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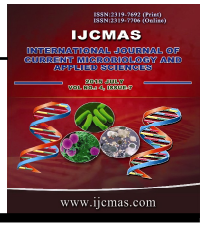

Original Research Article

Sero prevalence of *Helicobacter pylori* infection in patients of tuberculosis: Analysis by ELISA, western blot and indirect immune fluorescence assay

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Keywords

Helicobacter pylori, TB, ELISA, Western blot and IIFA

A B S T R A C T

To determine the authenticity of three serotechniques viz, ELISA, western blot and IIFA to establish the connection between *H. pylori* infection and tuberculosis. Samples were collected from Andhra Pradesh State Chest Diseases and Tuberculosis Centre. Immunological studies were carried out at Department of Stem Cell Transplant Biology, Global Hospital, Hyderabad, India. Studies were conducted between January and June 2009. Serum samples collected from tuberculosis patients and healthy control subjects were analyzed by three sero-based techniques viz, enzyme linked immune sorbent assay (ELISA), western blot and indirect immune fluorescence assay (IIFA). ELISA and western blot results revealed no connection between the *H. pylori* infection and tuberculosis. Antibody pattern also did not support the connection between the *H. pylori* and tuberculosis. However, IIFA tests revealed a positive relation between the *H. pylori* infection and tuberculosis. The results of the present investigations reveal an association of *H. pylori* infection in tuberculosis patients. IIFA is a reliable test and hence it is recommended. The results of the present investigations as revealed by enzyme linked immune sorbent assay and western blot test are showing poor association of *H. pylori* infection in tuberculosis patients. However, indirect immune fluorescence assay (IIFA) investigations revealed an association of *H. pylori* in tuberculosis patients. Hence IIFA is a reliable test and recommended for routine diagnosis.

Introduction

Helicobacter pylori is a gram - negative, oxidase positive, micro aerophilic, flagellate, curved or spiral bacterium that colonizes the mucous layer of the human gastric epithelium (1, 2). Approximately, worldwide half of the population has *H. pylori* infection and the prevalence is thought to be 80 – 90 % in developing countries (3). *H. pylori* infection now – a – days is recognized as a major predisposing

cause for peptic ulcer disease, peptic cancer, chronic active inflammation and a risk factor for gastric cancer. Recent studies on *H. pylori* have revolutionized our insights into the pathogenesis and treatment of peptic ulcer disease (4). It is casually related to chronic active gastrititis (5), primary low grade B-cell gastric lymphoma (6) and gastric carcinoma (7,8). Recently, a high *H. pylori* seroprevalence has also been observed in ischemic heart diseases (9), rosacea (10) and childhood growth retardation (11). An increased seroprevalence of *H. pylori* has also been reported in various extra gastrointestinal disorders including skin, vascular and autoimmune disorders as well as in some respiratory diseases such as bronchial asthma, chronic bronchitis, and lung cancer (12). The mean serum concentration of IgG antibodies against *H. pylori* was also significantly higher in rosacea patients than in control subjects (13). Although *H. pylori* and *Mycobacterium tuberculosis* infections share many risk factors, no studies have examined the relationship between *H. pylori* and *Mycobacterium tuberculosis* infection. A prior cross sectional study revealed an association between prevalence of *H. pylori* and having had clinical tuberculosis, suggesting the two infections may be related (14). In contrast, a case-control study found no difference in seroprevalence of *H. pylori* infection between the patients with and without clinical tuberculosis (15). However, this small study compared inpatient cases with outpatient controls. Furthermore, because clinical tuberculosis represents only a fraction of *Mycobacterium tuberculosis* infections, any true relationship between *H. pylori* and *Mycobacterium tuberculosis* relationship may have been obscured.

Therefore, the aim of this study was to investigate *H. pylori* seroprevalence in tuberculosis patients and control subjects and its relationship between *H. pylori* seropositivity and severity of tuberculosis.

Materials and Methods

Patients and sampling methods

The present investigations were carried out at the Andhra Pradesh (A.P) State Chest Diseases and Tuberculosis Centre, Hyderabad (India). Consecutive patients with diagnosed and confirmed tuberculosis disease attending the center were selected for the present study. The prospective study had the Institutional Ethical Committee (IEC) approval. A written consent was obtained from the selected patients. Control group included healthy subjects, well matched for age, sex, nutritional status and socioeconomic status from a health camp conducted specifically for this purpose. Socioeconomic status of the patients and control subjects was determined as per Kuppuswamy scale (16). None of the control subjects had a known history of tuberculosis or upper gastrointestinal tract pathology and any clinical manifested diseases. The subjects included in this study were tuberculosis (n= 58 including 38 males and 20 females) patients, and control (n= 42 including 21 males and 21 females). A question list included the demographic characteristics like gender, age, socio-economic status, and profession etc. was filled for each individual. Five ml of blood was collected from both diseased and healthy subjects for serological tests.

Serological tests

Enzyme linked immune sorbent assay: Seropositivity of *H. pylori* infection in tuberculosis subjects and control group was tested with commercially available anti-*H. pylori* IgG (EUROIMMUN Mediznische labordiagnostika, Lubeck, Germany) as per

the instructions of the manufacturers. The optical density (OD) of the resultant colour was calculated from the extinction value of the subjects sample over the extinction value of the calibrator 2. The ELISA recommended values more than 1.1 units were taken as positive; between 0.8 -1.1 units are treated as borderline positive and less than 0.8 units were treated as negative (17).

Western blot: Western blot was performed to detect the seropositivity of *H. pylori* infection in tuberculosis and control subjects. Anti-*H. pylori* IgG antibodies in the serum were detected using commercially available western blot strips (EUROIMMUN Mediznischel abordiagnostika, Lubeck, Germany) and according to the manufacturer's instructions. Western blot test strips consisted of antigen extracts with the following molecular weights: 120 kDa (CagA); 95 kDa (VacA); 67 kDa (flagellar sheath protein, nonspecific); 66 kDa (UreB); 57 kDa (heat-shock protein homolog); 33 kDa, 30 kDa, 29 kDa (UreA); 26 kDa, 19 kDa and 17 kDa. However, Anti-*H. pylori* IgG antibodies positivity was determined when the 120 kDa (CagA) band, as well as at least two distinct antigen bands from species-specific and highly specific antigens with the molecular weights of 95 kDa (VacA), 33 kDa, 30kDa,29 kDa (UreA), 26 kDa, 19 kDa and 17 kDa were present. Faint bands or no band were treated as negative (18).

Indirect immunofluorescence assay: This assay was conducted with Euroimmune Biochip slides coated with *Helicobacter pylori* bacterial smear tagged with fluorescent labeled anti-human IgG (goat)(which is designed exclusively for the *in vitro* determination of human antibodies in serum).The specific antibodies were labeled with a compound that makes them glow in an apple green color when observed microscopically under ultraviolet light. If the sample is positive, specific antibodies in the diluted serum sample attach to the antigens coupled to a solid phase and thus a distinct fluorescence of the bacteria covering the reaction areas becomes visible. The results are correlated with both positive and negative controls. Depending on the sample, the fluorescence pattern appears in parts circular or granular. In the case of a negative result, the cells show no fluorescence (19).

Statistical analysis

Results are expressed as mean and one standard deviation (SD). Significance of difference between groups was assessed by unpaired students't' tests for continuous variables and X^2 test for proportions. Correlation coefficients between variables were determined using conventional Pearsons correlation analysis. The statistical analysis was performed using the SPSS program (SPSS, Inc,IL,USA). P values less than 0.005 were considered statistically significant. In western blot test control and patients with 100% positive results, not found in negative results so for were not used for statistical analysis for the significant or not significant. Sensitivity and specificity of each method were calculated using the MedCale@version 12.7.7.0-64 bit software

Results and Discussion

ELISA

The results pertaining to *H.pylori* association with tuberculosis as revealed by ELISA tests are presented in table 1 and fig. 1. It is evident from the critical study of the table that out of 58 subjects suffering with tuberculosis, 56 (96.60%) have proved to be

H. pylori positive, whereas out of 42 control subjects 35 have proved to be positive (83.3%). Thus the results indicated no significant correlation (P<0.023) with regard to association of *H. pylori* infection in patients suffering with tuberculosis. Demographic –wise analysis results revealed that among males, out of 38, 36 (94.7%) patients have shown seropositivity, whereas in females there was 100% seropositivity for *H. pylori*. However, when compared with controls the correlation does not appear to be significant (P<0.093 and P<0.079). Tuberculosis versus control subjects within the age group of 20-40 years showed 97.3% vs 88.9% seropositivity with ELISA. However, in comparison with control there is no significant correlation (P<0.170). Overweight tuberculosis patients have shown 100% seropositivity for *H. pylori* infection. Similarly low income and high income groups people have also shown 100% seropositivity and thus in all the above cases there is no significant correlation. Thus, no definite correlation could be drawn between the relationship of tuberculosis and *H. pylori* infection.

Western blot

The results pertaining to investigations on *H. pylori* association with tuberculosis as revealed by western blot are presented in table 2 and fig. 1. A critical perusal of the table reveals that all the 23 subjects suffering with tuberculosis have proved to be *H. pylori* positive (100%). Similarly, all the 20 control subjects proved to be positive (100%). Thus, there is no significant relationship between the association of *H. pylori* infection and tuberculosis. Gender-wise analysis of western blot results revealed that out of 13 male patients with tuberculosis, 13 (100%) and out of 10 females all the 10 (100%) have proved to be positive for *H. pylori*, whereas out of 10 males control subjects 10 (100%) and out of 10 females all the 10 (100%) have shown positivity for *H. pylori*. Statistically, the correlation between *H. pylori* infection and tuberculosis is not significant both in males and females. With regard to different age groups, there is no significant relation between *H. pylori* infection and tuberculosis. Among normal, overweight and underweight populations, statistics did not show any significance. Similarly, socioeconomic status –wise results also did not reveal any correlation between *H. pylori* infection and tuberculosis. Thus analysis of the results pertaining to demography revealed that absolutely there is no correlation between tuberculosis and *H. pylori* infection.

Testing of serum antibodies against the standard antigens shows that cagA (p120) antibodies were found in tuberculosis as well as control subjects. Similarly, certain antibodies like p67, p57, p54, p50, p41 were found in detectable quantities in tuberculosis and healthy controls. Interestingly, antibodies against p33, p30, p29, p26, p19 and p17 which are considered to be positive for *H. pylori* were not observed in detectable quantities (table 3). All in all, western blot investigations did not reveal any connectivity between tuberculosis and *H. pylori* infection.

IIFA

Table 1 presents the results pertaining to *H. pylori* association with tuberculosis as revealed by IIFA tests. A critical study of the table revealed that out of 39 subjects suffering with tuberculosis, 31 (79.05%) have proved to be *H. pylori* positive; whereas out of 42 control subjects only 4 (9.5%) have proved to be positive. Thus, there is a valid significance (P<0.000) between the association of *H. pylori* infection and tuberculosis. Gender–wise statistical analysis revealed a highly

significant correlation between *H. pylori* infection and tuberculosis. In different age group people also a significant correlation was noticed, though there is some variation between the two age group people. Relationship between nutritional status, tuberculosis and *H. pylori* infection showed that in normal and underweight people there is a significant correlation between tuberculosis and *H. pylori* infections. A significant ($P<0.000$) correlation between *H. pylori* infection and tuberculosis was observed in low income and middle income groups. Thus, in all the cases IIFA investigations revealed a positive correlation between *H. pylori* infection and tuberculosis.

In this study, an attempt was made to evaluate the possible relationship between *H. pylori* infection in tuberculosis patients by ELISA, western blot and IIFA. The IIFA results suggest a significant association between *H. pylori* infection and tuberculosis diseases. Previously, a number of investigators reported that *H. pylori* infection might play a supporting role for tuberculosis (20). Data in the literature on the relationship between *H. pylori* infection and pulmonary TB are poor. A previous epidemiological study, conducted in southern China suggested that a history of tuberculosis might be associated with increased prevalence of *H. pylori* infection (21). Poor socio-economic and sanitary conditions during childhood could explain these results, as it is well known that in developing countries acquisition of both *H. pylori* and *Mycobacterium tuberculosis* occurs early in life (22,23). A recent study showed no difference in *H. pylori* seroprevalence between patients on antituberculosis chemotherapy and control subjects (24). However, in that study the decrease in serum concentration of *H. pylori* IgG antibodies due to the eradication of *H. pylori* by antituberculosis drugs could not be excluded. Rifampicin and streptomycin, the two drugs commonly used in anti-tuberculosis regimens, are also effective against *H. pylori* and therefore treatment with these two antibiotics is likely to decrease in *H. pylori* seroprevalence during antituberculosis therapy (25,26). IIFA tests indicated that *H. pylori* seroprevalence in patients with pulmonary TB is significantly higher than that of the control subjects. The socio-economic status, which is related to both *H. pylori* seroprevalence and risk of pulmonary TB, is similar in the two groups. Moreover, the airborne transmission of *M. tuberculosis* occurs even without physical contact, whereas *H. pylori* usually spreads via close physical contact. Thus, the observed association between *H. pylori* infection and pulmonary TB seems to be real and cannot be attributed to transmission-associated confounding factors. Seroprevalence of *H. pylori* has also been reported in patients suffering with asthma (27,28). On the other hand, the role of chronic *H. pylori* infection as a predisposing factor for development of pulmonary TB is unknown. An increased risk of TB for persons who had undergone partial gastrectomy or vagotomy for peptic ulcer disease has previously been reported (29). With regard to the pathogenic role of *H. pylori* infection in peptic ulcer disease, we hypothesize that *H. pylori* infection *per se* may be related to the risk of pulmonary TB.

A comparison of ELISA, western blot and IIFA with regard to sensitivity, specificity and both positive and negative predictive values revealed that ELISA technique did not show any correlation between tuberculosis and *H. pylori* infection. These results are in agreement with the observations made by earlier investigators (30). Similarly, western blot test results revealed that healthy and diseased subjects have shown similar *H. pylori* status.

Table.1 Demographic- wise IgG Anti-H. pylori association in tuberculosis patients and control subjects through ELISA, IIFA and Western blot

	Demographic	Versus	ELISA IgGAnti-*H.pylori* seropositivity	Significane value	IIFA IgGAnti-*H.pylori* seropositivity	Significane value	Western blot IgGAnti-*H.pylori* seropositivity	Significance value
Gender	Males	TB	36/38 (94.7%)		13/19(68.4%)		13/13(100.0%)	
		Control	17/21 (81.0%)	P < 0.093	02/21(09.5%)	P < 0.000	10/10(100.0%)	NA
	Females	TB	20/20 (100.0%)		18/20(90.0%)		10/10(100.0%)	
		Control	18/21 (85.7%)	P<0.079	02/21(09.5%)	P<0.000	10/10(100.0%)	NA
	Total	TB	56/58 (96.60%)		31/39(79.5%)		23/23(100.0%)	
		Control	35/42 (83.30%)	P < 0.023	04/42(09.5%)	P < 0.000	20/20(100.0%)	NA
Age group	20-40	TB	36/37 (97.3%)		22/27(81.5%)		16/16(100.0%)	
		Control	24/27(88.9%)	P < 0.170	03/27(11.1%)	P < 0.000	13/13(100.0%)	NA
	41-60	TB	15/16(93.8%)		07/9(77.8%)		04/04(100.0%)	
		Control	11/15(73.3%)	P<0.122	01/15(06.7%)	P<0.000	07/07(100.0%)	NA
	Above 61	TB	05/5(100.0%)		02/03(66.7%)		03/03(100.0%)	
		Control	00/0(00.0%)	NA	00/0 (00.0%)	NA	00/00(100.0%)	NA
Nutritional	Normal	TB	04/04 (100.0%)		03/3(100.0%)		01/01(100.0%)	
status (BMI)		Control	12/14 (85.7%)	P < 0.423	00/04(00.0%)	P < 0.000	07/07(100.0%)	NA
	Overweight	TB	03/03(100.0%)		03/3(100.0%)		01/01(100.0%)	
		Control	02/03(100.0%)	P<0.273	00/03(00.0%)	P<0.014	02/02(100.0%)	NA
	Underweight	TB	49/51(96.1%)		25/33(75.8%)		21/21(100.0%)	
		Control	21/25(84.0%)	P<0.067	04/25(16.0%)	P<0.000	11/11(100.0%)	NA
Socioeconmic status	Low income	TB	26/26 (100.0%)		11/17(64.7%)		14/14(100.0%)	
		Control	15/17(88.2%)	P < 0.073	01/17(05.9%)	P < 0.000	09/09(100.0%)	NA
	Middl income	TB	18/20(90.0%)		14/15(93.3%)		05/05(100.0%)	
		Control	11/13(84.6%)	P<0.643	01/13(07.7%)	P<0.000	05/05(100.0%)	NA
	High income	TB	12/12(100.0%)		06/07(85.7%)		04/04(100.0%)	
		Control	09/12(75.0%)	P<0.064	02/12(16.7%)	P<0.003	06/06(100.0%)	NA

Note: P values less than 0.005 are significant more than 0.005 are not significant; NA- statistically test not applicable

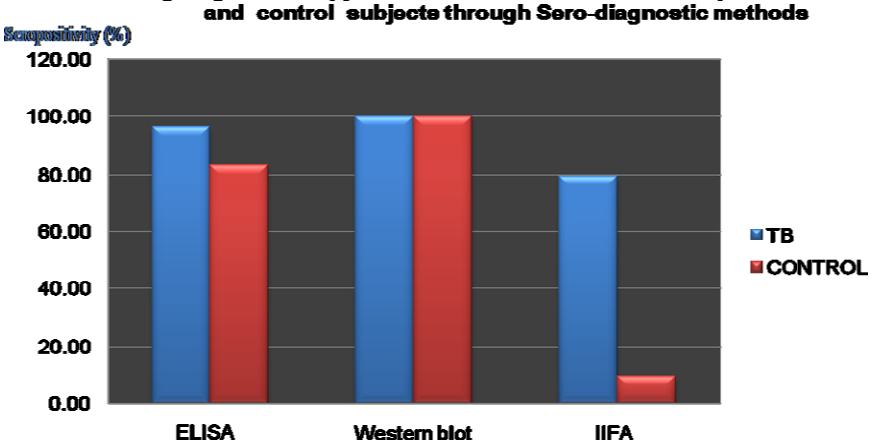
Table.2 IgG Anti- H. pylori antigens find in tuberculosis patients and control subjects by immuno blot test

Western blot IgG Antigen *H.pylori*	IgG-anti *H.pylori* TB seropositivity		IgG-anti *H.pylori* Control seropositivity	
	Number	Percentage	Number	Percentage
P120,cagA	18/23	78.3%	17/21	85.0%
P95,vacA	04/23	17.4%	01/21	05.0%
p75	04/23	17.4%	01/21	05.0%
P67,Flag	11/23	47.8%	12/21	60.0%
P66,ureB	11/23	47.8%	14/21	60.0%
P57	21/23	91.3%	15/21	75.0%
P54	12/23	52.2%	09/21	45.0%
P50	17/23	73.9%	13/21	65.0%
P41	11/23	47.8%	11/21	55.0%
P33	02/23	08.7%	04/21	20.0%
P30	07/23	30.4%	05/21	25.0%
P29,urea	12/23	52.2%	09/21	45.0%
P26	06/23	26.1%	09/21	45.0%
P19,omp	06/23	26.1%	03/21	15.0%
P17	01/23	04.3%	01/21	04.7%

Table.3 Comparision of sensitivity, specificity, positive predictive value, negative predictive values of ELISA, western blot and IIFA

Test	Tuberculosis				Control			
	Sensitivity %	Specificity %	Positive predictive value (PPV%)	Negative predictive value (NPV %)	Sensitivity %	Specificity %	Positive predictive value (PPV%)	Negative predictive value (NPV %)
ELISA	96.55	03.45	50.00	50.00	83.33	16.67	50.00	50.00
Western blot	100.00	00.00	50.00	00.00	100.00	00.00	50.00	00.00
IIFA	79.49	20.51	50.00	50.00	09.52	90.48	50.00	50.00

Fig.1. IgG Anti-*H. pylori* associations in tuberculosis patients and control subjects through Sero-diagnostic methods



A survey of literature showed that no such investigations were made earlier. Nevertheless, IIFA tests revealed a strong relationship between *H. pylori* infection in tuberculosis. In this regard, our studies are the first of its kind and as such no reports are available for comparison purpose.

Out of the present investigations, it can be concluded that western blot and enzyme linked immune sorbent assay test have not shown any link between *H. pylori* infection and tuberculosis disease. However, this relationship can be confirmed through indirect immune fluorescence assay. Moreover, we need a large number of samples for the clarification of association between *Helicobacter pylori* infections and tuberculosis patients by indirect immune fluorescence test.

Authors contributions

This work was carried out in collaboration among all authors. Authors CEP and LKC designed the experiment. Author SP made sample collection and carried out ELISA, western blot and IIFA tests. CEP wrote the manuscript. Author LKC helped in interpretation of results. Author SRR corrected the manuscript. All authors read and approved the manuscript.

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