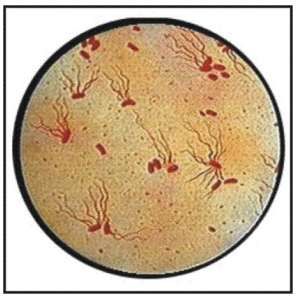
ORIGINAL PROF-895

SIGNIFICANT INCIDENCE OF TYPHOID IN HEPATITIS C PATIENTS



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ABSTRACT ... ahaq_nibge@yahoo.com **Background:** Only proven way of transmission of Hepatitis C is through blood. The origin is unknown in nearly half the cases. Pollution is suspected as a cause but it is impossible to prove this relationship directly. **Objectives:** we thought that typhoid being a proven pollution related disease, determination of its confection in Hepatitis C patients representing same Socio-economic group would be of interest. A typhoid in Hepatitis C patients can easily be overlooked because symptoms like fever and abdominal discomfort are present in both diseases. **Design of study;** Blood samples were collected from three groups of study as mentioned in materials and methods. These samples were processed for 4th generation HCV ELISA. PCR for HCV, PCR for typhoid. Blood culture for typhoid and widal test as required (details are given in methodology). Finally the data thus obtained was analysed and conclusions were drawn. **Place of study:** Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE) and Millat Laboratory Faisalabad. **Period:** April 2004 to Oct 2004. **Methods:** The parameters included were PCR, blood culture and widal test. There were three groups of study, PCR and ELISA positive patients of Hepatitis C (105) - further subdivided into two groups, with history of exposure to known causes of spread of HCV in last one year (65) and those without such history (40); clinically diagnosed cases of typhoid (30); and healthy controls (50). **Results:** In the three groups, PCR was positive in 9.5(7.7 and 12.5), 63.3, and 2.0%

cases respectively. Figures for blood culture were 4.7(3.1 and 7.5), 33.3, and 0% in the same order, and the respective figures for widal test were 34.2(33.8 and 35.0), 56.6, and 24.0%. The increase in PCR and blood culture positivity in Hepatitis C cases as compared with normal subjects is statistically significant (P< 0.05). **Conclusion:** These results clearly suggest that the source of infection for the two diseases is same in many cases, and therefore, provides a strong indication of a relationship between pollution and Hepatitis C.

Key Words: Hepatitis C, Typhoid, Pollution

INTRODUCTION

Hepatitis C is very common, potentially fatal disease. Globally, an estimated 170 million people are living with HCV[7]. This infection is very common in developing countries. In Egypt, prevalence ranges from 18-35% in different parts. Even in some developed countries like Australia, an estimated 200,000 people are living with HCV, with approximately 150,000 having chronic HCV infection[1].

HCV is undoubtedly the most important cause of chronic Hepatitis. It has also been reported to be associated with acute hepatitis, autoimmune chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma[2]. Because the infection becomes chronic in more than 80% of in the infected people, the disease is an important public health and economic problem[3].

There is significant association between acquiring disease and a history in the six months prior to illness of blood transfusion, an exposure to a contact who had hepatitis, and low socioeconomic level[4]. Other important risk factors include abuse of intravenous drugs[5], use of contaminated glass syringes during mass vaccination programs[6], hemodialysis[7], infection with HIV[8], breast feeding[9], mother to infant transfer[10], and tear fluid[11]. There is no conclusive evidence about sexual contact[12].

Some reports suggest that as many as 44% of HCV victims typically report no risk factors-such as having had a blood transfusion, having shared intravenous needles or having pollution is suspected to one of the main reasons for these unaccounted for cases. No direct linkage has yet been established, however, sewage workers have been described to be at higher risk of acquiring Hepatitis C from occupation exposure[14].

It is impossible to isolate HCV from polluted water or food because viruses unlike bacteria cannot grow out side a living cell and thus their number is too small even of they survive. The situation is made even more complex by the fact that HCV is very illusive. Therefore, the only option left is to find some indirect evidence that can link pollution with HCV spread.

Typhoid is a proven water and food pollution related disease. It has been clearly demonstrated that the incidence of typhoid fever decreases dramatically with the provision of clean water through chlorination and filtration[15].

We hypothesized that if the incidence of typhoid markers was significantly higher in Hepatitis C cases (indicative of co-infection of typhoid and HCV) belonging to a poor community where water pollution is rampant, it would be a strong evidence that the source of two diseases in many cases might be the same. Or in other words, water and food pollution may also be a significant cause of Hepatitis C, especially in developing countries where hygienic conditions are poor.

For this study, it was decided to check three typhoid parameters, blood culture, PCR for typhoid and widal test in HCV patients and control groups. PCR was included because of its proven superiority over other methods in terms of sensitivity; and blood culture, inspite of low sensitivity is very specific. Because both PCR and blood culture are techniques for direct direction and do not provide an insight into previous exposure, so Widal test

was included as an indirect test to detect antibodies produced against Salmonella typhi as a consequence of a previous infection. We preferred Widal test over Typhoid, a novel test with projected superiority, because some recent studies have established that the later has no significant advantage inspite of being relatively expensive[16].

MATERIAL & METHODS

Study groups

All the subjects included in this study belonged to low socioeconomic group (age 15-70 years). There were three study groups.

1. Hepatitis C patients

These patients cannot be clinically differentiated from other hepatitis patients. Therefore, we chose clinical tests, fourth generation ELISA and HCV, PCR as our criteria for identification for Hepatitis C patients. All patients included in these studies were positive for both parameters. ALT levels were not considered a criterion because these are confusing especially in patients taking therapy[4].

HCV patients were further divided into two subgroups:

a. With a history of known causes of HCV spread during last one year. It included blood transfusion, multiple use of syringes for injections, and hemodialysis. There were no intravenous drug users.

b. Without a history of foreign blood contact in last one year.

2. Clinically diagnosed cases for typhoid referred for PCR

Patients with fever for 3-20 days and having most of the following symptoms: enlarged spleen, headache, rose spots, malaise, abdominal discomfort, lethargy, constipation followed by diarrhea, fatigue, delirium and agitation.

3. Healthy controls

These were normal persons with no history of fever for last one year.

Bacterial strains

Two pure strains of Salmonella typhi and STK1 and STK2 provided by department of Microbiology, University of Karachi, were used for standardization of PCR conditions for typhoid according to the recommendations of song[18].

Sample collection

Five ml of blood in EDTA were collected for all study groups. Three ml of blood was centrifuged at 5000 rpm for 5 minutes within five hours (storage at 4°C) of collection. Plasma was separated for HCV ELISA, HCV PCR and Wide test. Cellular portion was processed for Typhoid PCR and blood culture.

ELISA Test for the Detection of Antibodies to HCV in Human Plasma

For the detection of HCV antibodies, ELISA Kit (Biokit, Spain) was used as per instructions of the manufactures.

Widal Test (Slide Method)

Widal test was performed by tube method according to manufacturer's instructions (Bio-systems, Spain).

Blood Culture

Two ml of blood was inoculated into a culture bottle containing 16 ml of trypticase soy broth (Difco) with 0.02% SPS (sodium polynethanol sulfonate) and incubated at 37°C for 72 hours. From both culture, biochemical identification was done by conventional procedures. Confirmation was carried out by PCR.

DNA extraction from blood samples

DNA from blood samples was obtained by conventional method[17]. Briefly cellular part from one ml of blood was obtained after centrifugation at 10,000 rpm for 5 minutes. After removal of plasma, one ml of lysis buffer (0.2% Triton X 100 in 10 mM Tris-HCl pH 8.0) was added followed by gentle aspiration several times to effect

hemolysis. The tube was centrifuged at 12,000 rpm for 6 minutes and supernatant was removed carefully so as not to disturb the pellet. The step was repeated twice. For two further steps, lysis buffer was replaced by distilled water. Finally, the pellet was suspended in 20-30 µl of distilled water. The tube was sealed and kept in boiling water for 20 minutes.

DNA Extraction from Bacterial Culture

DNA extraction was done by conventional phenol-chloroform method[17]. Extracted DNA was used for optimizing PCR conditions[18].

PCR for Typhoid

PCR for typhoid was carried out by targeting of fliC gene. This is the most specific and reliable method for detection of Salmonella typhi[17,18,19]. Primers were synthesized on pharmacia LKB, Gene Assembler special. Oligonucleotides ST1 and ST2 were used for regular PCR. For nested PCR, oligonucleotides ST3 and ST4 were used (Table I).

Table I. Nucleotide sequence of primers used for targeting of fliC gene of Salmonella typhi

Primer name	Sequence (5' – 3')	Size (amplicon)	Location
Regular PCR			
ST1	TATGCCGCTACATATGATGAG		1036-1056
ST2	TTAACGCAGTAAAGAGAG	495bp	1530-1513
Nested PCR			
ST3	ACTGCTAAAACCACTACT		1072-1089
ST4	TGGAGACTTGGGTCGCGCGTAG	363bp	1435-1416

Table II. Nucleotide sequence of primers used for targeting of 5' NCR region of HCV

Primer name	Sequence (5' – 3')	Size (amplicon)	Location
Antisense primer			
For cDNA			
AS1	GTCGACGGTCTACGAGACCT		1-21
Regular PCR			
S1	GCCATGGCGTTAGTATGAGT		260-241
AS1	GTGCACGGTCTACGAGACCT	260bp	1-21
Nested PCR			
S2	GTGCAGCCTCCAGGACCC		237-220
AS2	CCGTGAGCGTTCGTGGGATA	210bp	27-46

Conditions for PCR amplification were as follows: For regular PCR, 100 µl of DNA amplification mixture contained 10mM Tris HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 150 pmol of each primer, 95 nmol of each dNTP, 1 µl of Taq polymerase, 20 µl of DNA mixture and distilled water to make the volume.

The thermal cycler (Mastercycler, Eppendrof) conditions were 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute each followed by 7 min at 72°C. For nested PCR, conditions were similar except that a 1:5 dilution of amplified product and different set of primers (ST3, SR4) were used. A positive control representing 5 bacteria/ml of Salmonella typhi[17], and negative control without any DNA was also included in each lot.

cDNA Preparation for HCV PCR

RNA was extracted from plasma according to Petrelli et al[20]. cDNA was synthesized from all RNA extracted in previous step, by using reverse transcriptase enzyme following the protocol of Tisminetzky et al[21] with some modification. Briefly, total reaction volume (20µl) contained 10 µl of RNA sample, 1 x first strand buffer (5 x stock: 250mM) Tris HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$, 50mM DDT, 10mM dNTPs, 5.0 pmol of antisense primer ASI (Table II), 10U of ribonuclease inhibitor (10U/ul), 50U of Monoly murine leukemia virus (M-NLV) reverse transcriptase (RT) enzyme and 5mmol DTT. The reaction of mixture was incubated at 42°C for 1 hour in water bath. After incubation, the samples were stored at-20^0C till PCR amplification.

PCR for HCV

For regular PCR, sense primer S1 and anti-sense primer AS1 were used. For nested PCR, two internal primers, sense S2 and anti-sense AS2 were employed[21]. Sequences and other information are given in Table II.

Regular PCR was run with total reaction volume of 50µl containing 10 µl synthesized sample cDNA at template DNA and with final concentration of each, 1mmol dNTPs; 1 x PCR buffer (10 x stock PCR buffer: 10mM Tris HCl pH 8.8, 160mM (NH) SO, 0.1% Tween, 0.075mM EGTA, 20mM $MgCl_2$), 5U of Taq polymerase and 50 pmol each of both specific primers. The reaction was adjusted with sterile distilled water.

For PCR amplification, first cDNA reaction mixture was denatured for 1 minute at 94°C for the first cycle only, followed by 30 cycles each of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds. For nested PCR, 1 µl of regular PCR product was used as template. Others conditions were the same as in case regular PCR.

RESULTS

PCR for typhoid

The targeting of **flic** gene of Salmonella typhi for PCR is a very specific and the most sensitive method for diagnosis of typhoid[16,17,18]. Our results reflect these qualities. In the control group of 50 healthy persons, only one (2%) gave a positive PCR reaction. On other hand, in clinically diagnosed cases of typhoid the figure was as high as 63.3% in 30 cases. In HCV patients, the figure for positivity of typhoid PCR was 9.5% in 105 cases. The figure was significantly higher (12.5% in 40 cases) in persons with no history of known causes of HCV spread as compared with those with an exposure to known causes of HCV spread (7.7% in 65 cases). The results are summarized in Table III.

Blood culture for typhoid

Blood culture is the most specific test for detecting Salmonella typhi but it suffers from poor sensitivity. None of the control group was positive for blood culture as expected. In clinically diagnosed typhoid patients, 33.3% (10 out of 30 cases) were blood culture positive. Success I cultivating Salmonella typhi from blood of as many as 4.7% (5 out of 105 cases).

HCV patients conclusively shows a co-infection with typhoid bacillus. And these figures were higher in HCV patients without exposure to known causes of transmission, i-e, 7.5% as compared with 3.1% (Table III).

Widal test

Widal test is superceded by PCR and blood culture in terms of sensitivity and specificity, but being a serological test, it remains a very useful semi quantitative indicator for presence of antibodies against flagellar (H) and somatic (O) antigens of Salmonella typhi providing a clue to a past exposure. Its non specificity was evident by the

fact that it was positive (a titre of more than 160 for O antigen) in 24% (12 out of 50) healthy controls. In typhoid fever the patients was more than double as expected, i.e, 56.6% (17 out of 30 cases).

The figure for HCV patients were much higher as compared with healthy individuals, i.e, 34.2% (36 out of 105 cases). Again, within subgroups of HCV patients, those with out a history of exposure to known cases of spread had slightly higher figures, i.e, 35.0% as compared with 33.8% (Table III), although this difference is not statistically significant.

Among the major medical challenges of modern era. Hepatitis C is the one of the most formidable, especially in developing countries. This is because of sheer magnitude of its occurrence and stealthy manner of spread. It has been called the "Silent Killer" because in most causes, the disease is detected at an advanced stage when prognosis is poor.

There are several known causes of spread of this disease, mainly contact with blood and cross contamination with blood products. However, after considering all the known sources of infection, 44% of HCV victims remain unaccounted for[13], suggesting that there is some major link that has not been identified as yet.

Table III. Positivity of typhoid parameters in different groups of study

	PCR (%)	Blood culture (%)	Widal test (%)
Hepatitis C patients with history of blood contact in last two years (n=65)	7.7 (n=5)	3.1 (n=2)	33.8 (n=22)
Hepatitis C patients without history of blood contact in last two years (n=40)	12.5 (n=5)	7.5 (n=3)	35.0(n=14)
Clinically diagnosed cases of typhoid (n=30)	63.3 (n=19)	33.3 (n=10)	56.6 (n=17)
Healthy individuals with no history of fever in last one year (n=50)	2.0 (n=1)	0.0	24 (n=12)

Pollution is a strong candidate for this dubious identification. This hypothesis is supported by the fact that HCV is more common in developing countries, especially the areas with poor hygienic conditions and socioeconomic status. But, there has been no scientific evidence to prove it. However, a recent report has indicated that sewage workers are at greater risk of acquiring Hepatitis C[14].

It is impossible to obtain direct evidence because Hepatitis C virus cannot be isolated from polluted water of food. So, the only option is to find some indirect evidence that can relate it with pollution.

Typhoid is a proven water and food pollution related disease and the causative agent, Salmonella typhi gains access to the body via contamination from fecal material[15].

Both typhoid and HCV are very common in countries with high level of water and food pollution. We hypothesized that if we could find significant co-occurrence of these two diseases in an area where water and food pollution is prevalent, it would be a strong indicator that there was a common source. And as typhoid is a proven pollution related disease, it would strongly suggest relation of HCV with pollution as well.

We planned an extensive (positive control group) and healthy persons (negative control group), and to test them for typhoid parameters. The typhoid parameters we used were PCR (based on targeting of flagellant gene), blood culture and widal test.

One hundred and five (105) HCV patients, positive by PCR and fourth generation ELISA test were included in this study. There were further subdivided into two groups: with and without history of known causes of HCV spread during last one year. Two other groups, one of suspected typhoid patients and other of healthy persons were also included for comparison.

PCR targeting fliC gene was selected because it has proved to be the most sensitive and highly specific technique for diagnosis of typhoid [16,17,18]. High specific of PCR for typhoid was highlighted by the fact only one of the 50 healthy controls tested positive (Table III). On the other hand, as expected, 63.3% (19/30) clinically diagnosed cases of typhoid were PCR positive. However the most significant finding that was 9.5% (10/105) cases of Hepatitis C were positive for typhoid PCR. When comparison is made with healthy individuals, this result is statistically significant ($P<0.05$). When we split the results for HCV patients with ans without history of known causes of transmission, the figure in later group is significantly higher (12.5% as compared with 7.7%).

Blood culture is the most specific tool for diagnosis of typhoid, but unfortunately, its sensitivity is low due to various reasons including very low number of bacteria and antibacterial factors in blood. However, a positive blood culture represents a definitive diagnosis of typhoid. As expected, none of healthy control group tested positive and 33.3% (10 out of 30) of clinically diagnosed typhoid patients were blood culture positive. The isolation of Salmonella typhi from the blood of (4.7%) out of 105 cases proved co-existence of both diseases. The figures differed significantly in two groups of HCV patients, as blood culture positivity was more than double in the group without a history of known causes (7.5% as compared with 3.1%).

Widal test was used because it reveals the presence of antibodies against flagellar (H) and somatic (O) antigens of Salmonella typhi and can therefore point towards an infection in the recent pass that has been eliminated. We preferred widal test over Typhidot, because some recent studies[16] have shown that Typhidot has no significant advantage and is much more costly. In fact, both of these tests suffer from lack of specificity.

As shown in Table III, the figure of widal positivity for Hepatitis C patients was 34.2% (36/105). As is the pattern with other parameters, the figure is higher for the group without exposure to known causes of spread of HCV (35.0% and 33.8%). In comparison the percentages for clinically diagnosed cases of typhoid and healthy controls were 56.6 (17/30) and 24.0 (12/50) respectively. The figure for Hepatitis C patients was much closer to that of suspected cases of typhoid as compared for healthy individuals. Although widal test, due to its inherent non-specificity, could not provide statistically significant differences, it did show greater prevalence of typhoid antibodies in Hepatitis C patients which in turn means greater occurrence of co-infection.

These positive results of typhoid PCR and blood culture in Hepatitis C patients are statistically significant ($P<0.05$) when comparison is made with healthy controls. Results of widal test were also suggestive of co-infection. It is not surprising because fever and abdominal discomfort are common complaints in chronic Hepatitis C patients, so even if it is due to co-existence typhoid infection, it may easily overlooked.

We concluded that higher incidence of typhoid in Hepatitis C patients in communities with poor hygienic conditions is significant and provides a strong evidence for a link of Hepatitis C with water and food pollution, and this fact must be given importance for devising strategies for control of this dreaded disease.

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