates and 2 MDR of *H. parainfluenzae* were isolated. Antibiotic resistance is a serious public health threat and the genes encoding resistance in the absence of antibiotic selection further contribute to this problem. However, most human microbiota and environmental microorganisms, which are neutral for the host, do not only exchange resistance genes among each other but they might also interact and transmit them with other bacteria [49]. Increased resistance to antimicrobials commonly used for treatment of community-acquired infections, like beta-lactams or trimethoprim/sulfamethoxazole, may be associated with higher rates of their usage. Dissemination of antibiotic resistance is not only facilitated by the uncontrolled usage of antibiotics in clinics and hospitals, but it can be also transmitted to humans through the various environmental ways, e.g., in agriculture and aquaculture. The extensively drug-resistant (XDR) species of *H. parainfluenzae* nonsusceptible to ampicillin, amoxicillin-clavulanate, cefotaxime, cefepime, meropenem, cefuroxime, azithromycin, ciprofloxacin, tetracycline, and chloramphenicol were observed by Tinguely et al. [50]. According to CLSI M100-S24 recommendation Table 2E [23], some antimicrobials like amoxicillin-clavulanic acid, azithromycin, cefuroxime axetil "are oral agents that may be used as empiric therapy for respiratory tract infections due to *Haemophilus* spp. The results of susceptibility tests with these antimicrobial agents are often not useful for management of individual patients. However, susceptibility testing of *Haemophilus* spp. with these compounds may be appropriate for surveillance or epidemiologic studies". People with chronic diseases like CHC-positive patients are usually treated with multiple courses of antimicrobials. The presence of respiratory microbiota resistant to

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antimicrobials and the community-acquired infections seems to be harder to treat medically. Both our results and the literature data suggest that this problem requires further detailed studies, especially in CHC-positive patients.

Conclusions

This is the first study determining the association of chronic hepatitis C and the respiratory microbiota disturbance on the basis of decreased *H. parainfluenzae* colonization in CHC-positive patients. Haemophili may be regarded as a marker of changes in the composition of the respiratory microbiota in this group of patients. Colonization of these bacteria was changed significantly in CHC-positive patients together with an antiviral therapy. It is possible that *Haemophilus* spp., mainly *H. parainfluenzae*, may play an important protective role, e.g., against opportunistic diseases.

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