Original Article

Multiple infections with hepatitis A virus and development of rheumatoid arthritis among waste water treatment plant workers

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Abstract

Introduction: In the present work, we studied the association between multiple exposure of waste water treatment plant workers to infection with existing hepatitis A virus in waste water and development of rheumatoid arthritis, taking in consideration number of working years as an indicator for frequency of exposure to infection, compared to non waste water treatment plant workers.
Methodology: A total of 105 waste water treatment plant workers and 48 NWWTPWs were included in the study. Exclusion criteria were positivity for HBV and/or HCV IgG, negativity to HAV IgG and suffering from rheumatic diseases other than rheumatoid arthritis.
Results: 96.2% of waste water treatment plant workers were anti-HAV-IgG positive, of whom 5 had high antibody titer indicating ongoing infection and were anti-HAV-IgM negative excluding primary infection. These 5 samples were further subjected to quantification of liver enzymes, glutamate oxaloacetate trasaminase and glutamate pyruvate transaminase and HAV-RT-PCR to check viremia and results showed increase of glutamate oxaloacetate trasaminase and glutamate pyruvate transaminase as well as viremea in all of them. Rheumatoid arthritis diagnosis was carried out by detection of C-reactive protein, rheumatoid factor and anti-cyclic citrullinated protein. Rheumatoid arthritis development was 19% in the waste water treatment plant workers with >10 working years and 8% for < 10 working years. Also, disease development started earlier (Age 30-40 years) among the waste water treatment plant workers compared to non waste water treatment plant workers (age: 40-50 years).
Conclusions: Multiple exposures of waste water treatment plant workers to HAV might be one of the etiological stimuli of rheumatoid arthritis.

Key words: Waste water treatment plant workers; HAV infection; IgM; IgG; RT-PCR; rheumatoid arthritis.

Introduction

Wastewater treatment plants workers (WWTPWs) are subject to successive infections with various pathogens (viruses, bacteria and parasites) present in waste water [1,2]. In Egypt, hepatitis A virus (HAV) is commonly found in waste water [3] with prevalence rate of 63 % [4] and it is known to remain infectious up to three months at room temperature [5]. Worldwide, several studies showed that HAV infection rates among WWTPWs is significantly higher than non endemic population. [6-8].

Reports showed association between HAV infection and various extra-hepatic diseases as arthritis [9,10], allergy [11] and urticaria [12]. Appearance of extra-hepatic diseases might be due to the important role of the liver in regulating immune response and formation of immune complexes during infection. Those immune complexes are believed to play a role in diseases development, especially arthritis [13]. Also, in patients developing arthritis; deficiency in the liver enzyme cysteine dioxygenase occurs that results in decrease in production of, proteoglycans, heavily glycosylated proteins that combine with the collagen

forming the cartilage [14] and this causes erosive disease and persistence of joint symptoms. This indicates a possible link between changes in the liver metabolism and development of arthritis [13].

Abnormal liver tests were found in 18-50 % rheumatoid arthritis (RA) [15]. RA is an autoimmune disease causing chronic inflammatory polyarthritis that progressively destroys synovial joints and can cause systemic complications. It affects about 1% of the world's population [16].

It is hypothesized that viral infections triggers RA development [3,17]. Virus might act as an adjuvant in autoimmunity development by stimulating innate immune responses non-specifically including mast cells, dendritic cells, toll-like receptors and complement receptors [18]. Thus, theoretically there might be an association between exposure to, or infection by, HAV and development of RA.

Here we checked the prevalence of RA among different age groups of WWTPWs who are known to be under frequent exposure to HAV infection during their working years in comparison to non-endemic control individuals from the same age groups. Obtained results suggest that multiple exposures of WWTPWs to HAV might be one of the etiological factors triggering development of RA.

Methodology

Questionnaire

It contains all symptoms of HAV infection sample (during collection and in the past) and RA development considering number of working years for WWTPWs to reflect frequency of contact with contaminated water. Note: Vaccination status was included in the questionnaire, all workers were vaccinated against HBV but none was vaccinated against HAV.

Clinical examination

All participants were subjected to thorough clinical examination including musculoskeletal assessment.

Human samples

A total of 105 blood samples were collected from workers in three WWTPs namely Balas (52 samples), Arab Abou Saiid (37 samples) and El-Berka station (16 samples). 48 samples were collected from healthy persons as control, they are people going to clinics of National research center and work completely away from WWTPs, we call them non waste water treatment plant workers (NWWTPW). Exclusion criteria were infection with hepatitis B or C viruses and presence of autoimmune rheumatologic disease other than RA.

Blood collection was performed in compliance with relevant laws and institutional guidelines in accordance with the ethical standards of the Declaration of Helsinki and after taking the approval of the Ethical Committee of the National Research Centre. Serum was stored was separated from both groups in the same day of sample collection and store at -80 °C till use.

Sample classification and detection of HAV infection.

All sera were screened for hepatitis C antibodies and hepatitis B surface antigen using rapid detection test (Acon,California, USA).

Qualitative detection of serum anti-HAV-IgM detection was carried out by rapid test (ACRO BIOTECH, California, USA) according to the manufacturer instructions. Quantitative detection of serum anti-HAV-IgG was carried out by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions (Precheck, USA), In it, the ELISA plates are coated with HAV purified antigens, In case the tested sample contained IgG, it will compete with the monoclonal HAV-IgG antibodies labeled with horse radish peroxidase (HRP-conjugate) not allowing it to bind with the antigen and so no color would be produced but when the tested sample had no HAV-IgG, the HRP labeled IgG antibodies will bind to the coated antigens producing the color. Positive control reading was 0.018 and its cut off was 0.5

Serum samples with high anti-HAV-IgG values were further subjected for quantitative determination of both aspartate aminotransferase/glutamate oxaloacetate trasaminase, (AST\GOT) and alanine aminotransferase/glutamate pyruvate transaminase, (ALT\GPT) according to the manufacture instructions (Diamond Diagnostics, Cairo, Egypt), samples giving > 12U/L is considered positive.

Sera of high anti-HAV-IgG values were subjected to viral RNA extraction (QIAGEN, Hilden, Germany), reverse transcription (super script III Reverse transcriptase, Invitrogen, USA) then PCR (QIAGEN, Hilden, Germany) using forward primer: 5'-CCTCTGGGTCTCCTTGTACA-3', reverse primer: 5'-GAAACTGGTTTCAGCTGAGG-3'. Reaction mixture was subjected to the following temperature conditions, 50 °C for 30 seconds, 94 °C for 15 seconds., denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute., extention at 68 °C for 2 minutes., for 40 cycles followed by extension at 68 °C for 7 minutes. Positive HAV stool sample was used as control with expected molecular weight 278 bp [19].

Diagnosis of RA

According to the classification criteria of the American College of Rheumatology (ACR) 1987 [20], three tests were made: C reactive protein (CRP), Rheumatoid factor (RF) and Antibodies to CCP sera from both the WWTPWs and the non-endemic controls were measured.

According to kits, For CRP and RF (Minineph, Bimingham, UK) samples showing higher values than 3.5 and 30.92 unit/mL respectively were considered positive and for CCP (Orgentec diagnostika, Mienz, Germany) samples giving more than 20 unit/mL were considered positive.

Statistical analysis

Statistical analysis and plots were done using the GraphPad PRISM version 5 software. Results were expressed as medians ± standard deviations (SD). Statistical significance was calculated by comparing the differences between medians of different studied groups using the Student's t-test. Differences were considered significant when the *p*-value was < 0.05.

Results

Exclusion of samples

Among the 105 WWTPW: 16 HCV, 1 HBV positive and 4 anti HAV IgG negative samples and among the 48 non-WWTPW 8 HCV, 2 HBV positive and 3 anti-HAV IgG negative samples were recorded and excluded from the study.

Demographic characteristics of WWTPWs and NWWTPW group

For WWTPW: 84 samples were included in the study for males with age (Mean ± SD) equals 44.71 ± 1.053 and number of working years (mean ± SD) equals 18.9 ±1.02.

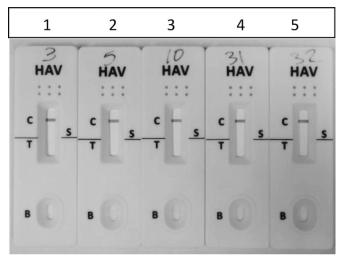For NWWTPW: 35 samples were included in the study for males with age (Mean ± SD) equals 42.14 ± 2.572.

Detection of anti-HAV-antibodies

Of the 84 IgG positive WWTPWs, 5 individuals had high IgG titer compared to positive control of ELISA kit as well as other samples under test and were classified as highly positive. Their questionnaire showed no HAV infection symptoms during the sample collection (Table 1). Sera of these 5 individuals were tested for presence of anti-HAV-IgM and the obtained results revealed that they were all uniformly negative (Figure 1) which indicates that the high IgG recorded in those 5 individuals was not due to first infection with HAV.
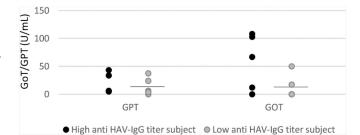
Figure 1. Detection of anti- HAV IgM in the 5 highly positive anti-HAV-IgG individuals showing that that they were all uniformly negative which indicates that the remarkably high IgG recorded in those 5 individuals was due to secondary infection due to absence of primary immune response.
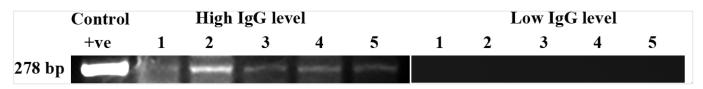

Table 1. HAV symptoms in people with high HAV IgG values.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	46	45	55	52	59
Working years	25	25	25	27	27
HAV infection symptoms					
Fatigue	-	-	-	√	√
Nausea and vomting	-	-	-	-	-
Clayed colored bowel movement	-	-	-	-	-
Abdominal pain	√	√	-	√	√
Loss of appetite	-	-	-	-	-
Low grade fever	-	-	-	-	-
Dark urine	-	-	-	-	-
Joint pain	√	-	√	-	√
Yellowing of skin and eye (Jaundice)	-	-	-	-	-
Diarrhea	-	-	-	-	-
Itching	√	√	-	-	-
Skin rash	-	-	-	-	-

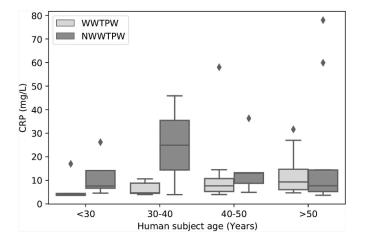(√): Presence of symptom; (-): absence of symptom.

Figure 2. Comparing levels of GOT and GPT liver enzymes between the 5 WWTPWs which had remarkably high anti-HAV-IgG with 5 other WWTPWs who showed lower anti-HAV-IgG levels (Nearly equal the cut-off of the kit), showed that levels of both the GOT and GPT were higher among those who had high anti-HAV-IgG titers. This suggested active liver involvement.
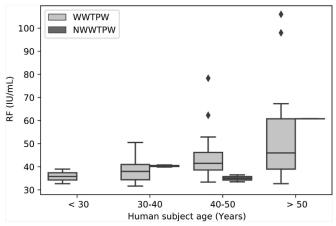
Figure 3. Purified RNA samples from the sera of the 5 WWTPWs who had high anti-HAV-IgG and 5 WWTPW who had low anti-HAV-IgG were checked for active viremia by RT-PCR with including RNA from HAV positive stool sample as positive control and results revealed amplification products at the expected molecular weight of 278 bp corresponding to the target HAV sequence in the RNA of the five samples with differential intensities that showed up very sharp in the RNA of the HAV positive control stool sample (lane 1), No bands were observed in samples with low anti-HAV-IgG.

Figure 4. Kinetics of the CRP levels in both the WWTPWs and NWWTPW group of various age groups showed that CRP levels did not significantly differ between the two groups, and the same was shown at the different age ranges.

Figure 5. Kinetics of the RF levels in both the WWTPWs and NWWTPW group of various age groups. Showed appearance of high RF readings in the WWTPWs starting from age 30-50. On the other hand, the highest RF levels within the NWWTPW group were recorded in the age group higher than 50 years and number of samples having high RF levels among the WWTPWs was significantly higher (P=0.04) than the NWWTPW controls.

Association between increased GOT/GPT levels and high serum anti-HAV-IgG

Liver enzymes of the 5 WWTPWs with high anti-HAV-IgG were compared to 5 other WWTPWs having low anti-HAV-IgG levels (Nearly equal the cut-off of the kit), results showed that levels of both the GOT and GPT were higher among those who had high anti-HAV-IgG titers (*p*-value equals 0.70 which was non-significant) (Figure 2). This suggested active liver involvement in high IgG samples.

Polymerase chain reaction (RT-PCR)

Active viremia in the serum of 5 WWTPWs having high anti-HAV-IgG was checked by RT-PCR. Results (Figure 3) showed appearance of the expected band with different intensities in the five samples.

CRP, RF and CCP levels among both the WWTPW and the NWWTPW group

CRP, did not significantly differ between the WWTPWs and the NWWTPW groups, and the same was shown at the different age ranges (Figure 4).RF results showed appearance of high RF readings in the WWTPWs starting from age 30-50 (Figure 5). On the other hand, the highest RF levels within the NWWTPW group were recorded in the age group higher than 50 years (Figure 5) and number of samples having high RF levels among the WWTPWs was significantly higher (*p* = 0.04) than the NWWTPW controls.

CCP results showed negative CCP in individuals younger than 40 years as its delectability started in individuals ranging in age 40-50 years old and increased by aging in both the WWTPWs and the

NWWTPW groups with recording higher values among the WWTPWs (Figure 6).

Figure 6. Kinetics of the RF levels in both the WWTPWs and NWWTPW group of various age groups showed negative CCP in individuals younger than 40 years as its delectability started in individuals ranging in age 40-50 year old and increased by aging in both the WWTPWs and the NWWTPW groups with recording higher values among the WWTPWs. Note: CCP data cannot be plotted as it was positive in 8/84 of WWTPW and in only 1/35 in NWWTPW, the values can be communicated written rather than plotted as all data relates to the above 40's group in both workers and non-workers. Note:one sample of value 1000 U/mL was removed from WWTPW age group 40-50 to allow data of other samples to appear better.
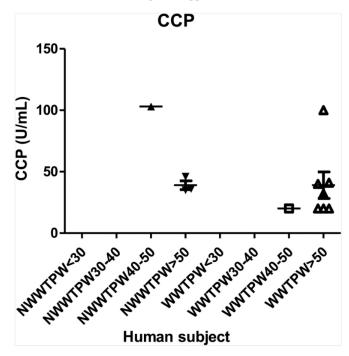

Table 2. Association between appearance of RA symptoms and levels of the CRP, RF and CCP among the confirmed WWTPW-RA patients.

Patients Data			Symptoms of rheumatoid arthritis disease								Tests for Rheumatoid arthritis diagnosis		
Patient	Age	Working years	Fatigue.	Joint tenderness	Joint redness	Joint stiffness	Joint pain	Joint swelling	Joint warmth	Loss of joint range of motion	CRP	RF	CCP
1	47	27	√	√	-	√	√	-	-	√	N*	78.3	N
2	52	26	√	√	√	√	√	-	-	-	4.9	106	N
3	48	26	√	√	√	√	√	√	√	√	5.2	286	< 1,000
4	39	11	√	√	√	-	√	√	√	√	4.5	N	N
5	34	7	√	√	-	√	√	-	√	√	N	N	N
6	46	9	√	√	-	-	√	√	√	-	58.2	N	20
7	55	24	√	√	-	√	√	-	√	-	4.84	51.7	N
8	47	23	√	√	√	-	√	√	√	-	10.8	60.8	40
9	59	41	-	-	-	-	-	-	-	-	11.3	39.4	100
10	57	33	√	√	-	-	√	√	√	-	N	65.3	N
11	52	25	√	√	√	√	√	-	√	-	31.6	47.2	N
12	46	23	-	√	-	-	√	-	-	-	11	288	N
13	47	25	√	√	-	√	√	√	√	-	N	62.3	N

N: Normal; (√): Presence of symptom; (-): absence of symptom. CRP: C reactive protein; RF: Rheumatoid factor; CCP: Cyclic citrullinated peptide) antibodies.
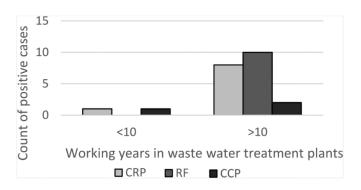
Levels of CRP, RF and CCP among the WWTPWs in relation to number of working years

Results in Figure 7 showed that the number of workers having positive CRP, RF and CCP levels increased with increasing number of working years (comparing those who spend more than 10 to less than 10 working years). Correlating both clinical investigations with results of the three tests, one can see a direct association between well identified RA patients and the number of working years. The association between appearance of RA symptoms and levels of the CRP, RF and CCP among the confirmed WWTP RA patients is presented in Table 2.

Comparing percentage of well identified RA patients among the WWTPWs and NWWTPW groups with respect to age

Results in Figure 8 showed appearance of the disease in younger age groups (30-40) years in case of WWTPWs and its complete absence in the same age range in NWWTPW group. However, the disease was recorded in the age group between 40- 50 in both the WWTPWs and the NWWTPW controls but with higher prevalence among the WWTPWs. Results of both age groups indicates presence of additional factor(s) other than aging that plays role in the disease development. At age over 50 years higher prevalence of RA was recorded within the NWWTPW group than in the WWTPWs. It can be concluded that aging might be the main reason for RA development in the NWWTPW controls but additional etiological factor(s) might play a role in the disease onset in the WWTPWs.

Discussion

HAV spreads among WWTPWs with higher rates than normal population [6-8] Joints symptoms are considered good extra-hepatic markers for hepatic diseases particularly viral hepatitis [21].

HAV infection induces life-long-lasting blood circulating IgG [22], which protects humans from developing symptoms upon re-infection [23]. It is known that IgG can only neutralize circulating viral particles in the blood [24] but has little impact on intrahepatic viral load [25]. This indicates that HAV must reach blood at detectable viral load to be neutralized by IgG. So upon each new infection with HAV, virus must enter the liver via portal circulation [26] where replication occurs leading to viral shedding into blood that induced production of anti-HAV-IgG which needs two weeks to reach its peak and become enough to neutralize the virus [27]. All this happens before appearance of symptoms that require 4-6 weeks post infection [28], and thus usually the reinfected person does not develop symptomatic infection.

Although it is thought that this memory stops disease development, the necessary two weeks duration to develop enough immunity to neutralize newly invading HAV are long enough to allow many viral

Figure 8. Percentage of well identified RA patients amongst the WWTPWs and NWWTPW group with respect to age showed appearance of the disease in younger age groups (30-40) years in case of WWTPWs and its complete absence in the same age range in the NWWTPW group. However, the disease was recorded in the age group between 40- 50 in both the WWTPWs and the NWWTPW group but with higher prevalence among the WWTPWs. Results of both age groups indicates presence of additional factor(s) other than aging that plays role in the disease development. At age over 50 years higher prevalence of RA was recorded within the NWWTPW group than in the WWTPWs. It can be concluded that aging might be the main reason for RA development in the NWWTPW controls but additional etiological factor(s) might play a role in the disease onset in the WWTPWs.

Figure 7. CRP, RF, CCP levels among confirmed RA patients in WWTPWs considering number of working years showed that the number of workers having positive CRP, RF and CCP levels increased with increasing number of working years (comparing those who spend more than 10 to less than 10 working years)
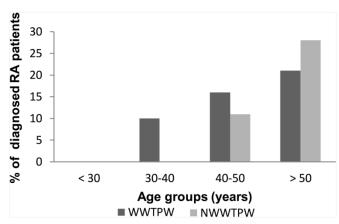
replication cycles, bearing in mind that the virus needs only eight hours for each replication cycle, which eventually lead to further damage of liver cells.

Detection of anti-HAV IgG by ELISA results showed that 5 WWTPWs had high IgG titers compared to the rest of the study subjects and their reactivity ranged from nearly around the maximum limit of the positive control provided with the detection kit. Although their high IgG titers, those 5 individuals did not have any HAV onset symptoms during sample collection and they behaved normally, reflecting that this particularly high IgG titers (2ry immune response) is not due to an old infection but due to recent re-infection [27].

To confirm that those 5 individuals experienced re-infection and are not just having long-lasting immunity due to past infection, they were further checked for serum anti-HAV-IgM which reports on primary immune response to infection and were found uniformly negative. Since it is well known that IgM is produced only in response to first infection and lasts in blood for 4-7 months after infection [29], both the absolute negativity of those 5 samples for IgM and their remarkably high IgG levels confirm their subsequent re-infection. Importantly, the 5 highly IgG-positive workers were from the same WWTP (Arab AbouSaiid) which may suggest 1) an increase in the HAV load in the waste water from this station around the time of blood collection, and 2) history of frequent contact of these particular 5 individuals to contaminated water with the virus. In concordance, it was reported that 14 % of HAV infected patients do not develop symptoms [30].

Results also showed that same 5 individuals showed increase in the liver enzymes and were all HAV-RNA positive in RT-PCR which confirms viremia. Although those patients were asymptomatic, the association between the remarkable elevation of their serum anti-HAV-IgG, increase in their liver enzymes and possessing circulating viral RNA proves liver involvement in every time a person is exposed to a new HAV infection and not only the initial infection which was always thought to be self-limiting.

Of note, the appearance of faint PCR products in the sera of those 5 patients is more likely due to the presence of high neutralizing IgG titers which is able to clear viremia by time. It is reported that viral clearance from serum starts at week 3 post infection and complete clearance is achieved by weeks 6-8 [31]. All together indicates that at each time a person receives HAV infection (even re-infection) the virus enters liver cells,

replicates leading to damage of infected cells and new virions are shed to blood leading to viremia.

To confirm RA diagnosis, in addition to clinical investigations, sera were tested for CRP, RF and CCP [20,32].Our results showed that the CRP, which is a non-specific inflammation marker [33] but reported to be high in 50-70% of RA patients, levels did not show remarkable difference between WWTPWs and NWWTPW individuals.

RF, which is known to be positive in 60-70 % of patients developing RA [34], was found to be high among the WWTPWs starting from age less than 30 with gradual increase with aging. On the contrary, the NWWTPW group showed high RF in about 50% of the individuals ranging in age between 30-40 years then a sharp decrease in its level was observed with progress in age. This might be due to that RF is not only high in case of RA patients but also in patients having other connective tissue diseases (e.g. Systemic lupus erythematosus and Sjögren's syndrome), some infectious diseases (e.g. Infectious hepatitis, syphilis, infectious mononucleosis, parasites and tuberculosis), liver disease and sarcoidosis. Also, RF can sometimes be present in healthy individuals without diseases specially those having family history for RA development [35].

In fact both the poor hygienic status of WWTPWs and the nature of their occupation make them subject for exposure to many other bacterial or parasitic infections that might explain the gradual increase in the measured RF with progress in age which was not the case within the NWWTPW group.

Anti-CCP is very useful for RA diagnosis in high-risk groups and is rarely found in individuals without RA [36]. On the other hands anti-CCP stared to be detectable starting from the age of 40.

Percentages of highly positive CRP, RF and CCP and correlating them with number of working years showed increase in the prevalence of the three markers in workers with more than10 years working experience than those having shorter work durations.

Correlating results of the CRP, RF and CCP with the clinical manifestations allowed appropriate (confirmed) diagnosis of RA patients and was directly proportional to the number of working years of the WWTPWs.

Among the WWTPWs, RA development started at age between 30-40 years which was not the case among the NWWTPW control individuals. Also, between age of 40- 50 years incidence of RA was higher among the WWTPWs compared to the NWWTPW control group. On the contrary, at age < 50 years incidence of the

disease becomes higher in the NWWTW group and if we attribute this to aging, there should be an additional etiological factor affecting the WWTPWs leading to RA development at a younger age.

Conclusions

Based on the relation between RA development and liver injury, complete exclusion of HCV and HBV infections from the WWTPWs can allow the conclusion that their continuous exposure to HAV due to their frequent contact with contaminated water might be among the major etiological factors triggering development of RA due their continuous disturbed liver metabolism. This is also, confirmed by appearance of the disease in young age among WWTPWs compared to NWWTPW individuals, and the positive correlation between RA development and number of working years.

Acknowledgements

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Conflict of interests: No conflict of interests is declared.