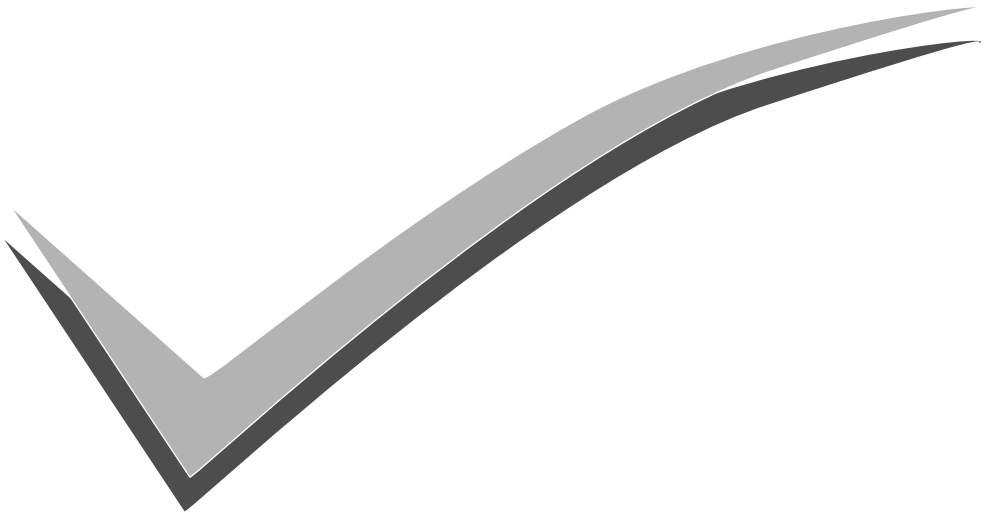
Performance Evaluations



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3.	http://www.tm.mahidol.ac.th/seameo/2010-41-3/10-4695.pdf	570-573
4.	Indian Journal of Medical Microbiology vol. 31, No. 3	314-315
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14.	Directorate General of Health Services, Drug Control Section, (DCGI), India

Performance Evaluations

AS A REFERENCE PRODUCT

RETROQUIC®-HIV

Rapid Immunoconcentration test for the detection of HIV1 & HIV2 antibodies

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Original Article

Human Immunodeficiency Virus Infection Among Tuberculosis Patients in Mumbai

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ABSTRACT

Background: Human Immunodeficiency Virus (HIV) is the most powerful risk factor for the progression of *Mycobacterium tuberculosis* infection to Tuberculosis (TB) disease. TB accelerates the progression of HIV infection to AIDS and shortens the survival of such patients.

Aim: To determine the seroprevalence of HIV infection among TB confirmed patients in a tertiary care center in Mumbai in view of the significance of HIV in TB. Its association with gender and age was also determined.

Materials and Methods: Blood samples were collected by venipuncture from 432 TB patients and their HIV status was determined. HIV antibody detection was carried out as per Strategy III, National AIDS Control Organisation (NACO) guidelines. Statistical analysis was carried out by applying the Chi-square test.

Results and Conclusion: Of the 432 patients screened, 9% (39) were HIV positive. The prevalence of co-infection was higher among females (9.4%) than the male (8.7%) patients and highest amongst those aged 21to40 years (13.7%). Co-infection was found to be statistically highly associated with age ($p < 0.05$). This high prevalence calls for routine screening of TB patients for HIV infection.

Keywords: Human immunodeficiency virus, seroprevalence, Tuberculosis

INTROdUCTION

The emergence of Human Immunodeficiency Virus (HIV) has paved way for the resurgence of *Mycobacterium tuberculosis* infection. While HIV is the most powerful risk factor for the progression of *M. tuberculosis* infection to Tuberculosis (TB) disease, TB accelerates the progression of HIV infection to Acquired Immunodeficiency Syndrome (AIDS) and shortens the survival of such patients.[1,2] The two are intricately linked to malnutrition, unemployment, poverty, drug abuse, and alcoholism and have also been referred to as the "Cursed Duet."[3] HIV is known to increase the risk of reactivation in people with latent TB and also increases the risk of subsequent episodes of TB from exogenous reinfection.[3,4] It has also corroborated by noting that HIV patients are highly vulnerable to TB because of their weakened immune systems and the latter is now their number one killer.[5] Surveillance of HIV among TB patients has been recognized to be important as the HIV epidemic continues to fuel TB epidemics. In many countries, HIV prevalence among TB patients is a sensitive indicator of the spread of HIV into the general population.[6]

TB and HIV epidemics are heavily intertwined, each increasing the morbidity and mortality of the other. In India, there are over 3 million annual prevalent active TB cases and over 5 million HIV-positive individuals. Although the countrywide prevalence remains less than 1% (0.91%),[7] a vast majority of HIVpositive patients do not know their status. Less than 5% of the approximately 7,70,000 patients in need of antiretroviral therapy currently have access to it.[8] Strategies to detect and treat HIV are thus of a pressing concern. Given that TB is the most common

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opportunistic infection in HIVinfected individuals in India, detecting HIV among TB patients presenting to the health sector represents an important public health opportunity. Programmatic planning requires the availability of up-to-date data on HIV seroprevalence among different populations of TB patients.

In the view of the aforementioned, a retrospective analysis was carried out to estimate the prevalence of HIV infection among TB patients visiting a tertiary care center in Mumbai.

MATERIALS ANd METHOdS

A retrospective analysis of TB patients referred for HIV testing over a period of 1 year was carried out in the Department of Microbiology at the B.Y.L. Nair Charitable Hospital, Mumbai. This institute is a tertiary care referral hospital**.** Only those TB patients whose HIV status was not known at the time of TB diagnosis were included in the analysis to draw the true prevalence of HIV infection among TB patients. TB diagnosis was done on the basis of smear microscopy, chest radiography, and clinical signs/symptoms as per the Revised National Tuberculosis Control Programme (RNTCP).

TB patients registered at the DOTS center of this hospital are routinely advised to undergo HIV screening after pretest counseling and informed consent. Our laboratory caters laboratory services to all such patients, and tests are carried out as per the guidelines laid down by the National AIDS Control Organization (NACO), India.[9] The results were collected from all TB patients tested in this laboratory and no selection bias was observed. The findings were analyzed over a period of 1 year from August 2009 to July 2010. All the tests were done after a written informed consent was obtained from the patients and in accordance with the institutional ethical guidelines.

Venous blood sample (5 ml) was collected in a plain container from all the patients who consented for testing. Blood was allowed to clot for 30 mins at room temperature (25–30°C) and serum was separated after centrifugation at low speed. The serum samples were then stored at 4°C and were tested within 48 hours.

HIV antibodies were tested by the three ELISA/Rapid/ Supplemental tests protocol as per the guidelines laid down by NACO (Testing strategy III) and positive test result was disclosed to the patients by post test. Antibodies to HIV (1 and 2) were tested initially using COMBAIDS test (Span Diagnostics Ltd, Surat, India). Samples found reactive by COMBAIDS were tested by RETROSCREEN test (Qualpro Diagnostics, Goa, India) and RETROQUIC test (Qualpro Diagnostics, Goa, India).

RESULTS

The study population comprised of 420 confirmed TB patients who were screened for presence of HIV antibodies. Of these, 191 (44.2%) were females and 241 (55.8%) were males. There were more patients in the 21–40 years age group (47.5%) than in any other age group. Patients between 1 and 20 years of age were the least (1.6%).

The overall prevalence of co-infection of *M. tuberculosis* and HIV in this population was 9%. In relation to gender, it was 9.4% and 8.7% among females and males, respectively [Table 1].

The prevalence of co-infection also varied with age of the patients. It was highest among TB patients aged 21–40 years (13.7%) followed by those aged 41–60 years (9.6%), more than 60 years (4.8%), and least among those aged 1–20 years (1.6%) [Table 2]. There was a statistically significant association between age and HIV infection among TB patients in this study ($p < 0.05$).

dISCUSSION

This study demonstrated that the HIV seroprevalence among TB patients presenting to the tertiary care hospital in Mumbai in 2009–2010 was 9%, as compared to previous reports from Delhi of 0.4% in 1995–1999,[10] 9.4% in 2000–2002,[11] and 8.3% in 2003–2005.[12] Ramachandran *et al* have reported a seroprevalence of 4.7% in Tamil

Table 1: Prevalence of HIV infection among tuberculosis patients in relation to gender

Gender	Number screened	Number positive	Percentage
Male	241	21	8.7
Female	191	18	9.4
Total	432	39	9

Table 2: Prevalence of HIV infection among tuberculosis patients in relation to age

Age (years)	Number screened	Number positive	Percentage
1–20	123	2	1.6
21–40	205	28	13.7
41–60	83	8	9.6
>60	21	1	4.8
Total	432	39	9

Nadu in 1997–1998.[13] The trend observed over the years highlights the importance of continuous surveillance and in-time appropriate preventive measures.

The HIV seroprevalence of 9% among TB patients in our study is a cause for alarm, especially in view of the fact that HIV seroprevalence among TB patients is a good indicator of the spread of HIV infection in the general population. This high prevalence might not be unconnected with the relatively high HIV prevalence in the area,[14] which is important in latent TB reactivation[3] leading to a preponderance of HIV/TB co-infection.

We found that HIV seroprevalence rates among TB patients were more in females (9.4%) as compared to males (8.7%). This is probably related to the higher incidence of HIV infection in females, which predisposed them to TB as HIV is known to activate dormant TB. Women also have a higher susceptibility to HIV infection and are usually exposed to sexual activities earlier than men mainly due to economic circumstances. Furthermore, most Indian women are so subordinated to their husbands that they have little or no say in issues related to sexual relationships.

HIV seroprevalence rates were highest in the age group of 21–40 years (13.7%). Our data suggests that it is better to target TB patients aged between 21 and 40 years for HIV screening, as 47.5% of the cases were found in this age group. The preponderance of HIV/TB co-infection among patients aged 21–40 years observed in this study ($p < 0.05$) is similar to some other reports.[1] This is a sexually active group in which both TB and HIV prevail the most.[14] Thus the significantly high prevalence of co-infection.

CONCLUSION

Knowledge of HIV status in a TB patient is critical from both patient and public health perspectives. In those patients who test seropositive for HIV, better care can be provided in the form of effective combined antitubercular (ATT) therapy and antiretroviral treatment. If a HIV-positive TB patient on ATT worsens or fails to improve with therapy, the possibility of other co-existing opportunistic infections or immune reconstitution syndrome should be considered. Knowledge of a person's HIV serostatus also provides the opportunity to administer prophylaxis for opportunistic infections and thereby reduces morbidity and mortality. The spouse and relatives of HIV-seropositve patients may also be counseled on HIV infection and its modes of transmission and prognosis, preventing the spread of infection. Spouses may be educated on safe sex practices and may be offered testing themselves.

In conclusion, there is a pressing need for the Revised National Tuberculosis Control Programme (RNTCP) and NACO to collaborate on the feasibility of making HIV counseling and testing routinely available to all DOTS and other TB patients throughout India.

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Immunofluorescence assay in India for confirmation of HIV-1 infection using a T-cell line infected with defective HIV-1

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SUMMARY

Background: In India, the enzyme immunoassay (EIA)/rapid test is used for screening and confirmatory antibody testing of HIV infection, and all HIV reactive samples are further confirmed by two other rapid tests working on different principles; however, Western blotting (WB) and immunofluorescence (IF) assays are not routinely performed in this country.
Methods: A total of 2104 sera from Indian subjects were tested for the presence of HIV-1 antibody using EIA/rapid tests, according to the guidelines of the National AIDS Control Organization of India, and were also subjected to IF test using L-2 cells persistently infected with defective HIV-1. WB and a nested reverse transcriptase polymerase chain reaction (RT-PCR) were performed on discrepant samples.
Results: IF results were 100% concordant with EIA/rapid tests for 212 HIV-1-positive samples and 1889 HIV-1-negative samples. Interestingly, three (0.14%) samples negative by EIA/rapid tests were weakly or moderately positive (1+/2+) by IF test. All three of these samples were confirmed to be negative by WB (reactive with Gag/Pol, but not with Env), but positive by RT-PCR with primers targeting the C2–V5 fragment of the *env* gene. These three samples were from individuals who voluntarily reported for HIV testing because of high-risk practices, and they may have been at an early stage of HIV infection.
Conclusions: These results confirm that the IF test using L-2 cells is a sensitive and specific alternative method for confirmation of HIV-1 infection and could be included in the diagnostic algorithm in reference laboratories in developing countries.

1. Introduction

The human immunodeficiency virus (HIV) epidemic continues to be a burden worldwide, especially in developing countries.[1] Though there has been dramatic progress in the diagnostic methodologies, the detection of antibodies continues to be the mainstay of diagnosis in most of these countries. Serologic assays for the detection of anti-HIV antibodies, rapid HIV tests, and enzyme immunoassays (EIAs) are universally used for initial screening. The currently available commercial rapid or EIA kits are more specific than previous ones, but they still produce false-positive reactions, necessitating the use of a confirmatory test. The US Centers for Disease Control and Prevention (CDC) recommend that all reactive screening tests be confirmed by supplemental testing using either a Western blot (WB) or indirect immunofluorescence (IF) assay.[2] Additionally, if the WB or IF assay is negative or indeterminate, confirmatory testing should be repeated with a follow-up blood specimen collected 4 weeks after the initial tests to rule out specimen mix-up or early seroconversion.[2]

The WB technique provides a specific approach for HIV serology. However, despite the proven advantages of WB, the use of this test has been limited to only a few reference laboratories. The reasons for the restricted use of WB include the technical complexity of the procedure, i.e., developing in-house strips, time-consuming procedures, high cost, and the requirement of technical expertise and specialized equipment and reagents. A number of HIV-1/2 WB strips are commercially available that are more rapid (16–20 h) than in-house tests and are easy to perform without any special equipment, however these kits remain cost-prohibitive. Furthermore, WB usually yields a high number of indeterminate results due to the restrictive criteria that require the presence of Gag, Pol, and Env antibody, usually lacking in many AIDS patients; this complicates the accurate interpretation of results, which must be followed by further serological examinations, thus further increasing testing costs, time, and technical requirements.

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In contrast to WB, the IF assay is a well-accepted and widely used confirmatory test in many laboratories. The primary advantages of the IF assay are that it is technically simple, more convenient, less expensive, and more rapid than WB. Commercial WB tests usually require overnight incubation for optimal results, while commercial IF assays can be performed within 2 h. Additionally, cells are maintained by dilution passage every 4 days, which takes less than 5 min. Moreover, the use of an uninfected cell control ensures high assay specificity by easily differentiating non-specific staining from specific staining.

The source of viral antigen in HIV IF assays has commonly been continuous lymphoid cell lines such as H9 or HUT78[3] and non-lymphoid cell lines such as the T4 HeLa cell line.[4,5] In this study, a previously developed human T-cell line persistently infected with defective HIV-1 (L-2 cell clone)[6] was used in an IF assay system along with MT-4 cells as control cells. The advantage of using this cell clone is that L-2 cells show exceptionally strong HIV-1 antigen expression compared with other persistently HIV-1 infected human T-cell lines[7] and continuously produce non-infectious, reverse transcriptase-negative doughnut-shaped particles.[6] The goal of the present study was to explore the suitability of L-2 cells as a potentially useful source of viral antigens in IF assays for HIV confirmatory testing.

2. Methods

2.1. Serum samples

From October 2006 to May 2008, 2104 serum samples were tested. Sera were obtained from 954 consecutive diagnostic specimens received for HIV antibody testing from individuals attending the Integrated Counseling and Testing Centre (ICTC) of a tertiary care hospital in India and 1150 consecutive samples collected during a sentinel surveillance program. Out of the 1150 samples, 400 were from antenatal clinic attendees and 250 each were from female sex workers, intravenous drug users, and men having sex with men (MSM). Each patient gave written informed consent for study participation and received pretest counseling prior to sample collection, in accordance with the guidelines of the National AIDS Control Organization (NACO), India.[8]

2.2. Routine HIV serology

In this study, screening and confirmatory antibody testing for HIV infection was performed as recommended by NACO, India, following a serial testing strategy (Figure 1). Briefly, the samples were initially screened for HIV antibody by EIA/rapid test, i.e., CombAids-RS (Span Diagnostics Ltd, India; Dot Immunoassay)/ Microlisa-HIV (J. Mitra & Co. Pvt. Ltd, India; Indirect ELISA principle). All samples found negative by the first screening test were reported negative. To reduce the costs of HIV testing as recommended by the World Health Organization,[9,10] all the HIV reactive samples were further confirmed by two more rapid tests working on different principles, such as ACON-HIV (Rapid Diagnostic Pvt Ltd, India; Tri-line rapid chromatographic immunoassay), Capillus test (Trinity Biotech plc, Bray, Co. Wicklow, Ireland; agglutination test), or Retroquic-HIV (Qualpro Diagnostics, India; rapid immunoconcentration test). Samples reactive in all three assays were reported as positive for HIV antibody. For

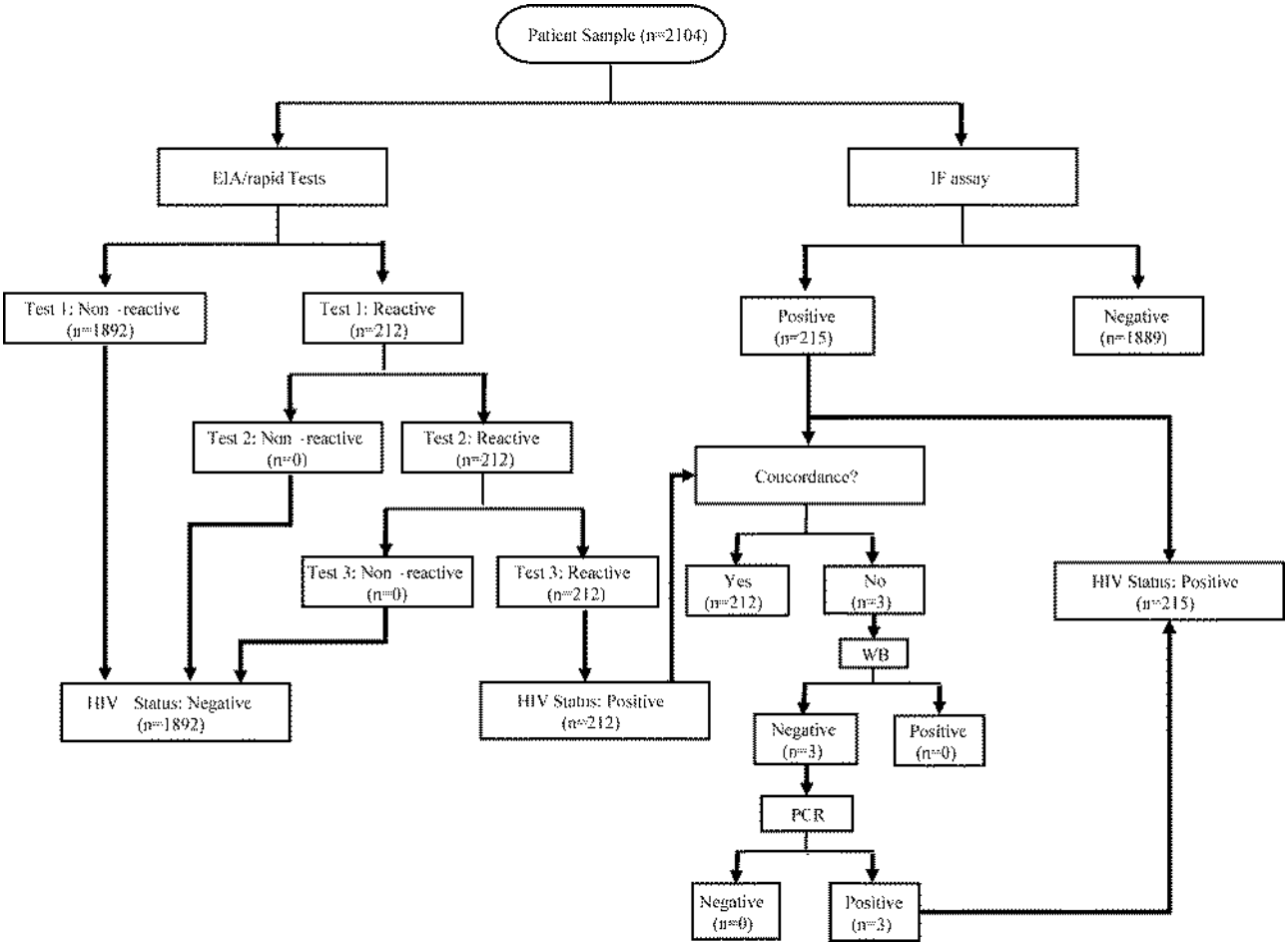

Figure 1. Diagnostic algorithm used for HIV positivity confirmation. On the left-hand side, EIA/rapid tests were performed as per National AIDS Control Organization, India HIV testing guidelines. On the right-hand side, the diagnosis procedure using the IF assay for the same samples is shown, including follow-up confirmation using WB and RT-PCR.

sentinel surveillance samples, the NACO testing strategy is screening and then confirmation of the positive samples by one test.[8] All the kits used for the detection of HIV antibody were approved and supplied by the Delhi State AIDS Control Society, India. In India, WB testing and IF assays are not routinely performed and are restricted to specimens with atypical serological profiles (discordant results by three EIA/rapid tests).

2.3. Cell lines

The uninfected MT-4 human T-cell line and the infected L-2 cell line were used. MT-4 was the parental cell line used for the preparation of the L-2 cell clone that was persistently infected with the defective HIV-1 subtype B LAI strain.[6] These cell lines were maintained at the Department of Virology, Research Institute of Microbial Diseases, Osaka University, Japan. L-2 and MT-4 cells were propagated by incubation in RPMI-1640 supplemented with 5–10% heat-inactivated fetal bovine serum at 37 °C in a CO_2 incubator. Cells were cultured at 5×10^5/ml until the cell density reached 2–3 $\times$ 10^6/ml on the 4th day post-incubation. Cells were maintained by subdividing 1:4 (v/v) in fresh medium every 4 days.

2.4. Slide preparation

The cells were harvested, washed with phosphate-buffered saline (PBS), and suspended in PBS at 1×10^6 cells/ml. One part L-2 cell suspension was mixed with three parts uninfected MT-4 cell suspension. The MT-4 and L-2 cells were smeared on a 12-well glass slide (Flow Laboratories). Approximately 5×10^3 cells (5 μl of a 1×10^6 cells/ml suspension) were placed in each well. The slides were air-dried and fixed in cold acetone for 10 min at 4 °C. The slides were packed in heat-sealed plastic bags and stored at −20 °C until use.

2.5. IF assay procedure

All patient sera were heat-inactivated at 56 °C for 30 min and diluted 1:100 in PBS. Ten microliters of each diluted serum were applied to wells and incubated in a humid chamber at 37 °C for 30 min. PBS and HIV-positive and HIV-negative sera were used as internal controls each time. After washing three times with PBS at room temperature, 10 μl of the fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc.) was applied to the wells; these were again incubated at 37 °C for 30 min. The slides were rinsed three times with PBS and mounted in 90% glycerol in PBS and read independently by two trained persons, who were blinded to serological results, under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). This test was performed as a single assay (without replicates). Any ambiguous or discordant samples were retested in duplicate.

2.6. Interpretation of IF assay results

The typical cytoplasmic staining patterns of L-2 cells were graded from 0 to +4 according to the intensity of FITC in approximately 25% of L-2 cells present in a monolayer of the HIV-infected wells (approximately 1.25×10^3 L-2 cells/well), as described elsewhere.[11] A false-positive reaction was detected when both the L-2 and MT-4 cells were stained.

2.7. Western blot

WB was performed on the samples with discordant rapid test and IF assay results. WB testing was performed with the HIV WB kit from J. Mitra & Co. Pvt Ltd, India, following the manufacturer's instructions. Results were interpreted according to the criteria suggested by the manufacturer; interpretation criteria for a positive test were 2 Env + 1 Gag ± 1 Pol band and for an indeterminate test were 1 Env ± Gag ± Pol or Gag ± Pol or only Gag or only Pol.

2.8. RNA extraction and envelope PCR amplification

Viral RNA was extracted from the patient serum samples using the QIAamp blood kit (Qiagen, Chatsworth, CA, USA). A reverse transcriptase polymerase chain reaction (RT-PCR) was performed to amplify a 708-base pair C2–V5 fragment of the *env* gene, using previously described primer sets.[12] We used an infectious molecular clone of Indian subtype C HIV-1 – Indie-C1 – as a positive control.[13] Briefly, viral RNA was initially reverse-transcribed at 50 °C for 50 min using the ThermoScript kit (Invitrogen, Carlsbad, CA, USA). Subsequently, for the cDNA amplification, the EX Taq PCR kit (Takara, Kyoto, Japan) was used as per the manufacturer's instructions. A nested PCR reaction protocol was used. In the first round reaction, the forward primers were ED31 (CCTCAGCCATTACACAGGCCTGTCCAAAG) and BH2 (CCTTGGTGGGTGCTACTCCTAATGGTTCA). Five microliters of the first round reaction product was subjected to a second round of amplification, with the primers DR7 (TCAACTCAACTGCTGTTAAATGGCAGTCTAGC) and DR8 (CACTTCTCCAATTGTCCCTCATATCTCCTCC). The cycling conditions for both amplifications consisted of initial activation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 7 min. Amplified PCR products were visualized by electrophoresis in 1% agarose gel.

3. Results

Out of the 2104 samples, 1892 tested HIV-negative and 212 HIV-positive by EIA/rapid test (Table 1). Specific IF patterns of reactivity to HIV antigens were clearly demonstrated in all 212 sera positive by EIA/rapid test, as well as the positive control serum from an HIV-1-infected individual (Figure 2, G). Of the 212 serum samples, 184 were IF-strongly positive (3+ or 4+), 21 were moderately positive (2+), and seven were weakly positive (1+). These seven samples weakly positive (1+) at a dilution of 1:100, were strongly positive (3+ or 4+) at a dilution of 1:20, which gave negative staining in MT-4. In addition, three sera (termed X, Y, and Z) from the ICTC that were negative by EIA/rapid test, were also weakly to moderately positive (one sample 2+, two samples 1+) by IF assay (Figure 2, A, C, and E). The positive reactions of the three sera by IF were confirmed on repeat testing. We also observed that another nine (seven from the ICTC and two from MSM) of the sera negative by EIA/rapid test were also weakly positive by IF. However, all the sera showed negative reactions on repeat testing with the IF test. Hence we concluded that the results were negative in these nine serum samples. The other 1880 sera negative by EIA/rapid test were also negative by IF.

Table 1
Results of EIA/rapid tests and IF assay in different subject groups

Subject group	Number tested	Number reactive by EIA/rapid test	Number reactive by IF assay
Integrated counseling and testing centre	954	202	205
Antenatal clinic	400	1	1
Female sex workers	250	3	3
Intravenous drug users	250	2	2
Men having sex with men	250	4	4
Total	2104	212	215

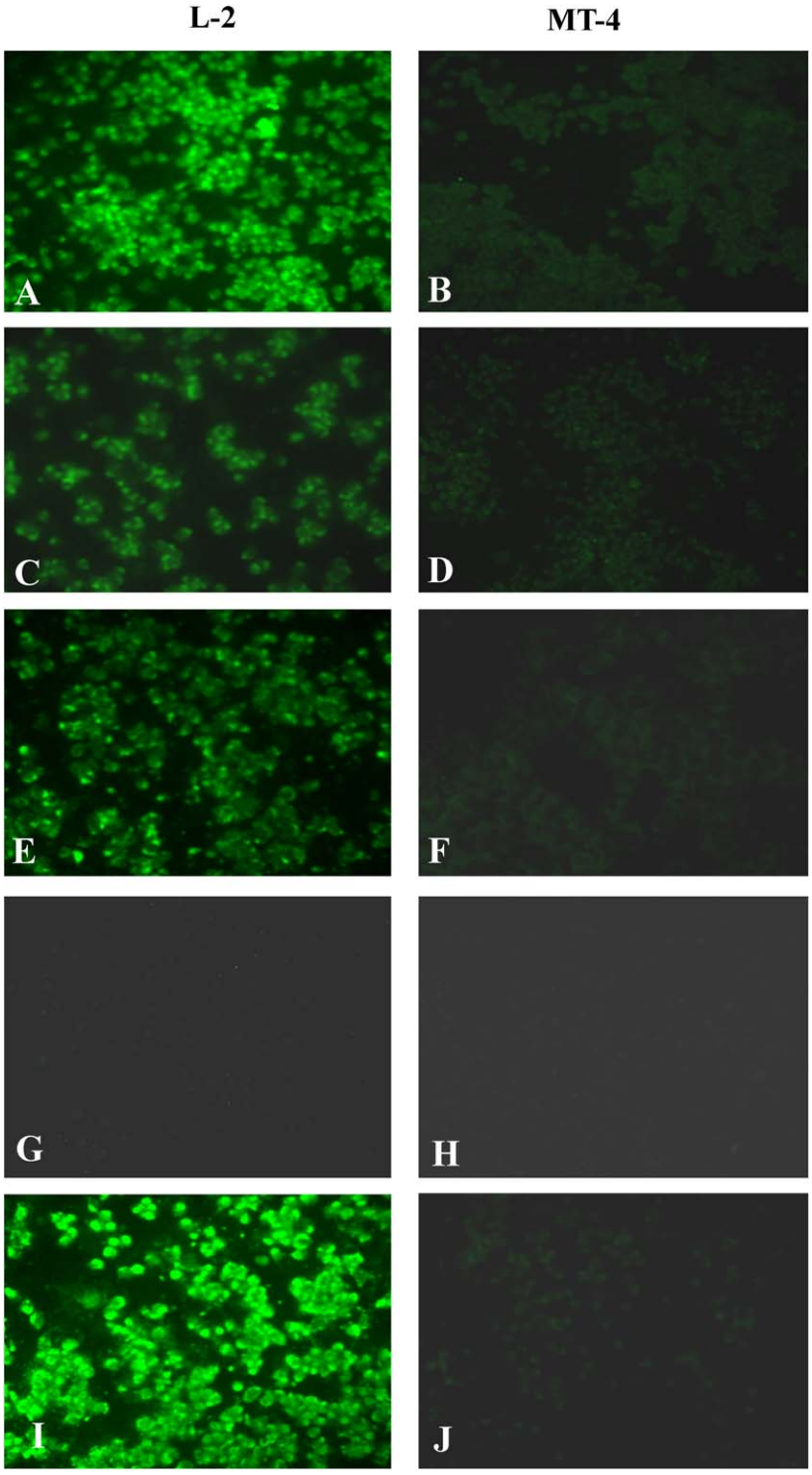

Figure 2. IF of the three discordant serum samples. The three serum samples (X, Y, and Z) showed discordant results: negative by EIA/rapid test but positive by IF assay. The L-2 cells (A, C, E, G, and I) and uninfected MT-4 cells (B, D, F, H, and J) as negative control were similarly reacted with 1:100 dilutions of sera from X (A and B), Y (C and D), and Z (E and F), and negative control serum from a healthy individual (G and H) and a positive control serum from an HIV-1-infected individual (I and J).

The above-described X, Y, and Z sera were determined to be negative by WB because reactions with Gag and Pol proteins were observed, however there were no reactions with Env proteins in any of the three serum samples (Figure 3, A). When we subjected these three plasma samples to RT-PCR analysis for the detection of HIV-1 RNA in plasma, it was found that all the samples were positive (Figure 3, B).

4. Discussion

The IF assay has been used successfully in diagnostic laboratories for three decades and has proven to be an acceptable test for the detection of anti-HIV-1 antibodies.[14–20] We have described here the performance of one such IF assay using L-2 cells as an alternative antigen source, within the context of a state reference

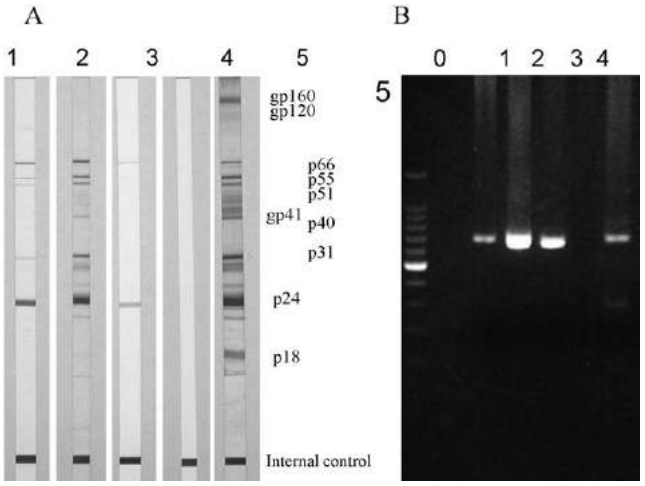

Figure 3. Confirmation of the three discordant serum samples by WB and RT-PCR. The three serum samples (X, Y, and Z) were subjected to WB (A) and nested RT-PCR (B). For WB: lane 1, X serum; lane 2, Y serum; lane 3, Z serum; lane 4, reagent blank as a negative control; lane 5, Indie-C1 as a positive control.

laboratory that is accustomed to screening large numbers of suspected cases of HIV infection.

The IF assay was compared with EIA/rapid tests for the ability to detect antibodies to HIV. A total of 2104 specimens were tested by both systems. When both assays were used, 212 serum samples were positive and 1889 samples were negative (99.86% concordance). Only three (0.14%) specimens gave discrepant results, i.e., IF produced weakly to moderately positive results, which required retesting for resolution. The three discrepant samples were also negative on WB but were positive on PCR. These samples were from direct walk-in, asymptomatic individuals attending the ICTC for HIV testing, who were involved in high-risk practices. The IF assay and PCR positivity in these cases denotes an early stage of infection, indicating the high sensitivity of the IF assay in comparison to WB. No false-negative results were observed with the IF assay on single testing of 212 HIV-positive sera. The IF assay was found to be almost as sensitive and specific as ELISA as reported in earlier studies.[17,18] However, in this study it was observed to be slightly more sensitive and equally specific to the EIA/rapid test. Lennette et al.[17] tested 181 sera by both IF assay and ELISA and there were 91 positive and 85 negative sera. They reported that an additional five sera were positive by ELISA only. All five were shown to be negative on retesting and were considered as false-ELISA positives. Gallo et al.[18] observed 100% agreement between EIA and IF on 142 serum specimens from homosexual men and on 88 sera from frank AIDS cases.

It has been reported that if the IF assay is performed in a diagnostic laboratory with experience in IF, the false-positive rate should be less than 1% on initial testing and should approach zero on repeat testing.[14] In the present study no false positivity was observed.

Additionally, the cost–performance ratio must be considered when deciding which confirmatory test to use for the evaluation of sera that show discrepant results in two screening assays. Previous studies[21,22] have suggested both WB and IF to have comparable performance characteristics, but confirmation by IF assay can be implemented at a cost that is on average five times less than WB. Therefore IF constitutes a better cost–performance alternative to WB, especially in the case of healthcare centers in India where immunofluorescent microscopy is routinely done to evaluate other infectious disease, and thus can be implemented at no additional cost.

To conclude, the evaluation of IF using HIV-1-infected L-2 cells as a source of antigen shows it to be a rapid, reliable, and less expensive test. As it was observed to be slightly more sensitive than WB after evaluating the results of discordant samples with PCR, we feel that it is well suited for use in reference laboratories for confirmation of HIV infection, replacing or complementing WB. However, its sensitivity for the diagnosis of early infection needs to be evaluated further. The IF assay reagents are simple to prepare within the capacity of most reference laboratories. The reagents are also quite stable with a shelf life of many months.

Ethical approval

Study samples were obtained from HIV-1-infected patients attending the Integrated Counseling and Testing Centre at Safdarjang Hospital, New Delhi, India. The study was approved by the local ethics committee, and all patients provided their written informed consent to participate.

Conflict of interest

No conflict of interest to declare.

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CASE REPORT

DELAYED PROGRESSION AND INEFFICIENT TRANSMISSION OF HIV-2

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Abstract. We report a case of HIV-2 infection with delayed progression, taking approximately one and a half decades to develop HIV related symptoms. The spouse was still negative for HIV with a history of having regular unprotected sex with the index case which highlights the inefficient transmissibility of HIV-2. Continued surveillance is needed in screening of HIV-2 infection, especially in cases with a high index of suspicion and risk factors for HIV-2, as these patients develop AIDS related symptoms quite late due to delayed progression.

Key words: HIV-2 infection, delayed progression, inefficient transmissibility, India

INTRODUCTION

In 1984, 3 years after the first report of the disease that would become known as AIDS, researchers discovered the primary causative viral agent, HIV-1. In 1986, HIV-2, was isolated from AIDS patients in West Africa. Aproximately 2.4 million people are currently living with HIV AIDS in India (UNAIDS, 2008). The first documented HIV infection in India was among sex workers in Chennai in 1986 (Simoes *et al*, 1987). Infection rates soared throughout the 1990s, the epidemic being most extreme in the southern half of the country and in the far north-east. Today the epidemic affects all sectors of Indian society, not just specific groups – such as sex workers and truck drivers – with which it was originally associated. The first evidence of HIV-2 infection in India was provided in 1991 (Rubsamen-Waigmann *et al*, 1991). Since then it has been sporadically reported from various states of India (Kulkarni *et al*, 1999). A recent 3 year study at two HIV centers in southern India reported HIV-2 prevalences of 0.32% and 0.13% (Murugan and Anburajan, 2007). HIV-2 appears to be transmitted principally by sexual contact, with prostitutes being the well-studied group. The virus can also be spread by contact with infected blood as a transfusion. Early evidence indicates the transmission pattern of HIV-2 differs in at least one significant manner from HIV-1: at least 30% of babies born to mothers infected with HIV-1 become infected themselves, but no more than 10% of infants born to HIV-2 infected mothers become infected (Kanki, 1991).

We report a case of HIV-2 infection in which delayed progression of disease and

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inefficient transmissibility are suspected.

CASE REPORT

A 49 year old mechanic presented to an integrated counseling and testing center (ICTC) as a direct walk-in for HIV testing in October 2008 with unexplained chronic diarrhea for longer than one month and weight loss of >10% of presumed body weight. He was asymptomatic until January 2008 when he developed oral plaque-like lesions, which later healed after receiving treatment from a local medical practitioner. The patient was specifically questioned about having experienced acute retroviral syndrome, but he was unable to recall any symptoms. The ICTC is in the Department of Microbiology, Maulana Azad Medical College and is associated Lok Nayak Hospital, which is a tertiary care hospital in New Delhi, India. The patient was a resident of Kerala and stayed with his spouse in Mumbai from 1994 to 2006 and then moved to Delhi. The patient gave a history of frequent contacts with commercial sex workers (CSW) and multiple male sexual partners. He revealed he was first tested for HIV in 1995 in a private laboratory. At that time he had a positive HIV test. To confirm his HIV status he gave another blood sample at another private laboratory and that test was negative; no copies of these test results are available. The patient stated he did not indulge in any high risk sexual behavior after that. Most private laboratories at that time were performing single HIV antibody tests and did not use the rapid test to detect HIV-2.

Following the guidelines of the National AIDS Control Organization (NACO) (WHO, 2007), after informed consent and pre-test counseling, his blood sample was tested for HIV using a rapid test (Immunocomb, J Mitra, Delhi) following manufacturer's instructions. Test was reactive for HIV, following which two more rapid tests (Retroquic QUALPRO Diagnostics Goa; Acon Trilene ACON BIOTECH, China) were carried out on the same sample (one test was able to differentiate between HIV-1 and 2). The sample was reactive on all three tests and was further confirmed by Western blot. The patient was found to be HIV-2 positive and was registered with the anti-retroviral therapy (ART) Center. His CD4 and CD3 counts (FACSCount system, Becton Dickinson) were 364 cells/μl and 1,749 cells/μl, respectively. Other investigations were within normal limits. The patient was investigated for chronic diarrhea, but a definitive diagnosis was not made since three stool samples revealed no pathogens. The case was clinically diagnosed as a case of unexplained chronic diarrhea, per NACO guidelines (WHO, 2007) since he had watery stool more than three times daily for longer than one month. VDRL and HCV test were negative but the HBsAg was positive. The patient's repeat CD4 and CD3 counts were 247 cells/μl and 1,650 cells/μl, respectively, on 14-02-09.

Since the patient was in an advanced stage of HIV, WHO clinical stage 3, and his CD4 count was <350 cells/μl, he was started on ART (ZDV + 3TC + NVP). Since the index case gave a history of regular unprotected sex with his spouse, we suggested the wife to be tested for HIV. She was found to be negative for HIV per NACO guidelines (WHO, 2007). She tested negative for HIV infection 3 years earlier under the Prevention of Parent to Child Transmission (PPTCT) program. Under NACO guidelines, pregnant women are offered the HIV test under the PPTCT program and those who give consent for HIV testing are tested after pre-test counselling. Their $2^{1}/_{2}$ year

old daughter was also tested for HIV and found to be negative. Adherence to ART was >95% for the index case and he reported no side effects due to ART. The index case was monitored for ART per NACO guidelines (WHO, 2007). He reported weight gain and his diarrhea resolved by 3 and 4 months after initiation of ART, respectively. He had an adequate immunological response per NACO guidelines (WHO, 2007) and his CD4 count increased to 400 cells/μl after six months of ART.

DISCUSSION

Although HIV-2 infection is mostly confined to West African countries it has been identified in other continents following sexual contact with foreigners with a history of frequent contact with CSWs or multiple male sexual partners, as in our case. These CSWs provide a bridge for transferring HIV-2 infection from high prevalence regions, such as West African countries, to low prevalence countries, such as India. In our case there is a possibility the CSW may have had contact with a sexual partner from Western Africa, who then transferred the disease to the index case. One study (Leaño *et al*, 2003) found a large number of travelers to and from different regions of the world plays an important role in introduction of diverse HIV strains into a country, and further suggests overseas contract workers be included in surveillance programs.

Studies of HIV-2 infection are limited, but to date comparisons with HIV-1 show both viruses share properties, such as CD4 cell tropism, mode of transmission and morphology, but differ at molecular, clinical and epidemiological levels.Though human infection with HIV-2 is associated with eventual immunologic failure, disease progression has been reported to be much slower. A prospective 8 year study concluded HIV-1 infected women had a 67% probability of AIDS-free survival 5 years after seroconversion, in contrast with 100% probability for HIV-2 infected women. The rate of developing abnormal CD4+ lymphocyte counts with HIV-2 infection was also significantly lower, which demonstrates HIV-2 has lower virulence than HIV-1 (Marlink *et al*, 1994). Our patient also presented with unexplained chronic diarrhea for longer than one month and weight loss of >10% of presumed body weight almost 14 years after a history of a first sexual contact with a CSW, before which he was apparently healthy. Persons infected with HIV-2 are less infectious early in infection. Infectiousness increases as disease progresses, however, the duration of this infectiousness is shorter than with HIV-1. Knowing this incidence is epidemiologically significant; >95% of infected individuals followed for at least 8 years fit a clinical definition of long-term non-progression.

With HIV-2 infection, both heterosexual and vertical transmissions have been reported to be rare. More discordant couples exist with HIV-2 infection than HIV-1 infection as was seen in our case and his spouse in spite of having regular unprotected sex with her husband for almost over 10 years. One study recently reported the levels of viral RNA in plasma are much lower in HIV-2- infected individuals despite similarities in age at infection and time infected (Popper *et al*, 2000). Another study showed HIV-2 infection is characterized by a significantly lower plasma viral level (Andersson *et al*, 2000).

Given the slower development of immunodeficiency and the limited clinical experience with HIV-2, little is known about the best approach to manage these

patients. More clinical experience is needed to determine the most effective treatment for HIV-2 patients, the optimal timing for ART is unclear.

Although the prevalence of HIV-2 infection is very low, the potential risk for obtained HIV-2 infection in some populations justifies routine HIV-2 testing for all people from areas with high HIV-2 prevalence, people sharing needles or having sexual partners known to be infected with HIV-2, people receiving transfusions or other non-sterile medical care from endemic areas and children of women with risk factors for HIV-2 infection (O'Brien *et al*, 1992).

In conclusion, continued surveillance is needed to monitor for and develop special guidelines for HIV-2 in the Indian population. Physicians involved in screening for HIV need to have a high index of suspicion in patients with risk factors for HIV-2 infection, to appropriately diagnose and treat the disease since these patients develop AIDS related symptoms late due to delayed progression. Guidelines should be created for HIV testing of spouses with HIV-2 infection to diagnose and treat the disease, since these cases have inefficient transmission.

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Seroprevalence of human immunodeficiency virus type 2 infection from a tertiary care hospital in Pune, Maharashtra: A 2 year study

Dear Editor,

Human immunodeficiency virus type 2 (HIV-2) belongs to the family *Retroviridae* and is morphologically similar to HIV-1. However, HIV-2 shows considerable difference in course of the disease and treatment modality.

The transmission rate for HIV-2 compared to HIV-1 is very low both by heterosexual route and mother to child transmission.[1] India is one of the few countries outside the African continent, in which a dual epidemic of HIV-1 and HIV-2 is occurring, though HIV-1 dominates. The first report of HIV-2 in India was from the port city of Mumbai in 1991 and soon after, infected individuals were identified from various parts of the country.[1]

We undertook a retrospective study to find seroprevalence of HIV-2 infection in patients from a tertiary care hospital in Pune, Maharashtra. The study group included a cross-section of patients attending out and in-patient departments of the hospital. Consent was taken from all patients and pre-test counselling was done. Ananalysis of HIV data was carried out over a 2 years period from March 2010 to March 2012. A total 21720 serum specimens were processed on ARCHITECT i1000 (fourth generation enzyme-linked immunosorbent assay-Abbott laboratories U.S.A) as per manufacturer's instruction. The ARCHITECT HIV Ag/Ab combo assay is a two-step immunoassay to determine the presence of HIV p24 antigen and antibodies to HIV-1 and/or HIV-2 using the chemiluminescent microparticle assay technology. However, it cannot differentiate between HIV-1 and HIV-2.

Reactive specimens were confirmed by HIV TRIDOT (J. Mitra and Company Ltd., New Delhi, India) and Retroquic (Qulpro Diagnostics, India) as per National AIDS Control Organization (NACO) guidelines and lab. protocols. Both these kits are approved by NACO and can distinguish HIV-1 and HIV-2. Results were interpreted as per product kit insert.

Out of total 21720 serum specimens tested, 242 (1.11%) were reactive by architect [Table 1]. (Indicating that these samples were positive for either HIV-1 or HIV-2 or both) These reactive specimens were further retested by TRIDOT and retroquic to distinguish if it was HIV-1 or HIV-2 infection, there was complete agreement between results of two tests. In our study, seroprevalence of HIV-2 was found to be 0.03%. Studies published previously give seroprevalence of HIV-2 in high-risk group only. The current study does not look

Table 1: Year wise results of HIV testing of serum specimens from March 2010 to March 2012

Year	Specimen number	Total specimens reactive for HIV-1 and/or HIV-2	Specimenreactive for HIV-1 only	Specimen reactive for HIV-2 only	Specimen reactive for HIV-1 and 2 (dualinfections)
March2010-February2011	10160	98	94	2	2
March2011-February2012	11560	144	140	2	2
Total	21720	242	234	4	4

HIV: Human immunodeficiency virus

into high-risk population of HIV such as sex workers or intravenous drug abusers as regular attendees of this corporate tertiary care hospital does not exclusively include such population. The exact figure of prevalence rate of HIV-2 infection in general Indian population is not available so far. Serological estimates on the prevalence of HIV-2 infection vary from 0.33% to 2.05% of the total HIV infection in various regions of India.[2]

A study conducted in Mumbai, Maharashtra found seroprevalence of HIV-2 to be 0.35%.[3] Seroprevalence of 0.29% for HIV-2 infection was observed in South Tamil Nadu.[4]

The Southern States of Tamil Nadu have found seroprevalence of HIV-2 at 0.03%.[5]

According to our lab protocols, all the serum specimens for HIV testing are processed first on ARCHITECT, which has sensitivity and specificity of 100%. It detects both antigen and antibodies to HIV, so it is expected to pick-up patients in the window period. However, the test cannot differentiate between HIV-1 and HIV-2. This differentiation becomes essential in the light of existence of dual HIV infection in India.

All reactive specimens were tested by HIV TRIDOT and HIV Retroquic (which have a sensitivity and specificity of almost 100% according to the manufacturers) to identify pure HIV-2 infections and dual infections. Results of both tests match and thus confirm HIV-2 seroreactivity.

It is important to differentiate between HIV-1 and HIV-2 virus as clinical course and treatment modalities differ. Non-nucleoside reverse transcriptase inhibitor, which are first line of drugs against HIV-1 virus and are usually given in government and antiretroviral therapy centres are not effective against HIV-2.

To conclude, it is the need of the hour to use cost-effective tests to differentiate between these two viruses and to assess the exact prevalence and incidence of HIV-2 infection in India so as to frame specific guidelines and treatment modalities for management of HIV-2 infections.

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Hepatitis B virus and human immunodeficiency virus infections are a public health problem even in rural communities of Vellore district, Tamil Nadu

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Dear Editor,

Hepatitis B virus (HBV) and Human immunodeficiency virus (HIV) infections have been reported from many parts of India, especially from urban centers. [1] There are only a few reports from centers on the problem of these infections in rural communities. [2]

We report here data collected over three years starting from 2005 on HBV and HIV prevalence. This study was carried out at a 150-bed tertiary hospital. The hospital serves a large rural and tribal population of about 130,000 in a 50 km radius. The HBsAg screening was carried out by Quick Chek-HBsAg (flow through device, Morepen Laboratories Ltd, New Delhi). HIV antibody was screened by Retroquik HIV (flow through device, Qualpro Diagnostics, Goa), and the antibody status confirmed by a supplementary test ELISCAN HIV (RFCL limited, Haridwar) as per strategy IIB of the NACO guidelines. The presumed HIV positive individuals were referred to the Integrated Counseling and Testing Center, at the Government Vellore Medical College Hospital, Vellore for management. These tests were carried out as part of preoperative screening, antenatal screening, and on patients suspected to have these infections, after their verbal consent.

In our study the frequency was quite high. Of the 6233 individuals screened between 2005 and 2007, 106 (1.7%) were HBsAg positive. Among these, 2493 were from rural areas with HBsAg positive rate being 1.97%. In the periurban population the frequency was 1.52% of 3740 individuals. There was no statistically significant difference between the two groups (Chi square test; $P > 0.05$). Studies from rural areas on the frequency of HBV infection as measured by detection of HBsAg have shown a high frequency in Tamilnadu, [1] Punjab, [2] and Maharashtra. [3] The percent frequency has been in around 5%. In rural areas, outbreaks of HBV infections have also been reported to be associated with injection practices. [4] The reasons for the high frequency of HBV infections in our rural population needs to be investigated. An earlier study reported that poor injection practice, high risk sexual behavior, and transmission in childhood could be important factors. [1]

In our hospital between the years 2005 and 2007, 94 (1.85%) of 5085 individuals from rural and periurban communities tested were HIV antibody positive. Among these, 2034 individuals were exclusively from rural communities. The HIV antibody frequency was 1.72%. In the peri group, the frequency was 1.93% of 3051 individuals. There was no statistically significant difference between the two groups (Chi square test; $P > 0.05$). There are only a few reports on the frequency of HIV in rural communities. [5],[6] One particular study from Tamil Nadu, [5] carried out in the mid-1990s indicated 7% frequency among rural population. Another study from Tamil Nadu carried out on samples collected in 1999 and 2000, indicated a relatively low frequency of only 0.66% among rural people. The NACO statistics presently indicates a country wide reduction in the frequency and the estimate for the general population is 0.3-0.4%. However, the rates are ten times higher among high risk groups. The rural communities in our service area have high frequency of HIV, suggesting a need to intervene with an AIDS awareness program.

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Role of Interferon Gamma Release Assay in Active TB Diagnosis among HIV Infected Individuals

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Abstract

Background: A rapid and specific test is urgently needed for tuberculosis (TB) diagnosis especially among human immunodeficiency virus (HIV) infected individuals. In this study, we assessed the sensitivity of Interferon gamma release assay (IGRA) in active tuberculosis patients who were positive for HIV infection and compared it with that of tuberculin skin test (TST).

Methodology/Principal Findings: A total of 105 HIV-TB patients who were naïve for anti tuberculosis and anti retroviral therapy were included for this study out of which 53 (50%) were culture positive. Of 105 tested, QuantiFERON-TB Gold in-tube (QFT-G) was positive in 65% (95% CI: 56% to 74%), negative in 18% (95% CI: 11% to 25%) and indeterminate in 17% (95% CI: 10% to 24%) of patients. The sensitivity of QFT-G remained similar in pulmonary TB and extra-pulmonary TB patients. The QFT-G positivity was not affected by low CD4 count, but it often gave indeterminate results especially in individuals with CD4 count $<$200 cells/µl. All of the QFT-G indeterminate patients whose sputum culture were positive, showed $\leq$0.25 IU/ml of IFN-γ response to phytohemagglutinin (PHA). TST was performed in all the 105 patients and yielded the sensitivity of 31% (95% CI: 40% to 22%). All the TST positives were QFT-G positives. The sensitivity of TST was decreased, when CD4 cell counts declined.

Conclusions/Significance: Our study shows neither QFT-G alone or in combination with TST can be used to exclude the suspicion of active TB disease. However, unlike TST, QFT-G yielded fewer false negative results even in individuals with low CD4 count. The low PHA cut-off point for indeterminate results suggested in this study ($\leq$0.25 IU/ml) may improve the proportion of valid QFT-G results.

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Introduction

Tuberculosis (TB) remains the single infectious disease, causing the highest mortality in humans, leading to 3 million deaths annually. Approximately 8–10 million people are infected with this pathogen every year [1]. The vast majority of TB cases are reported in Africa, South East Asia and Western Pacific countries. The recent increase in the number of cases even in developed countries, associated with the spread of human immunodeficiency virus (HIV) infection, has had a major impact on the current situation of TB. India accounts for a huge number of HIV-TB cases and ranks first in the world in terms of incident of TB cases. [2].

Individuals with HIV infection are at increased risk of rapid progression of a recently acquired tuberculous infection, as well as of re-activation of latent TB infection (LTBI). Delayed diagnosis of TB and initiation of appropriate treatment more than 3 weeks after presentation, are associated with 45–85% of deaths in HIV infected patients [3]. Early diagnosis and prompt treatment for TB are the key elements to control the mortality rate of HIV infected subjects. The clinical features of HIV-infected patients with TB are often non-specific. Decreased tuberculin reactivity, lower sensitivity of acid fast staining, atypical radiographic presentations, and similarity in presentation with other HIV related infections hinder the diagnosis of TB in HIV infected patients [4].

Recently introduced Interferon gamma release assays (IGRA) are promising tests for the diagnosis of TB infection. IGRA is available in two commercial formats: (i) Quantiferon TB Gold (QFT-G) (Cellestis Ltd, Victoria, Australia), which measures the quantity of IFN-γ secreted by T cells and (ii) T-Spot.TB assay (Oxford Immunotech, Oxford, UK) which enumerates the number of IFN-γ secreting T cells after *in vitro* stimulation with TB-specific antigens. T-Spot.TB assay uses only Early Secreted Antigen Target (ESAT)-6 and Culture filtrate protein (CFP)-10, whereas an additional antigen TB7.7 is incorporated in Quantiferon TB Gold. Several studies have been conducted in various clinical settings on the accuracy and utility of IGRA and these have been reviewed elsewhere [5]. Most of these studies have reported that sensitivity of IGRA is modest to detect active TB disease [6–12] and also suggested that IGRA alone [13,14]

or in combination with TST [15] can be used to exclude the suspicion of active TB disease. Nevertheless, in general there is a concern about the sensitivity of IGRA in immunosuppressed (especially HIV) patients, as these assays are T-cell based whose number is often compromised in these patients [16]. Only a very few studies have been conducted on IGRA assays in immunocompromised patients, especially from TB endemic countries [17,18], emphasizing the need for more such studies to test the validity of the IGRA assays in such settings. Hence, in this study, we aimed to measure the sensitivity of IGRA in diagnosing of active TB among HIV positive subjects in a country like India where both the diseases are co-existing and are endemic.

Materials and Methods

This study was approved by the Scientific Advisory Committee and Institutional Ethical Committee of Tuberculosis Research Centre, Chennai. A written and informed consent was obtained from all the study participants before drawing blood.

Study subjects recruitment

The recruitment of study subjects were done at Government Hospital of Thoracic Medicine, Tambaram, Chennai during April 2007 and March 2008. The demographic details and information on previous tuberculin skin test (TST) results were collected. Individuals with previous history of TB, silicosis, end stage renal disease, leukemia/lymphoma, who had TST in the past 16 months, under ATT for more than two weeks or ART or immunosuppressive therapy were excluded from the study. Pregnant and lactating patients were also excluded.

After registering the eligible patients, the radiological examination was carried out. A total of six sputum samples were collected from each study subject and they were stained for acid fast bacilli (AFB) microscopy. The staining for acid fast bacilli was done by Ziehl-Neelsen method [19]. Three sputum samples of each subject were cultured in conventional Lowenstein Jensen (Biomerieux Inc., Marcy I'Etoile, France) and also in liquid MP BacT medium (Biomerieux Inc., Marcy I'Etoile, France). The presence of *M. tuberculosis* in the positive culture samples was further confirmed by Gen-probe based PCR (Biomerieux Inc, Marcy I'Etoile, France) method.

The presence of active TB was defined as positive for sputum smear microscopy and/or identification of *M. tuberculosis* in sputum culture and/or abnormality suggestive of TB in chest x-ray. In addition, Fine Needle Aspiration Cytology (FNAC) was carried out in the individuals with extra pulmonary manifestations who were clinically suspected but had negative sputum results and normal chest x-ray. The diagnosis of pulmonary TB for the individuals with culture and smear negativity was based on chest x-ray finding and the clinician's opinion based on the clinical manifestations.

Blood was drawn from all the HIV-TB patients for total blood count, HIV serology and QFT-G. Then the TST was carried out. After the diagnosis, the patients were referred to respective treatment centers.

HIV testing

The HIV status was confirmed by 2 rapid tests (Retroquic Comb Aids-RS, Span Diagnostics, India and HIV TRI-DOT, J. Mitra & Co, India). When a serum was positive for both tests, it was considered as HIV positive. If a serum was positive for only one EIA (which was rare), Western Blot was done as confirmatory test.

CD4 count

The CD4 cell count was estimated in blood samples of HIV positive individuals by flow cytometry. 100 µl of whole blood was labelled with saturating concentrations of anti CD3-FITC, anti CD4-PE and anti CD8-APC (BD Biosciences, CA, USA). After 30 min incubation at 4°C, the red blood cells were lyzed using FACS lysing solution (BD Biosciences, CA, USA) and then fixed with 1% paraformaldehyde (Sigma Chemicals Co., MO, USA). The acquisition was done on FACS Calibur (BD Biosciences, CA, USA) and the percentages of CD3, CD4 and CD8 cells among the total lymphocytes were obtained using Flowjo Software (Tree star, Inc., CA, USA). The absolute CD3, CD4 and CD8 counts were calculated by multiplying the percentage with the total lymphocyte count.

Interferon gamma release assay

The IFN-γ release assay was performed using Quantiferon TB-Gold In-tube (QFT-G) test (Cellestis Ltd., Victoria, Australia). One ml of blood was taken in each of the three tubes precoated with TB–antigen, phytohemaglutinin (PHA) for the positive control or no antigen for the negative control. The blood samples were drawn between 10 and 11 AM and taken to the lab within 2 hrs of phlebotomy. The tubes were incubated for 16–24 hrs at 37°C and plasma were collected after centrifugation and stored at 4°C until assayed. Within two weeks of time, QFT-G enzyme linked immunosorbant assay (ELISA) was carried out. The test results were interpreted using the software given by the manufacturer (Cellestis Ltd., Victoria, Australia) and the cut-off point for the diagnosis was determined as per manufacturer's instructions. If the IFN-γ secretion in response to TB antigen was ≥0.35 IU/ml, after subtracting NIL control IFN-γ, it was considered as positive for QFT-G and if the value was <0.35 IU/ml, it was considered as negative. If the negativity was associated with poor PHA response (i.e. IFN-γ secretion in response to PHA, after subtracting NIL control IFN-γ was <0.5 IU/ml), it was considered as indeterminate or invalid result for QFT-G.

Tuberculin Skin Test

The 2 TU (tuberculin unit) of purified protein derivative (PPD) RT23 (Staten Serum Institute, Copenhagen, Denmark) was injected intradermally by Mantoux method and the induration was measured between 48–72 hrs after PPD injection by trained professionals. The cut-off point for TST positivity was considered as 5 mm for this study [20,21].

Statistical Analysis

Data were analyzed using SPSS 15.0 and Graphpad Prism 4.0 software. Mann-Whitney "U" test was carried out to calculate the difference between the groups. Logistic regression analysis was performed to examine the effect of potential variables on the odds of presenting a positive QFT-G test response. Odds ratios (OR) and their 95% confidence intervals (CI) were estimated by multivariate analyses. In these analyses, continuous variables were taken as dichotomous variables using the following arbitrary cut-off values: Age-36 years (median of the study population) and BMI-18.5 $kg.m^2$. Based on the CD4 count, the patients were classified as <50 (Advance stage), 50–199 (AIDS defining) and >200 cell/µl to assess QFT-G and TST results.

Results

A total of 300 HIV positive subjects were assessed during the study period. Among them 112 (37%) were identified as HIV-TB positive patients. 7 (6%) out of 112 patients refused to participate in this study and hence the remaining 105 were included. Among 105 patients who all were naïve for ATT and ART, 53 (50%) were culture confirmed TB patients. The demographic and baseline characteristics of all the 105 study subjects are given in **Table 1**.

Table 1. Demographic and baseline parameters of all the 105 HIV-TB patients.

Category	Subcategory	N (%) or Median (Range; IQR)
Sex, Number (%)	Male	84 (80)
	Female	21 (20)
Age, Median in years (Range; IQR)		36 (18–63; 25, 36)
BMI, kg/m^2 Median (Range; IQR)		19 (13–31; 17,21)
HIV strain, Number (%)	HIV-I	94 (90%)
	HIV-I&II	11 (10%)
TB types, Number (%)	PTB	66 (63%)
	EPTB	39 (37%)
Smear, Number (%)	Positives	47 (45%)
	Negatives	58 (55%)
Culture, Number (%)	Positives	53 (50%)
	Negatives	52 (50%)
CD4, Median (Range; IQR) (Available for 81 subjects)		116 (11–2062; 48, 209)

BMI – Body mass index.
PTB – Pulmonary tuberculosis.
EPTB- Extra-pulmonary tuberculosis.
IQR-Inter quartile range.
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Sensitivity of QFT-G and TST

The results of QFT-G and TST are given in **Table 2**. Of 105 patients tested, 68 (65%, 95% CI: 56% to 74%) were positive and 19 (18%, 95% CI: 11% to 25%) were negative for QFT-G. The remaining 18 (17%, 95% CI: 10% to 24%) patients showed indeterminate results. All the indeterminate or invalid results were due to poor response to PHA. Among the 105 patients, 33 showed ≥5 mm induration for TST yielding the sensitivity of 31% (95% CI: 22% to 40%). When comparing QFT-G and TST results, the former was 33% more sensitive than the latter (P<0.001). None of the TST positives were negative or indeterminate for QFT-G (**Figure 1**). Among the 72 TST negatives, 50% were positive and 25% were negative for QFT-G.

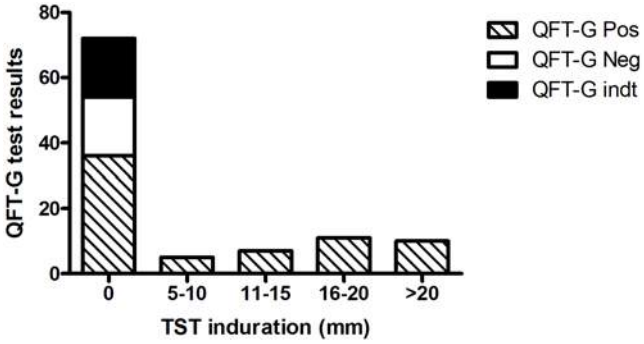

Figure 1. Agreement between TST and QFT-G test results. Among the 105 subjects tested, 72 were negative for TST. Of the 72 TST negative, 50% were positive for QFT-G. None of the TST positive was negative or indeterminate for QFT-G. TST-Tuberculin Skin Test, QFT-G-Quantiferon TB Gold in-tube, Pos - Positive, Neg - Negative, Indt – Indeterminate.
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In a sensitivity analysis to investigate whether inclusion of highly probable cases (which was diagnosed based on abnormalities found in the chest X-ray and clinician's opinion) affected the sensitivity of the tests, we calculated the sensitivity in culture positive and negative cases separately. The positivity obtained in culture positive PTB, culture negative PTB and EPTB patients were 66% (95% CI: 52%–80%), 45% (95% CI: 24% to 66%) and 77% (95% CI: 64%–90%) for QFT-G and 25% (95% CI: 12%–38%), 27% (95% CI: 8%–46%) and 41% (95%CI: 26%–56%) for TST respectively. The positivity of QFT-G was significantly higher than TST in culture positive PTB and EPTB groups (P<0.001 and P = 0.002 respectively) but not in culture negative PTB group (P = 0.347). The proportion of QFT-G indeterminate results obtained in these groups was 23%, 18% and 10% respectively.

Influence of Immunosupression on QFT-G and TST

The median total lymphocyte, CD3, CD4 and CD8 counts did not differ significantly between QFT-G positive and negative subjects but was significantly lower in indeterminate subjects when compared to QFT-G positives (P = 0.0013, P = 0.003, P = 0.0418

Table 2. Performance of QFT-G and TST in subgroups of HIV-TB patients.

Groups	QFT-G			TST	
	% Pos (95%CI))	% Neg (95%CI)	% Indt (95%CI)	% Pos (95%CI)	% Neg (95%CI))
Overall (N = 105)	65 (56–74)	18 (11–25)	17 (10–24)	31 (22–40)	69 (60–78)
PTB (N = 66)	59 (47–71)	20 (10–30)	21 (11–31)	26 (15–37)	74 (63–85)
Sputum culture positive (N = 44)	66 (52–80)	11 (2–20)	23 (11–35)	25 (12–38)	75 (62–88)
Sputum culture negative (N = 22)	45 (24–66)	37 (17–57)	8 (2–34)	27 (8–46)	73 (54–92)
Sputum smear positive (N = 43)	68 (54–82)	9 (1–18)	23 (10–36)	26 (13–39)	74 (61–87)
Sputum Smear negative (N = 23)	44 (24–64)	39 (19–59)	17 (2–32)	26 (8–44)	74 (56–92)
EPTB (N = 39)	77 (64–90)	13 (2–24)	10 (1–19)	41 (25–56)	59 (44–74)

Pos – Positive.
Neg – Negative.
Indt – Indeterminate.
Sen - Sensitivity.
PTB – Pulmonary tuberculosis.
EPTB- Extra-pulmonary tuberculosis.
doi:10.1371/journal.pone.0005718.t002

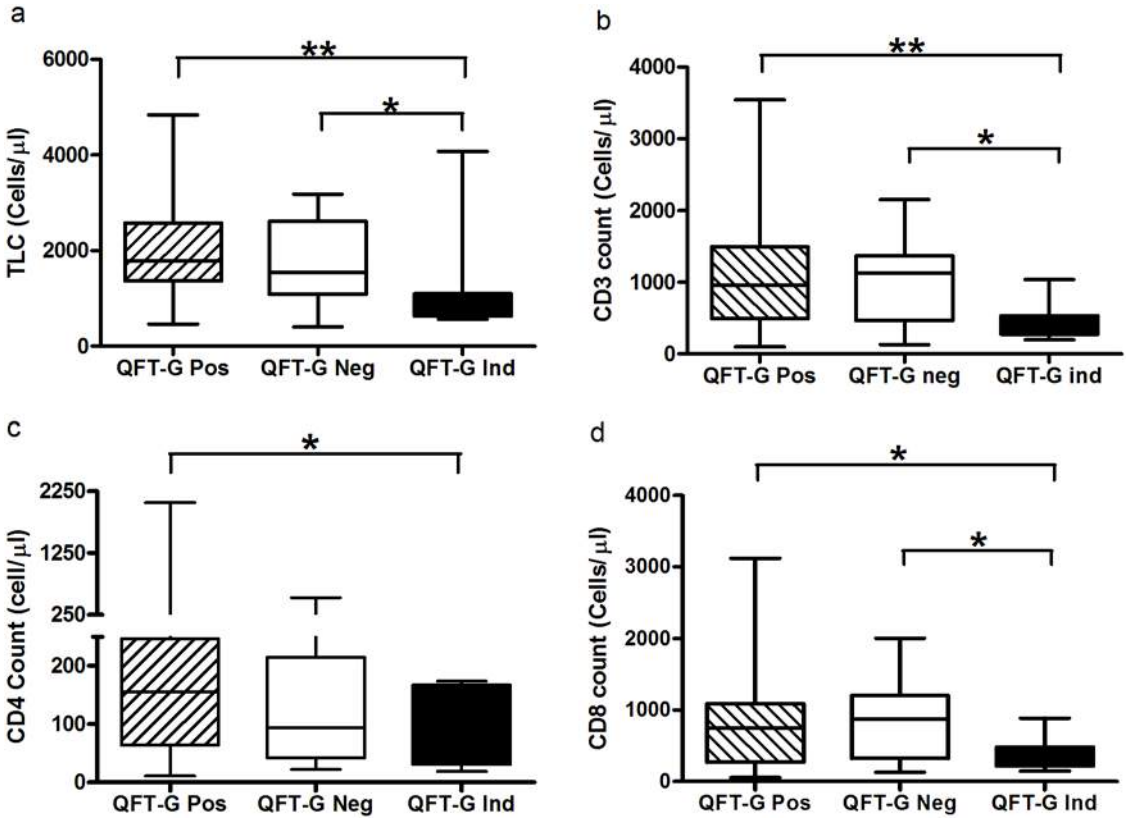

Figure 2. The level of total lymphocyte and T cell counts in QFT-G positive, negative and indeterminate subjects. The decline of total lymphocytes (a) or CD3 count (b) or CD4 count (c) or CD8 count (d) was associated with QFT-G indeterminate only but not with QFT-G negative. Box and Whisker plots show range, inter-quartile range and median. QFT-G – Quantiferon TB Gold (in-tube), * significant difference $p<0.05$, **significant difference $p<0.01$ by Mann-Whitney U test.
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and $P = 0.0221$ respectively) (**Figure 2**). When comparing the TST positive and negative subjects, the cell counts were significantly lower in TST negative than positive subjects ($p = 0.0403$, $p = 0.0344$ and $P = 0.017$ respectively) (**Figure 3**).

Further to confirm the influence of CD4 count on the positivity of QFT-G and TST, we developed a logistic regression model based on the CD4 counts (**Table 3**), for which, we stratified the CD4 cells counts as <50 cells/μl, 51–200 cells/μl and >200 cells/μl. When the QFT-G indeterminate results were considered as negative, the sensitivity of the assay was impaired in subjects with CD4 count <200 cells/μl ($P = 0.047$). This trend was not observed, if the indeterminate results were excluded ($P = 0.124$). TST positivity was decreased when the CD4 cell count dropped to <200 cells/μl, the ($P = 0.04$) (**Table 4**). The other tested parameters HIV strain, BMI, age, sex and types of TB were not found to influence the performance of QFT-G and TST.

QFT-G cut-off values

We analyzed the cut-off values for secretion of IFN-γ in response to TB antigens and PHA in culture confirmed TB cases. Applying the cut-off ≥0.13 IU/ml for TB antigens as suggested by Harada *et al* [22] to our data, did not improve the sensitivity of QFT-G. Only one indeterminate subject became positive for QFT-G.

A total of 36 subjects showed IFN-γ secretion <0.35 IU/ml in response to TB antigens. Among them 18 subjects were negative (i.e. IFN-γ secretion to PHA was >0.5 IU/ml) and the remaining 18 were indeterminate (i.e. IFN-γ secretion to PHA was <0.5 IU/ml) for QFT-G. The ranges of IFN-γ levels to PHA were 0.5 to 15.23 IU/ml in QFT-G negative subjects and 0–0.39 IU/ml in QFT-G indeterminate subjects. Of 18 indeterminate subjects, 12 (67%) showed ≤0.1 IU/ml IFN-γ response and 16 (89%) showed <0.25 IU/ml IFN-γ response to PHA (**Table 5**). Interestingly, all the culture positive HIV-TB cases who were indeterminate for QFT-G showed <0.25 IU/ml IFN-γ response to PHA.

Discussion

Early diagnosis of the active tuberculosis disease will help in earlier treatment, which is especially needed in HIV infected cases, since there is an accelerated progression of TB and higher mortality. However, there are lacunae in existing methods to diagnose TB in HIV positive subjects, which urge the development of alternative, rapid and accurate method. In this study, we have assessed the sensitivity of an emerging diagnostic method, IGRA among newly diagnosed HIV-TB patients.

QFT-G (In-tube) was recently approved by US Food and Drug Administration (FDA) in October 2007 and is the only commercially available IGRA test in India. Hence, in this study we used QFT-G (in-tube) method to analyze the role of IGRA.

In this study, we found that QFT-G can detect only 65% of active cases among the HIV infected individuals. Even in the culture confirmed TB cases, the QFT-G sensitivity was maintained as 66%. All the TST positives were positive for QFT-G. Hence, combining TST with QFT-G also could not enhance the overall sensitivity. If the indeterminate results were excluded, QFT-G

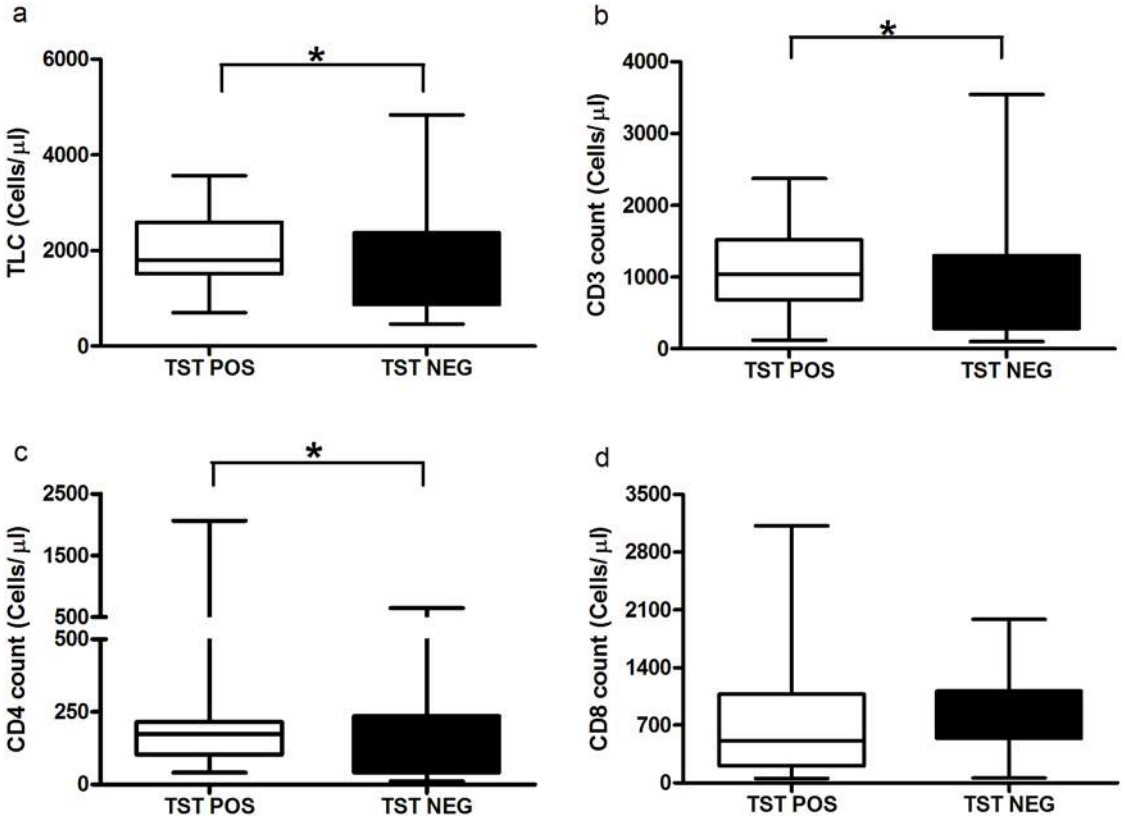

Figure 3. The level of total lymphocyte and T cell count in TST Positive and negative subjects. The level of total lymphocytes (a), CD3 count (b), CD4 count (c) were significantly low in TST negatives than TST positives whereas CD8 count (d) was not significantly different. Box and Whisker plots show range, inter-quartile range and median. TST – Tuberculin skin test, * significant difference $p<0.05$ by Mann-Whitney U test.
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showed 78% overall sensitivity and 88% sensitivity in culture confirmed cases. Hence, we conclude that QFT-G alone or in combination with TST cannot be used to exclude the active TB disease among HIV infected individuals. Of note, due to the inefficiency in discriminating the active and latent TB, the specificity of QFT-G and TST will always be low for active TB diagnosis in endemic countries like India (5).

The major concern in using T-cell based assay is the influence of CD4 count on the sensitivity. In QFT-G, the overlapping peptides of ESAT-6, CFP-10 and TB7.7 antigens are used, which are MHC class II restricted and largely recognized by CD4 T cells. Hence, it can be presumed that the sensitivity of QFT-G would be affected when CD4 count drops severely. While some of the studies showed IGRA was less influenced by HIV infection [23,24], other study results contradict the observations [25,18]. However, in our study we did not find any significant difference in CD4 count between QFT-G positive and negative subjects. The logistic regression model also confirmed that QFT-G negative results are CD4 count independent.

Another drawback of T-cell based assays is its poor sensitivity in EPTB patients. It was reported that in EPTB patients, the secretion of IFN-γ to *M. tuberculosis* antigens is poorer than the

Table 3. Analysis of variables associated with positive QFT-G test results.

Variables	With Indeterminate results			Without Indeterminate results		
	N	Odds Ratio (95%CI)	P	N	Odds Ratio (95%CI)	P
CD4 ≥200	81	7.058 (1.196–41.656)	0.047	70	4.596 (0.658–32.095)	0.124
Sex female	105	0.423 (0.107–1.673)	0.220	87	0.712 (0.147–3.456)	0.673
Age >36	105	1.282 (0.392–4.196)	0.681	87	0.960 (0.222–4.145)	0.956
BMI >18.5	91	1.005 (0.293–3.441)	0.994	78	1.890 (0.448–7.972)	0.386
EPTB	105	3.066 (0.765–12.284)	0.114	87	2.567 (0.564–11.691)	0.223
HIV strain I	105	1.295 (0.239–7.014)	0.764	87	0.836 (0.118–5.916)	0.858
Presence of Oral candidiasis	84	0.628 (0.150–2.625)	0.628	81	1.174 (0.203–6.783)	0.858

BMI – Body mass index.
N – Number of patients tested.
doi:10.1371/journal.pone.0005718.t003

Table 4. Analysis of variance associated with TST positive results.

Variables	N	Odd Ratio (95% CI)	P
CD4 >200 cells/μl	81	10.881 (1.010–117.277)	0.04
Sex female	105	0.412 (0.084–2.032)	0.276
Age>36 years	105	2.226 (0.603–8.210)	0.230
BMI>18.5	91	0.740 (0.204–2.686)	0.647
EPTB	105	1.263 (0.357–4.464)	0.717
HIV strain I	105	2.079 (0.430–10.051)	0.363
Presence of Oral candidiasis	84	1.112 (0.294–4.212)	0.876

BMI – Body mass index.
N – Number of patients tested.
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PTB patients [26]. Lee *et al* [27] reported that QFT-G is poor in detecting EPTB cases. Dewan *et al* [28] have shown only 16% sensitivity for QFT-G in EPTB patients. In contrast, we obtained 77% sensitivity using QFT-G in EPTB patients, in our study. Exclusion of indeterminate results yielded 86% sensitivity, which is similar to what we observed with culture confirmed PTB (88%). Hence, we conclude that the sensitivity of QFT-G is not compromised in EPTB when compared to PTB patients. However, since most of the EPTB subjects in our study group were TB lymphadenitis cases, further evidence is needed on the sensitivity of IGRA in other forms of extra pulmonary TB.

The major disadvantage we found in using QFT-G for HIV-TB diagnosis is the high number of QFT-G indeterminate results. In order to rule out the technical errors, the stimulated as well as unstimulated plasma samples which were used for QFT-G testing were centrifuged at high speed and measured for IFN-γ level once again using QFT-G ELISA plates. However, none of the indeterminate results became positive. The occurrence of indeterminate results is often pointed out in earlier studies as drawback of IGRA in TB diagnosis especially among individuals with strong immunosuppression [29]. Ferrea *et al* reported that QFT-G showed 20% indeterminate results and it was associated with severity of immunosuppression [30]. In our study, we observed 17% indeterminate results and all the subjects who showed indeterminate results had CD4 count <200 cells/μl. Our observations corroborate Ferrara *et al* study results.

To improve the sensitivity and reduce the indeterminate results of QFT-G, we re-analyzed our data with different cut-off points. Applying the reduced cut-off point suggested by Harada et al, [22] (0.13 IU/ml) for TB antigen specific IFN-γ levels, did not improve the QFT-G sensitivity in our study. When we analyzed the levels of IFN-γ in response to PHA, we found that all the culture confirmed QFT-G indeterminate subjects secreted IFN-γ in the range of 0–0.25 IU/ml and in particular most of the subjects showed ≤0.10 IU/ml.

A valid and lower cut-off point for PHA is required to overcome the misclassification of true negatives as indeterminate for QFT-G, when it is applied for active TB diagnosis in a clinically suspected population. Based on our study results, since the subjects with >0.25 IU/ml of IFN-γ secretion to PHA were able to give valid QFT-G results, we suggest that the IFN-γ level <0.25 IU/ml would be a better cut-off point for PHA. However, this needs further evaluation with a large sample size.

There are some limitations in our study. The diagnosis of active TB in patients with smear and culture negative results, was made only by clinical and radiology based methods and they were not followed up to find their response to anti-TB treatment. Clinical and radiological evidence have the inherent limitation of misclassifying other lung diseases as TB, especially in an endemic setting, which might be one of the reasons for obtaining the low positivity (65%) for QFT-G. However, similar positivity was observed among culture confirmed patients. Thus this limitation is less likely to affect our conclusions. Most of our study patients (73%) had CD4 count <200 cells/μl, which is considered as AIDS defining advanced stage. This might be alternative reason for obtaining low positivity for QFT-G and TST. The previous study which evaluated QFT-G in HIV-TB patients from an endemic country also reported a similar sensitivity (63%) [18]. In addition, another study from India also showed a low QFT-G sensitivity of around 70% in active TB patients [31]. Another limitation of our study is it did not report the specificity of QFT-G in our population since HIV positive TB negative subjects were not recruited. In this study, we used QFT-G and not another commercial IGRA test, T-SPOT. TB. It is reported that T-SPOT. TB is more sensitive than QFT-G [9,27,32]. Hence, further studies on the sensitivity of T-SPOT.TB in HIV-TB patients are needed to assess the role of IGRA in finding active TB among suspected cases.

Table 5. The level of IFN-γ secretion in response to PHA in QFT-G indeterminate subjects.

Subjects	Sputum culture results	Mitogen – Nil (IU/ml)
1	Negative	0
2	Positive	0.089
3	Positive	0.356
4	Positive	0
5	Negative	0.095
6	Positive	0.073
7	Negative	0.099
8	Negative	0.16
9	Negative	0.121
10	Positive	0.073
11	Positive	0.24
12	Positive	0.021
13	Positive	0.045
14	Positive	0.008
15	Negative	0.1
16	Negative	0.39
17	Positive	0.24
18	Positive	0.06

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Conclusions

To conclude, the QFT-G alone or in combination with TST cannot be used to exclude the suspicion of active TB among HIV infected individuals. Considering 65% sensitivity obtained using QFT-G alone, we suggest that the combination of QFT-G with any other test which detects active TB disease in the later stage such as detection of antigen in the urine samples could be better approach to exclude the active TB disease. In addition, the low IFN-γ cut-off point for PHA in indeterminate results, suggested in this study (≤0.25 IU/ml), may improve the proportion of valid QFT-G results, when QFT-G is applied to detect active TB cases among clinically suspected population.

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Author Contributions

Conceived and designed the experiments: AR. Performed the experiments: BSAK. Analyzed the data: VP. Contributed reagents/materials/analysis tools: RS SS PP. Wrote the paper: BSAK. Clinician responsible for patient recruitment: RS PP. Coinvestigator in R03 Grant: SS PP. Clinician involved in subject recruitment: SS. PI in the NIH R03 Grant: AR. Corrected and modified the manuscript: AR.

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Immuno-proteomic identification of human T cell antigens of *Mycobacterium tuberculosis* that differentiate healthy contacts from tuberculosis patients.

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IFN-γ- Interferon gamma

LPA- Lymphocyte proliferation assay

CFP- Culture filtrate protein

IL-4- interleukin-4

Abstract:

Identification of *Mycobacterium tuberculosis* antigens inducing cellular immune responses are required to improve the diagnosis and vaccine development against tuberculosis. To identify the antigens of *M. tuberculosis* that differentiated between tuberculosis patients (TB), and healthy contacts based on T cell reactivity the culture filtrate (CF) of in vitro grown *M. tuberculosis* was fractionated by two dimensional-liquid phase electrophoresis (2D-LPE) and tested for the ability to stimulate T cells in a whole blood assay. This approach separated the CF into 350 fractions with sufficient protein quantity (at least 200μg of protein) for mass spectrometry and immunological analyses. High levels of interferon-γ (IFN-γ) secretion were induced by 105 fractions in healthy contacts compared to TB patients ($p<0.05$). Most interesting was the identification of ten fractions that specifically induced strong IFN-γ production in the healthy contact population, but none in TB patients. Other immunological measurements showed 42 fractions that induced significant lymphocyte proliferative responses in the healthy contact group compared with the TB patients. Tumor necrosis factor-α (TNF- α) response for most of the fractions did not significantly differ in the tested groups and interleukin-4 (IL-4) response was below the detectable range for all fractions and both study groups. Proteomic characterization of the 105 fractions that induced significant IFN-γ response in the healthy contacts as compared to the TB patients led to the identification of 59 proteins, of which 24 represented potentially novel T cell antigens. Likewise, the protein identification in the 10 healthy "contact specific fractions" revealed 16 proteins that are key candidates as vaccine or diagnostic targets.

Introduction:

Tuberculosis (TB) is a major health problem throughout the world. Recent World Health Organization report shows that TB has been increasing at a rate of 1% per year and an estimated 9.2 million new cases arise each year (1). Though TB is preventable, there is an increase in its incidence in the recent years. Re-emergence of TB is mainly due to its association with human immunodeficiency virus infection (2), and also due to the occurrence of multi-drug resistant strains of the causative agent, *Mycobacterium tuberculosis* (3).

Vaccination, that involves limited contact with the patient population and is cost effective and represents one of the best biological measures for disease control. The current vaccine against tuberculosis, Bacille Calmette–Guérin (BCG) has been administered to more people than any other vaccine. The side effects of BCG are tolerable, and it prevents miliary and meningeal tuberculosis in young children. In striking contrast, it affords limited and highly variable protection (0 to 80%) against pulmonary TB (4). Thus, BCG does not seem to be a satisfactory vaccine (5, 6), and necessitates exploration of newer strategies to improve BCG or to develop a more effective vaccine.

One of the potential strategies for the development of an improved TB vaccine involves the use of the proteins secreted by *M. tuberculosis* during growth. There is evidence that proteins actively secreted by *M. tuberculosis* during growth induce cell mediated immune responses by causing expansion of specific interferon gamma (IFN-γ) producing T lymphocytes that are capable of recognizing and exerting antimicrobial effects against infected macrophages (7). The

importance of IFN-γ pathways in host defense against *M. tuberculosis* was clarified by experimental studies on IFN-γ knockout mice, as well as the identification and characterization of humans with mutations in IFN-γ receptor (8, 9).

Several studies have been carried out to define the secreted proteome of the *M. tuberculosis*. The earliest study aimed at the identification of mycobacterial culture filtrate proteins, used chromatography and an N-terminal sequencing to identify 8 culture filtrate proteins (10). Later, many studies used 2-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) combined with sensitive mass spectrometric methods for identification of proteins. The above mentioned approaches have identified nearly 300 culture filtrate proteins (11-13).

Identification of T cell antigens in a complex mixture was first done by a T cell Western blot method (14). Later, two-dimensional separation methods were used which involved protein separation by either isoelectrofocusing (IEF) (15) or chromatography (16) in the first dimension and preparative SDS-PAGE followed by whole gel elution (17) in the second dimension. Mouse T-cell antigens of *M. tuberculosis* were identified using this method (15). Mycobacterial antigens which induce immune response in healthy household contacts and treated TB patients were also mapped using this approach (16).

In the present study, 2-D liquid phase electrophoresis (LPE) along with an *in vitro* IFN-γ assay and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were used to identify potential human T cell antigens. Systematic screening of the *M. tuberculosis* culture filtrate (CF) proteome and comparative evaluation of cellular immune responses between TB patients and healthy contacts led to the identification of 59 proteins in the most immunogenic 2D-LPE

fractions. Twenty four potentially novel T cell antigens were identified and 16 proteins were identified in ten 2D-LPE fractions that differentiated healthy contacts from TB patients based on IFN-γ responses.

Materials and Methods

Growth of *M. tuberculosis* and preparation of culture filtrate protein (CFP):

M. tuberculosis H37Rv (ATCC 27294) colonies were transferred from LJ slants to 2 ml of Sauton's medium (Himedia Laboratories, India) and the cells dispersed using glass beads under sterile conditions. The bacterial cell suspension was transferred to 10 ml of Sauton's medium in a McCartney bottle for incubation at 37°C for 2 weeks. The bacilli were then transferred to 200 ml of Sauton's liquid medium and grown in shaker culture for 4 weeks, and transferred to a 4-L culture flask containing 2 L of Sauton's medium and grown as stationary culture for 4 weeks at 37°C. The bacilli in the culture were harvested by centrifugation at 3000 rpm for 30 min. The culture supernatant was filter sterilized using 0.20 μm filter (Pal Gelman Laboratory, Saint Germain en Laye, France) and the filtrate was concentrated using Quixstand Bench top Hollow Fiber System (GE Healthcare, Pittsburgh, PA, USA). The protein content in the CF was estimated using a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL, USA). The proteins were then distributed into smaller volumes along with sodium azide, at a final concentration of 0.2%, and stored at –80°C for later use.

Separtation of CFP by 2D-LPE:

CFP (300 to 350 mg) was solubilized in 60 ml of a buffer containing 8 M urea, 1 mM dithiothreitol (DTT), 5% glycerol, 2% digitonin, and 2% ampholytes (pH 3.0 to 10.0 and pH 4.0 to 6.0 at a ratio of 1:4) (Bio-Rad Laboratories, Hercules, CA, USA). The sample was loaded onto a liquid IEF system (Rotofor, Bio-Rad Laboratories) maintained at 4°C. Separation was achieved

by applying constant power (12W) until the voltage stabilized at approximately 1400V. Focusing was continued for an additional 30 min and terminated. The individual IEF fractions were harvested and their pH value determined. Each aliquot was subjected to SDS-PAGE and the proteins visualized by staining with silver nitrate. A total of three IEF runs were performed and fractions having similar pH from each run were pooled.

Preparative SDS-PAGE was performed with individual IEF fractions. Briefly, each IEF fraction (protein quantities ranged from 0.08 mg to 43 mg) was prepared for SDS-PAGE by the addition of 6X SDS-PAGE sample buffer (18) and heating at 95^{o}C for 5min. The reduced samples were loaded on 16 x 20 cm polyacrylamide gels which comprised of a 4% stack over a 12.5% resolving gel. The stacking gel contained a single 13 cm long sample well. Electrophoresis was performed at a constant current of 50 mA per gel until the dye front was approximately 2 cm from the bottom of the gel. The gel was equilibrated in elution buffer (60 mM Tris (pH 9.4), 40 mM CAPS) for 10 min and transferred to a Whole Gel Eluter (Bio-Rad Laboratories) according to the manufacturer's instructions. The proteins were eluted from the gel using 250 mA constant current for 1h. Thirty protein fractions (approximately 2.5 ml each) were harvested, and the protein concentration of each fraction was determined using the BCA assay (Pierce Biotechnology). Prior to testing for T cell stimulation, the 2-D LPE fractions were filter sterilized using a 0.2 μm filter. An aliquot (10 μg) of each eluted fraction was analyzed by SDS-PAGE and silver stained (19).

Identification of proteins

Proteins were digested in-gel or in solution with modified trypsin (Roche Molecular Biochemicals, Indianapolis, USA.). For in-gel digestions, 10 µg of selected 2D-LPE fractions was resolved by SDS-PAGE using 4 to 12% NuPAGE bis-Tris polyacrylamide gels and morpholine ethanesulfonic acid (MES) SDS running buffer (Invitrogen Corporation, Carlsbad, CA, USA). The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) and the resulting bands excised from the gel. Digestion of proteins with modified trypsin was performed as described previously (20, 21). For in-solution digestions, 5 µg of each fraction was digested with 0.8 µg of modified trypsin in 10% acetonitrile, 0.2 M ammonium bicarbonate and incubated overnight at 37°C. The reactions were terminated by the addition of 3 µl of 10% TFA. The resulting peptides from the tryptic digestions were dried , suspended in 15 µl of 5% acetonitrile, 0.1% acetic acid, and applied to a 0.2 x 50 mm C_{18} reversed-phase high-performance liquid chromatography (HPLC) column (Agilent Technologies , Santa Clara , CA). The peptides were eluted with an increasing gradient of acetonitrile at a flow rate of 5 µl per min using an Agilent 1100 capillary HPLC solvent delivery system. The effluent was introduced directly into a LTQ electrospray ion-trap mass spectrometer (Thermo-Finnigan San Jose , CA). The electrospray needle of the mass spectrometer was operated at 4 kV, with a sheath gas flow of nitrogen at 30 lb/in^2 and a heated capillary temperature of 200°C. Data-dependent tandem mass spectrometry (MS) was employed to generate fragment ions of individual peptides. The 5 most intense ions from the full MS scan were selected for fragmentation. Tandem MS was acquired for each precursor ion a maximum of two times before being placed on the dynamic exclusion list for 1 min. Ion fragmentation was achieved with 35% normalized collision energy.

MS/MS data were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA; Version 27, Rev. 12) and X! Tandem (www.thegpm.org; version 2006.04.01.2) software packages with data interrogation against the annotated *M. tuberculosis* genome (3912 entries). Fragment ion mass tolerance of 0.50 Da, parent ion tolerance of 1.5 Da, oxidation of methionine and acrylamide adduct of cysteine were specified in Sequest and X! Tandem, as part of the search criteria and variable modifications. The allowance for missed cleavages was two. Scaffold (Version Scaffold-01-06-05, Proteome Software Inc., Portland, OR) was used to validate peptide and protein identifications using a two peptide minimum and 95.0% peptide and protein probabilities as specified by the Peptide and Protein Prophet algorithms (22, 23).The Universal Protein Resource (UniProt) database was searched for getting the sub cellular localization information (www.uniprot.org) of identified proteins.

Study population:

The study was approved by the Institutional Ethics Committee of Tuberculosis Research Centre (TRC) and informed consent was obtained from all the persons who were enrolled in this study.

Ten patients with pulmonary TB were enrolled at the TRC clinic. The subjects of this group had not undergone anti-tuberculosis treatment when recruited for the study. Their age ranged from 26 to 52 years and the male to female ratio was 7:3. Two spot and one overnight sputum specimens were collected from each patient. Sputum specimens were examined for AFB by fluorescent microscopy (Auramine O phenol staining) and the samples were examined microscopically. All the TB patients were positive by sputum smear microscopy. For culture, the sputum specimens were processed by modified Petroff 's method and inoculated onto

Lowenstein Jensen (LJ) medium and incubated for up to 8 weeks at 37°C (24). Blood was collected from these subjects before treatment.

Seven individuals who shared living quarters with the tuberculosis patient agreed to join the study as healthy contacts (contacts) whose age ranged from 28-55 years. The male to female ratio was 5:2. Three heavily exposed health care workers, working closely with pulmonary tuberculosis patients for at least 2 years at TRC were also included in the study. Their age ranged from 28-35 years, and all were male. These individuals had no history of tuberculosis on the basis of personal history, physical examination, chest X-ray, and negative acid fast bacilli sputum smear microscopy.

Tuberculin skin test using 2 Tuberculin units of purified protein derivative (PPD) (Statens Serum Institut, Copenhagen, Denmark) was performed on all subjects, and an induration of 15 mm or more after 48 h was considered positive. All the ten healthy contacts enrolled in this study were PPD skin test positive. Out of the ten TB patients, seven were skin test negative and three were positive. All subjects were HIV negative as determined by Tridot (J.Mitra & co, India) and Retroquic (Qualprodiagnostics, India) assays with serum.

Heparinized blood (21 ml) was collected from all the subjects for the immunological assays presented in this study.

Lymphocyte proliferation assay (LPA):

A LPA was perfomed by diluting whole blood 1/10 in RPMI-1640 medium (Sigma Chemical Company, St. Louis, MO, USA), supplemented with glutamine (0.29 g/l), penicillin (100 IU/ml),

streptomycin (0.1 mg/ml) and amphotericin B (5 mg/ml), and cultured in 96-well flat bottom tissue culture plates. These cultures were stimulated with the 2-D LPE fractions, and as controls purified protein derivative (PPD) and phytohaemagglutinin (PHA). Cells cultured under similar conditions without any stimulation served as the negative control. Each antigen or fraction was added in triplicate wells to a final concentration of 5 μg/ml. The antigen stimulated cells were cultured for 6 days at 37^{o}C in 5% CO_2 atmosphere (Hera Cell, Kendro Laboratories, Germany). Sixteen hours before the termination of the cultures, 1 μCi of tritiated thymidine (^{3}H), (Board of Radiation and Isotope Technology, Mumbai, India) was added to each well. The cells were harvested onto glass fiber filters using a cell harvester (PHD, Cambridge Technology Ltd, Watertown, MA, USA), and the filter discs were dried overnight. An aliquot (2 ml) of scintillation fluid (0.05 mg/ml POPOP and 4 mg/ml PPO in toluene) was added to each filter disc and counted using a liquid scintillation beta counter (Wallac oy, Torku, Finland). The proliferation was measured as uptake of tritiated thymidine (^{3}H) by the cells and expressed as counts per minute (cpm). The mean cpm value of the triplicate culture was calculated. Proliferation was expressed as Stimulation Index (SI):

Stimulation Index (SI) = mean cpm with antigen / mean cpm without antigen.

Two analyses were performed with the lymphocyte proliferation data. First, the mean cpm value obtained for the ten healthy contacts were compared with ten TB patients, for each of the fractions using Mann-Whitney test (Graphpad Software, Sandiego, CA, USA) and p values <0.05 were considered significant. Based on the p value the fractions were grouped as "very highly significant" ($p<0.0005$), "highly significant" ($p<0.005$), "significant" ($p<0.05$), and "non-significant" ($p>0.05$).

In the second analysis, Subject showing a cut-off value of ≥3 SI was classified as a responder and the number of responders in each group was counted.

Cytokine measurements:

For quantification of cytokines (IFN-γ, TNF-α, and IL-4), cell-free culture supernatants were harvested after 6 days of *in vitro* stimulation by fractions and stored immediately (at -80 °C) until assayed. Cytokine production was determined by a standard ELISA technique using commercially available BD opt-EIA Kit (BD Biosciences, Franklin Lakes, NJ, USA) as per the manufacturer's instructions. The optical density (OD) values were read at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Two types of analyses were performed with the IFN-γ results. In the first analysis, the actual amount of IFN-γ secreted (pg/ml) in response to each fraction was calculated. The levels induced by each fraction was compared in the TB patient and healthy contact group using Mann-Whitney test (Graphpad Software, Sandiego, CA, USA), and p values <0.05 were considered significant. Based on the p value, fractions were grouped as "very highly significant" ($p<0.0005$), "highly significant" ($p<0.005$), "significant" ($p<0.05$) IFN-γ inducing, and "non-significant" ($p>0.05$).

In the second analysis, cut-off value for IFN-γ was fixed for each fraction by employing mean + 2 SD of IFN-γ levels in TB patients (susceptible population). Any subject with IFN-γ value above the cut-off point was classified as a responder. The number of responders to each fraction in each study group was calculated.

Results

2D-LPE separation of the CFP

The yield of *M. tuberculosis* CFP after 4 weeks of culture was 50 ± 5 mg per litre. A large quantity of culture filtrate protein (1g) was used as the starting material to ensure sufficient protein for immunological analysis and molecular identification of fractions. Initial experiments showed that when quantities of CFP greater than 350 mg were applied to preparative IEF excessive precipitation resulted. Therefore, three technical replicates of preparative IEF runs were performed, using 300 to 350 mg of protein per run. The 20 fractions collected from each replicate were analyzed by SDS-PAGE (data not shown). Fractions from each replicate were pooled based on their pH values resulting in a total of 20 pooled fractions, each corresponding to a specific pH range. The pH of the separated IEF fractions ranged from 2.5 to 12.9 and the protein content varied from 0.08 mg to 43 mg.

The second dimension preparative SDS-PAGE performed on each IEF fraction resulted in 30 sub-fractions, for a cumulative total of 600. All the fractions were numbered by IEF fraction number first, followed by whole gel elution fraction number (for eg. 1_1, 3_4 etc). Initially, the fractions were analysed by analytical SDS-PAGE On analysis, it was observed that each fraction showed 1–3 bands. Protein quantity of each fraction ranged from 50µg to 4mg. A minimum of 200 µg of protein was required for immunological as well as proteomic characterization. Out of the 600 2D-LPE fractions, 350 possessed 200 µg or more protein and these were selected for further analyses.

Definition of immunodominat fractions for TB healthy contacts.

IFN-γ, an important effector cytokine in tuberculosis infection (7) was used as a marker of antigen specific T cell activation in whole blood assays with the 2D-LPE fractions. In general, the antigen induced IFN-γ levels were higher in the healthy contact group when compared to the TB patients. The level of IFN-γ secreted by TB patients for all 2D-LPE fractions ranged from 0 to 2000 pg/ml whereas for healthy contacts, it ranged from 0 to 8000 pg/ml. Of the 350 2D-LPE fractions screened, 105 induced significant IFN-γ levels in the healthy contact group, compared to TB patients group (**Tables S1**). Based on the IFN-γ levels and the significance in difference between the two study populations the reactive fractions were sub-divided into three groups: (i) 32 fractions induced "very highly significant" levels of IFN-γ ($p < 0.0005$); (ii) 34 fractions stimulated "highly significant" levels of IFN-γ ($p < 0.005$); and (iii) 39 fractions produced "significant levels" of IFN-γ ($p < 0.05$) in the healthy contacts as compared to the TB patients. There were no fractions at all which induced significantly higher levels of IFN-γ in TB patients as compared to the healthy contacts.

Interestingly, nine of the 2D-LPE fractions designated "very highly significant" and one designated "highly Significant" for IFN-γ production in the healthy contact population showed a positive IFN-γ response in all healthy contacts (N=10), but a negative response in all TB patients (N=10), based on the mean IFN-γ response plus 2SD cut off value (**Fig. 1)**. These "contact specific fractions" are listed in **Table 1**.

Other cytokines such as TNF-α and IL-4 are known to influence the outcome of TB (7, 25). Thus, these two cytokines were also measured in the whole blood assays with the 2D-LPE fractions to determine if there were other immunological markers that could differentiate the healthy contacts and TB patients. All the 2D-LPE fractions induced a TNF-α response, but this response was not significantly different between the two study populations. It was noted that the spontaneous as well as antigen induced TNF-α level was found to be higher in TB patients when compared with healthy contacts for all the fractions, of which three fractions (6_7, 6_8, 9_9) showed a statistically significant difference **(Data not shown)**. In contrast to the TNF-α response, the IL-4 response was found to be below the detectable range in all the cell culture supernatants from both the groups **(Data not shown)**.

A fourth immunological parameter evaluated was induction by the 2D-LPE fractions of lymphocyte proliferation (LP) in the whole blood. Of the 350 fractions screened, 42 (12 %) induced significant proliferative response in the healthy contacts as compared with the TB patients (**Table S2**). Eight of these fractions were designated "highly significant" ($p<0.005$) and 34 were designated "significant" ($p<0.05$). All of the "highly significant" LP inducing fractions, except 8_30, showed positive LP responses in all 10 healthy contacts, and LP positivity in TB patients ranged from four to eight individuals depending on the 2D-LPE fraction (**Table S2**). The "significant" LPA fractions gave a positive LPA stimulation index for 7 to 10 individuals in the healthy contact group and in the TB patient group 4 to 10 individuals were LPA positive for the same fractions. Thus, unlike the IFN-γ assay no 2D-LPE fractions were identified that could differentiate all healthy contacts from TB patients by the LPA. There was also variability in the

correlation between the IFN-γ and LP responses. All but three of the 2D-LPE fractions inducing significant LP responses also induced significant IFN-γ secretion **(Table S2)**. However, several of the fractions not inducing a significant LP response did induce significant IFN-γ responses (Data not shown). The variability in these two immunological measurements carried over to the ten "contact specific fractions" defined through the IFN-γ response. Specifically, five of the "contact specific fractions" (8_2, 8_29, 8_30, 9_26, and 12_21) induced "highly significant" or "significant" LP responses in healthy contacts as compared to the TB patients (**Fig. 2)**, However, the remaining five "contact specific fractions" did not result in significant differences in the LP responses between healthy contacts and TB patients (**Table 1)**.

Molecular characterization of immunodominant 2D- LPE fractions

Given that current *in vitro* T cell based diagnostic assays for TB are based on the measurement of IFN-γ responses (26), and the majority of anti-TB vaccine candidates were selected based on their ability to induce an antigen specific IFN-γ response (27), we focused protein identification efforts on those 2D-LPE fractions that resulted in a significant IFN-γ response in the healthy contact population. Specifically, the IFN-γ inducing 2D-LPE fractions were analyzed by in-gel and in-solution proteolytic digestions followed by LC MS/MS and data interrogation via Sequest and collation via Scaffold was used to identify the dominant proteins in these immunologically reactive fractions (**Tables S3, S4, and S5**).

When all of the data were combined a total of 59 proteins were identified for the 105 2D-LPE fractions that had significant IFN-γ responses for the healthy contact population (**Table 2**). For

those proteins in the10-30 kDa molecular range, most were identified with 1 to 7 peptides, with a maximum of 7 unique peptide sequences found for the protein Rv2626c, followed by CFP17 (Rv1827) with 6 unique peptides. Proteins in the molecular range of 31-120 kDa were identified by matching 1-12 distinct peptides, with 12 distinct peptide sequences found to match KatG (Rv1908c) in fraction 13_12 (**Tables S6, S7, S8, and S9** contain the detailed proteomic data for each LPE fraction inducing a significant IFN-γ response). Among the 59 identified novel T cell antigens, UniPort database had showed that 12 were secretory, 4 proteins localized cell membrane, 15 proteins to cytoplasm and 1 protein has integral membrane location. Sub cellular localization information for 27 proteins was not annotated in UniPort database **(Table-2).**

The most relavent group of 2D-LPE fractions are those designated "contact specific fractions". Proteomic characterization of these ten fractions demonstrated 16 proteins **(Table 3)**, 13 of which (CFP-10, DnaK, FpbB, ESAT-6, GroEL2, GroES, HspX, KatG, PhoS1, PstS1, Rv2626c, TB8.4, TrxC), were previously reported as human T cell antigens. Three proteins (AcpM, Adk, and Rv3716c) identified in these fractions are potentially new human T cell antigens. Proteins could not be identified in two "contact specific fractions" (8_2 and 8_30) even though these fractions induced notable IFN-γ responses. Among the 16 proteins identified in "contact specific fractions" 4 proteins were secretory, 2 proteins were cell membrane associated and 4 protein have cytoplasmic location. Sub cellular localization of the remaining six proteins was not annotated in UniPort database **(Table-3)**.

The other 22 "very highly significant" IFN-γ inducing fractions that were not "contact specific" demonstrated 20 proteins (**Table S3**). Of these, ten were already reported as T cell antigens

(Table 2), and 10 (BfrB, GgtB, LpdC, MmsA, Pks13, Rv1910c, Rv1558, Rv2204c, Rv2251, and Rv2721c) are reported here as potentially novel human T cell antigens. Mass spectrometric analyses identified that 15 proteins in the in the 34 "highly significant" IFN-γ inducing fractions and 24 proteins in the 39 "significant" fractions. In the "highly significant" and "significant" IFN-γ inducing fractions, an additional 11 potentially new human T cell antigens were identified: (Ald, FabG4, ProA, Tal, Rv1324c, Rv3169, Acn, Fba, Frr, Pgi and SahH) (**Tables S4 and S5**).

A comparison of identified proteins across all of the IFN-γ inducing fractions ("very highly significant", "highly significant", and "significant") revealed only modest overlap in the proteins identified for each group. Specifically, only one protein (Rv2465c) were found in a 2D-LPE fraction of each group, and a total of eight proteins were shared between two of the groups (**Fig. 3**). These data demonstrate that immunological responses are stratified with respect to different antigens and it is this stratification that has allowed for the identification of the ten "contact specific fractions" and their corresponding proteins.

Discussion:

This present study was developed to identify the antigens of *M. tuberculosis* that have a potential use in TB vaccine or diagnostic development. Like many previous T cell antigen discovery efforts (15, 16, 28), we focused on the secreted proteins of *M. tuberculosis*. However, our current efforts differ from earlier studies which have used experimental animals (15), where as our study aims at immunological responses to human TB. Moreover, the present study used the differential immune response of TB patients and healthy contacts to identify those proteins of *M. tuberculosis* with the greatest potential as protective or diagnostic antigens.

Even though the immunological mechanisms of protection against tuberculosis are not fully understood, consistent evidence shows a dependence on antigen specific T lymphocytes and ability to stimulate the anti-mycobacterial activity of macrophages through the release of IFN-γ. The central role of IFN-γ in the control of TB is clearly demonstrated by the experiments which show that disruption of the IFN-γ gene in mice and mutation of IFN-γR gene in humans result in increased susceptibility to TB infection (8, 9). Therefore, the ability to stimulate T-cell release of IFN-γ has been used as a critical criterion for the identification of protective antigens for tuberculosis.

In our current studies medical, paramedical, laboratory staff and healthy household members that are in close contact with TB patients, but remain healthy with no evidence of disease are viewed as the "protected" population (contacts). Multiple studies provide evidence that antigens recognized by the "protected" group, but not active TB patients, can be considered for vaccine

development strategies by using IFN-γ response as a protective correlate (29-31). Although, donor to donor variation exists in antigens recognition and magnitude of response, this approach is considered as a highly viable method to identify the protective antigens (28). In agreement with a previous study (32), healthy contacts in our study displayed a strong IFN-γ response to the 2D-LPE fractions as compared with that of TB patients. Additionally, the stratification of the IFN-γ response allowed for the identification of ten 2D-LPE fractions possessing at least 16 proteins that are specific for the protected (contact) population verses the susceptible (TB patient) population.

The Quantiferon TB Gold assay is a well established T cell based *in vitro* diagnostic assay for infection with *M. tuberculosis* and is a significant improvement to PPD skin testing in a non-endemic population for tuberculosis surveillance (33). However, in endemic settings its ability to distinguish between infected and diseased individuals is severely limited (33). In fact our unpublished data with healthy controls and healthy household contacts of TB patients in Chennai, India reveals that 36% and 79% of these two populations, respectively, were positive to the Quantiferon TB Gold assay. This is compared to 96% positive response to the same assay in smear positive TB patients for the same area. The ten "contact specific fractions" identified in this study and the corresponding proteins show a positive IFN-γ response for 100% (10 of 10) healthy contacts and no reactivity for TB patients. These data provide evidence of proteins that if used in conjunction with the current Quantiferon TB Gold assay would allow for differentiation of infected and diseased individuals. Further, the proteins of the "contact specific fractions" are

also potential tools to monitor infected-healthy individuals for the development of active tuberculosis.

Many proteins in the CFP have been identified as potent T cell antigens including ESAT-6 (34), TB 8.4 (16), HspX (35), Ag85 complex (36) and CFP-10 (37). However, there is no direct correlation between the relative concentration of these antigens in CF and their immunological relevance. For instance, ESAT-6, which is present in low amounts in culture filtrate, acts as one of the most potent T cell antigens (34). Of the 350 2D-LPE fractions screened, we defined 105 fractions that induced significant levels of IFN-γ in healthy contacts as compared to the TB patients. In these fractions, 59 different proteins were identified. Among these proteins, 35 observed as immunodominant antigens in our study, were reported in earlier studies (**Table 2**), either as T-cell antigens in animal experiments or human studies or as B-cell stimulating antigens. Such concordance of our results with the pre-existing reports shows the reliability of the method used. Equally exciting is the fact that this methodology identified 24 proteins as potentially novel T cell antigens.

Among the 105 fractions inducing IFN-γ in healthy contacts, 10 fractions containing 16 proteins were considered "contact specific". Out of these, 3 proteins have been identified as novel human T cell antigens (Adk, AcpM, Rv3716c), along with the 13 already reported T cell antigens. Adk reported as a mouse T cell antigen (15) is identified as human T cell antigen in the present study. AcpM, previously identified as a weak B cell antigen is reported here as strong T cell antigen (38). The presence of Rv3716c protein in culture supernatants was reported earlier in proteomic

studies (11, 39) and it has been identified as an immunodominant molecule for the first time in the present study.

The novel proteins identified in this study can be classified into five groups: (i) the metabolic enzymes aconitase (Acn) (13), GgtB (40), Pgi (41), Tal, and FabG4; (ii) proteins with reduced presence or that are absent in BCG such as Ald (42), Rv1558 (43); (iii) proteins whose genes are up regulated during various stress conditions that include Fba (44), Rv1324 (45), MmsA (46), ProA (47), Rv2204c (48), Rv2721c (49): (iv) proteins which are absent in hyper virulent Beijing isolates, Rv1910c (50), Rv3179 (51); and (v) proteins identified as virulence factors such as LpdC/Rv0462 (52), SahH (53), Pks13 (54), (55), Frr (56), and Fba (53).

The presence of metabolic enzymes has been previously reported in the CF or extra cellular milieu of *M. tuberculosis* and various pathogens (57). The extracellular localization of these proteins may be due to autolysis, given the prolonged culture time (4 weeks) of *M. tuberculosis*. However, it is also possible they are present due to specialized secretion systems (58). The extra cellular metabolic enzymes of *M. tuberculosis* also have been identified as virulence factors (53, 59). A recent study (53) identified a list of proteins having plasminogen binding activity in *M. tuberculosis*. Interestingly this included the immunodominant antigens Ag85 complex, DnaK, MPT51, and GlnA1, as well as those of the potentially novel T cell antigens (LpdC, SahH, and Fba) identified in the immunodominant fractions of the current study.

Interaction between virulence factors and IFN-γ has been clearly documented in case of LpdC, a protein identified in this study. One of the activities of this protein is participating in the inhibition of phagolysosomal fusion by binding with coronin (a protein involved in

phagolysosomal fusion). Experiments showed that IFN-γ was able to inhibit LpdC protein association with coronin (60). In the present study, Lpd containing fraction induced increased IFN-γ secretion in "protected" population compared to TB patients, thus, corroborating that Lpd is produced *in vivo*.

It is generally believed that low molecular weight proteins of *M. tuberculosis* are predominantly recognized by the human T cells (61, 62), and most of the studies on human T cell antigens have focused on low molecular weight or selective pooling of fractions (16, 28) and reported that antigens <15 kDa were highly immunogenic. In the present study, only 14.8% of the immunodominant T-cell antigens were represented by antigens with a predicted mass of less than 15 kDa. A stronger immune response to the proteins below 25 kDa was observed in the present study (48.6%), which is in agreement with previous reports (16, 63). However, a sizeable number of recognized proteins (~31%) were also found to be greater than 40 kDa. Earlier studies have shown that secreted and membrane associated mycobacterial proteins were immunogenic (16, 79) and can be tested as vaccine targets. In this study we have found that 12 secretory and 5 cell membrane associated proteins to be immunogenic **(Table-2).** These proteins can be tested as potential vaccine targets.

Further animal and human studies are needed to explore the vaccine and diagnostic potential of the candidate antigens identified in this study. Experiments are underway to test the endemic normal population and TB patients who have successfully completed chemotherapy and remained quiescent for 1 year. These studies will help define the utility of the 16 "contact specific" antigens in distinguishing between "protected" and "susceptible" populations, and

whether IFN-γ responses to these antigens return after successful chemotherapeutic treatment of TB.

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Role of complement activation and antibody in the interaction between *Mycobacterium tuberculosis* and human macrophages

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Mycobacterium tuberculosis-specific antibodies possess immunomodulatory effects during tuberculosis infection. Prior sensitization to environmental mycobacteria is known to suppress immune responses against BCG and *M. tuberculosis*. Mycobacteria-induced antibodies can influence events such as complement activation and phagocytosis during infectious process. In the present study role of anti-*M. tuberculosis* IgG (anti-*M. tb* IgG) antibody during interaction between *M. tuberculosis* and human macrophages mediated through complement has been examined *in vitro*. Anti-*M. tb* IgG antibody significantly enhanced complement activation by *M. tuberculosis*. Phagocytosis of *M. tuberculosis* by macrophages increased significantly in the presence of complement and/or antibody. Moreover, antibody enhanced phagocytosis in the presence of complement. Addition of antibody alone or in combination with complement also augmented intracellular viability of bacilli within macrophages. Results of this study showed that anti-mycobacterial antibody enhances complement activation and anti-*M. tb* IgG antibody probably modulates effects of complement during early stages of tuberculosis infection.

Keywords: Complement, Immunoglobulin, *M. tuberculosis*, Phagocytosis

Mycobacterium tuberculosis remains one of the most common causes of infectious disease morbidity worldwide. It is estimated that 9.2 million new tuberculosis (TB) cases and approximately 2 million tuberculosis-related deaths are caused by this pathogen. One-third of the world's population is infected with *M. tuberculosis*[1]. *M. tuberculosis*, the causative agent of tuberculosis, is a facultative intracellular pathogen, which uses macrophages as its primary host cell and survives and replicates inside these cells. Therefore, invasion of macrophages by tubercle bacillus is a vital aspect in the establishment of tuberculosis infection; consequently, *M. tuberculosis* has evolved several strategies to parasitise its host cell. Multiple distinct macrophage receptors have the potential to recognise and bind *M. tuberculosis*; these include complement receptors (CRs) 1, 3 and 4; mannose receptor; CD14; surfactant protein A receptors and scavenger receptors[2].

Complement system comprises a network of more than 30 proteins, belonging to both innate and adaptive arms of the immune system. Complement receptor 1 (CR1) (CD35) is a single-chain glycoprotein that binds complement fragments C3b and C4b[3]. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are heterodimers belonging to leukocyte β_2-integrin family. These two receptors bind complement fragment C3bi and also contain a polysaccharide-binding site[4]. *M. tuberculosis* can bind to complement receptors *via* both complement-dependent and -independent pathways[5] and is subsequently phagocytosed by the phagocytic cell. Presence of human serum containing active complement components was found to enhance binding of *M. tuberculosis* to CR1, CR3 and CR4 on the surface of human monocytes and monocyte-derived macrophages (MDMs)[6]. Complement component C3 identified as the major component in human serum is involved in enhancing the adherence and uptake of *M. tuberculosis* by mononuclear phagocytes[7].

It is well documented that mycobacteria can activate the complement system[8-10]. Antibodies are known to enhance complement activation[11]. It has been demonstrated that intra-dermal BCG vaccination induces antibodies of immunoglobulin G1 (IgG1), IgG2 and IgG3 isotypes[12]. An important target of antibody responses induced by intra-dermal BCG

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vaccination was found to be lipoarabinomannan (LAM), a major component of the mycobacterial cell wall[13]. Several studies suggest that anti-LAM antibodies may have an important protective role. Passively administered monoclonal anti-arabinomannan antibody increased survival of mice after challenge with *M. tuberculosis*[14]. Antibodies induced by vaccination with arabinomannan-protein conjugates were partially protective in experimentally infected animals[15]. BCG could induce secretory mycobacterium-specific antibodies[16]. In the context of infections with other microorganisms it has been shown that antibodies could enhance immunity through many mechanisms, including neutralisation of toxins, opsonisation, activation of complement, promotion of cytokine release, enhanced antibody-dependent cellular cytotoxicity and enhanced antigen presentation[17]. Anti-mycobacterial antibodies were found to have enhancing effect on complement component C3 binding to the mycobacteria. The homologous antibodies should have more enhancing effect in comparison to heterologous antibodies[11].

Therefore, the aim of the present investigation was to study *in vitro* the role of anti-*M. tb* IgG antibody in interaction of *M. tuberculosis* with human macrophages in the context of complement system.

Materials and Methods

Study subjects—Thirty-five normal healthy laboratory volunteers aged 21 to 50 years (23 males and 12 females), having no clinical history of TB and other respiratory disorders, and 20 untreated pulmonary TB (PTB) patients (13 males and 7 females) aged 21 to 50 years were included in the study. All the PTB patients were naive for anti-tuberculosis treatment and were positive for sputum smears and culture. Examination of smear and culture was done according to the methods already established[18].

All the subjects recruited into this study were negative for HIV infection as ascertained by Tridot assay (J Mitra & Co., New Delhi, India) and Retroquic (Qualprodiagnostics, Goa, India). Informed consent was obtained from the subjects before drawing blood. This study was approved by the Institutional Ethics Committee.

Growth and preparation of mycobacteria—*M. tuberculosis* H37Rv bacilli were cultured in Middle brook 7H9 broth medium enriched with albumin, glucose and catalase ADC (Difco Laboratories, Detroit, Mich., USA) at 37°C for 3–4 weeks and harvested by centrifugation. The bacilli were washed and aliquoted in vials containing phosphate-buffered saline (PBS; *p*H 7.4). These aliquots were frozen in PBS containing 10% glycerol. During the experiments, vials were thawed and enumerated for viable colony forming units (CFU) on Middle brook 7H11 agar plates. Before inoculation onto the agar plates, bacterial aliquots were thawed at 37°C and diluted in PBS to the desired concentration.

Preparation of human sera for assaying complement activation—Normal human serum (NHS) was prepared using 10 ml blood individually drawn from 15 normal subjects and 20 PTB patients[8,11]. The obtained serum samples were stored for further use. During further experiments, demonstration of classical pathway (CP) and alternative pathway (AP) using sera samples was performed as described previously[8,11]. Briefly, sera samples were diluted in phosphate buffers, namely, PBS-Tween 20 (0.05%; *p*H 7.4) (PBST) containing $Ca^{++}Mg^{++}$ [supplemented as $CaCl_2$ (0.3 m*M*) and $MgCl_2$ (2 m*M*)] or PBST-MgEGTA to demonstrate, respectively, CP and AP. Addition of PBST-EDTA blocks complement activation and this was used as control.

M. tuberculosis sonicate antigen preparation—*M. tuberculosis* H37Rv bacilli (100 ml packed volume) harvested from the broth cultures were subjected to lysozyme treatment (1 mg/5 ml of cell suspension) for 15 min at 37°C with gentle stirring. The bacilli were then suspended in twice the volume of breaking buffer (20 m*M* Tris with 8.5% NaCl of *p*H 7.4 supplemented with 1 m*M* PMSF, 10 m*M* EDTA). The bacterial cells in the breaking buffer were disrupted using Fisher Sonic Dismembrator (Model 300) (Artek systems corporation, Port Washington NY, USA). The sonication was carried out for 30 cycles; each cycle lasting 1 min, with an interval of 1 min. The process of sonication was carried out under ice-cold conditions, since the procedure results in the release of heat that would otherwise denature the proteins.

Measurement and evaluation of purity of anti-M. tb IgG antibody—Antibody titration against *M. tuberculosis* sonicate antigen was done using ELISA technique described elsewhere[19]. After titration, isolation of anti-*M. tb* IgG antibody from patient sera was done as described by Cox *et al*.[20], using ammonium sulphate precipitation and affinity chromatography methods. Then, agarose column purification method[19] was used to purify IgG

antibodies. In addition, purity of anti-*M. tb* IgG antibody was evaluated by electrophoresis and immunoblot analysis[21]. Finally, the obtained anti-*M. tb* antibody fractions were aliquoted and stored at −20 °C.

Opsonisation of M. tuberculosis with anti-M.tb IgG antibody—For complement binding and infection experiments, *M. tuberculosis* bacilli were opsonised with anti-*M.tb* IgG antibody. Briefly, 2×10^7 *M. tuberculosis* bacilli were incubated with 20 µg/mL anti-*M. tb* IgG in RPMI-1640 for 1 h at 37°C. After incubation, the opsonised bacilli were washed with RPMI-1640 by centrifugation at 4000 *g* for 10 min. After washing, the bacterial pellet containing pre-opsonised bacilli was resuspended in 1ml of RPMI-1640 for use in complement activation and macrophage infection experiments.

C3, C4 and factor B binding assay for complement activation—Solid phase ELISA was performed to assess the pattern of complement activation by *M. tuberculosis* at levels of C3, C4 and factor B as described earlier[9] and also to determine the effect of anti-*M. tb* IgG antibody on complement activation. Briefly, heat-killed anti-*M. tb* IgG-opsonised or-unopsonised mycobacteria (at 10^8/mL, 10^7/mL and 10^6/mL numbers) were coated onto microtiter plates and incubated overnight at 4 °C, followed by blocking the wells with 1% BSA in PBST. NHS diluted in various buffers (*p*H 7.4) viz., PBST-$Ca^{++}Mg^{++}$ or PBST-MgEGTA or PBST-EDTA to demonstrate, respectively, CP, AP and as control was added to the wells and incubated for 1 h at 37 °C. C3 activation was measured through both CP and AP, with serum dilutions 1/50, 1/100 and 1/250 (for CP) and 1/10, 1/20 and 1/50 (for AP). C4 activation was measured through CP using 1/50, 1/100 and 1/250 serum dilutions. For factor B (through AP), serum dilutions used were undiluted sera, 1/2 and 1/10. Anti-human complement antibodies raised in rabbit (anti-C3c and anti-C4c; DAKO, Glostrup, Denmark) or goat (anti-factor B; R&D Systems, Minneapolis, MN, USA) conjugated with HRP were added and incubated at 37 °C for 1. After washing the plate, the substrate tetra methyl benzidine (TMB) was added and incubated for 15 min at room temperature in dark. Finally, the reaction was stopped using 0.5M H_2SO_4 and the plate was read for OD values at 450 nm in an ELISA Reader (Spectramax 250).

Macrophage culture and their infection with M. tuberculosis and evaluation of phagocytosis—Blood samples (40 mL) individually drawn from the remaining 20 normal healthy subjects were used for cell culture and infection experiments. Peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample as previously described[22]. For infection experiments, 2×10^5 MDMs/coverslip were incubated in a microtiter plate with RPMI-1640 containing CP components (NHS 1/50 dilution in PBST-$Ca^{++}Mg^{++}$) or AP components (NHS 1/10 dilution in PBST-MgEGTA) or components to block complement activation (NHS 1/50 dilution in PBST-EDTA). The macrophages were infected with anti-*M. tb* IgG-opsonised mycobacteria or -unopsonised mycobacteria at a multiplicity of infection (MOI) of 1:10 (macrophage:mycobacteria) and incubated for 2 h at 37°C, after which the macrophage monolayer was washed using warm RPMI-1640 to remove non-adherent bacilli. Plain MDMs (i.e., without treatment with complement or anti-*M. tb* IgG) were used as control. Finally, after formalin fixation of the macrophages, phagocytosed bacteria were quantitated by light microscopy in triplicates for each experimental condition (~300 MDMs/coverslip).

Phagocytic index was calculated using the formula:

$$\frac{\text{No. of macrophages containing intracellular mycobacteria}}{\text{Total no. of counted macrophages}}\times100$$

Measurement of intracellular mycobacterial growth—Intracellular growth of *M. tuberculosis* in macrophages, in the presence or absence of complement and/or anti-*M. tb* IgG antibody, was assessed as described elsewhere[23]. MDMs at a concentration of 1×10^5/well were used for infecting *M. tuberculosis* at an MOI of 1:10 under the same conditions as done for phagocytosis assay.

Statistical analysis—Statistical analysis of the data was carried out with the SPSS software program (SPSS 13. Chicago, IL, USA). The statistical significance of antibody titers and complement binding was determined by unpaired *t* test. One-way ANOVA with Tukey's test was used to compare the phagocytic ability of macrophages and intracellular viability of *M. tuberculosis*. Results are expressed as mean ± standard errors. *P* values less than 0.05 were considered as statistically significant.

Results

Determination of antibody titer against M. tuberculosis sonicate—Antibody titration against *M. tuberculosis* sonicate was done using ELISA. As a preliminary step, the titers of patient serum antibodies against *M. tuberculosis* sonicate were estimated and

these were compared with those from the NHS samples. Both IgG and IgM antibody titers against *M. tuberculosis* sonicate were higher in PTB patients compared to NHS (Fig. 1A and B). The antibody titers were significantly higher in PTB patients than normal healthy controls ($P< 0.001$). It was also found that IgG antibody titers were higher than IgM titers in serum samples from both patients and controls ($P<0.05$).

Isolation of anti-M. tb IgG antibody—Sera from 20 active PTB patients were used to prepare anti-*M. tb* IgG antibody. A total of 4 mg anti-*M. tb* antibody was obtained and this was checked for purity on SDS-PAGE. Heavy and light chains characteristic of IgG antibody in PTB patient sera are shown in Fig. 2A. On Western blot analysis of this preparation, it was found that the antibodies from the patient sera were predominantly against 24, 30, 45, 65, 71, 82 and 96 kDa antigens (Fig. 2B).

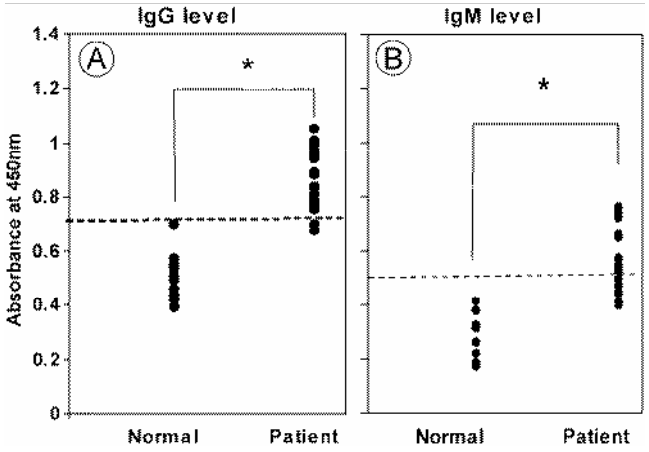

Fig. 1—Levels of antibody responses to *M. tuberculosis* sonicate: (A)-IgG; (B)-IgM. Sera were collected from untreated pulmonary tuberculosis patients (n=20) and normal healthy volunteers (n=15). Dotted line: 3 SD ± mean, **P*< 0.05.

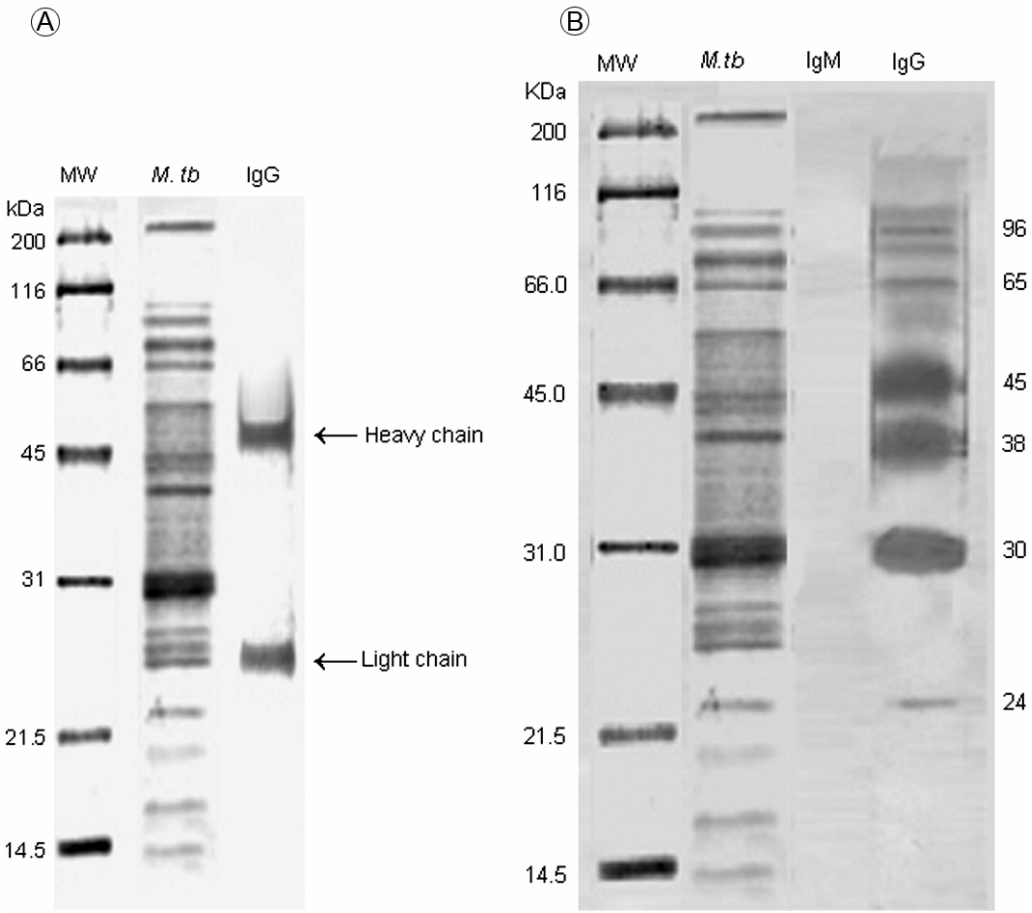

Fig. 2—(A)-Affinity purification of anti-*M. tb* IgG antibody. IgG antibodies against *M. tuberculosis* in pulmonary tuberculosis patients' serum were purified by affinity purification. The eluted IgG was analysed by Coomassie brilliant blue R-250 staining after SDS-PAGE;(B)-Western blot analysis of *M. tuberculosis*-specific IgG antibody. Bands from low molecular weight (24kDa) to the highest (96kDa) were observed for *M. tb* sonicate proteins probed with purified IgG. These were compared with protein profiles of *M. tb* sonicate and MW markers (in KDa), indicated on the left. [MW:Molecular weight marker; *M. tb*:*M. tuberculosis* sonicate protein; IgM: IgM antibody; IgG: Purified IgG].

Complement activation pattern by M. tuberculosis—Results of ELISA experiments to assess complement activation are shown in Fig. 3. Both C3 binding (through both CP and AP) and C4 binding (through CP) to *M. tuberculosis* are dependent on serum concentration as well as on the number of bacilli (Fig. 3A–C). Results of factor B binding to *M. tuberculosis* showed that factor B binding was highest only at 10^8/ml numbers of bacilli with all 3 serum dilutions as shown in Fig. 3D. There was negligible or almost no factor B binding at 10^7/ml and 10^6/ml bacilli numbers for all the 3 serum dilutions.

Effect of anti-M. tb IgG antibody on activation of complement by M. tuberculosis—To study the effect of anti-*M. tb* IgG antibody on complement binding, 10^8/ml of heat-killed bacilli (opsonised or unopsonised) were used to activate C3, C4 and factor B using NHS 1/50 dilution for CP, 1/10 for AP and undiluted sera for factor B binding using ELISA (Fig. 4). Anti-*M. tb* IgG antibody significantly enhanced the binding of C3 through both CP (P<0.01) and AP (P<0.05) and C4 (P<0.05) compared to that observed with unopsonised bacilli. However, the level of binding of factor B was not enhanced by prior

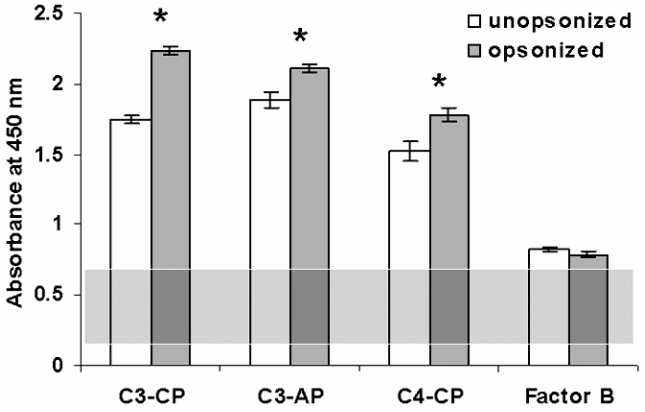

Fig. 4—Effect of anti-*M. tb* IgG antibody on complement binding of *M. tuberculosis* at levels of C3, C4 and factor B. [Shaded bar: Limits of complement binding measured using NHS diluted in PBST-EDTA. Anti-*M. tb* IgG-opsonised *M. tuberculosis* showed a significant increase of complement binding compared to unopsonised bacilli. *P< 0.05. CP: Classical pathway; AP: Alternative pathway].

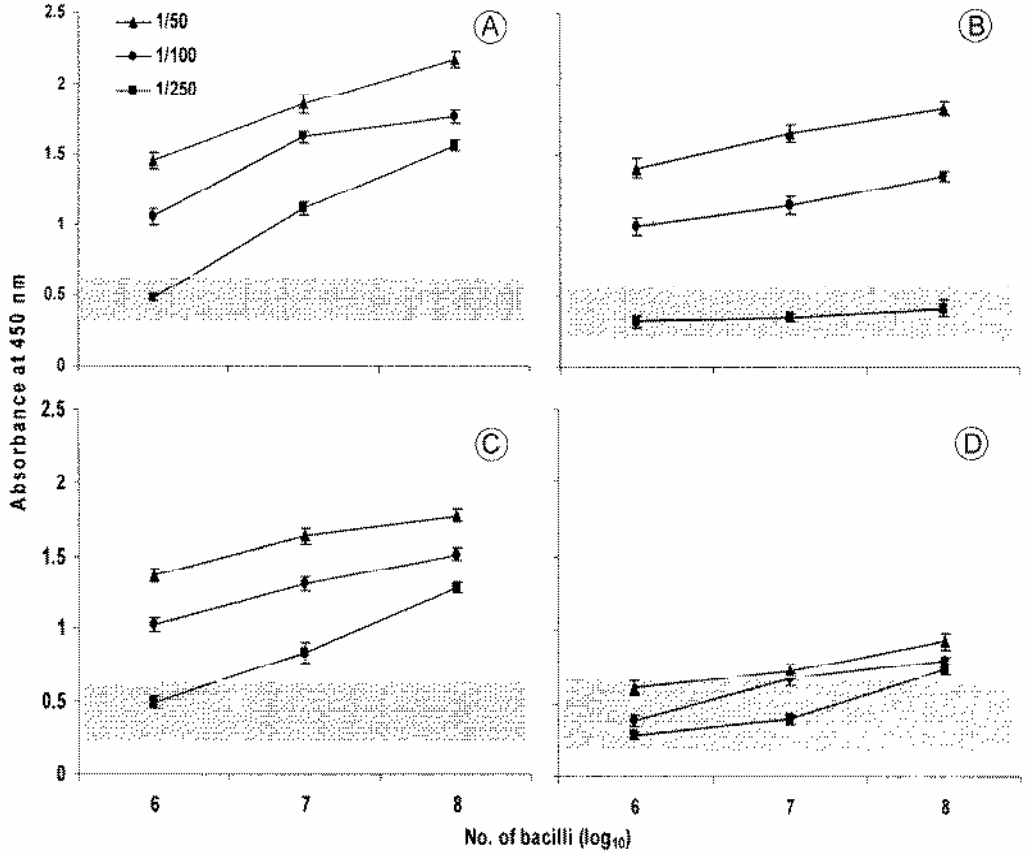

Fig. 3—Solid-phase ELISA was performed to determine patterns of complement binding to *M. tuberculosis* assessed by C3 binding through (A)-classical and (B)-alternative pathways, (C)-C4 binding through CP and (D)-factor B binding. [Shaded bar: Limits of complement binding measured using NHS diluted in PBST-EDTA. Complement binding was directly proportional to serum concentration and numbers of bacilli].

opsonisation and showed similar results to that obtained without *M. tuberculosis* opsonisation.

Effect of anti-M. tb IgG on phagocytosis of M. tuberculosis—Effect of anti-*M. tb* IgG antibody on the phagocytosis of *M. tuberculosis* by human MDMs under various conditions is shown in Fig. 5. Phagocytosis of unopsonized bacilli was >45%, while addition of CP components significantly increased the percentage phagocytosis to >70% (P<0.05) and with AP components it significantly increased to >60% (P<0.05), compared to that of the unopsonised bacilli. Addition of *anti-M. tb* IgG antibody alone also significantly increased the phagocytic ability of macrophages to about 60% (P<0.05%). Further, addition of anti-*M. tb* IgG antibody along with CP and AP components significantly increased phagocytosis by >80% and >70%, respectively compared to unopsonised bacilli. These results indicate that complement and antibody independently act as good augmenters of phagocytosis of *M. tuberculosis* and when in combination, the two opsonins probably have a synergistic effect.

Intracellular growth of M. tuberculosis in macrophages—Intracellular growth of *M. tuberculosis* in MDMs was assessed by counting the CFU on the 7H11 agar plates on which the lysed macrophages containing the bacilli were inoculated and grown. At 1 h post-infection, it was found that there were significantly more viable *M. tuberculosis* bacilli (P< 0.05) in the macrophages treated with *M. tb* + CP, *M. tb* + IgG and/or CP and AP compared to the macrophages treated with *M. tuberculosis* alone (Fig. 6A). At 24 h post-infection, significantly higher number of viable bacilli were found inside the macrophages (P< 0.05) at all conditions, compared to the macrophages treated with *M. tuberculosis* alone (Fig. 6B). Moreover, addition of anti-*M. tb* IgG in the presence of complement (CP and AP) significantly increased the intracellular growth of *M. tuberculosis*

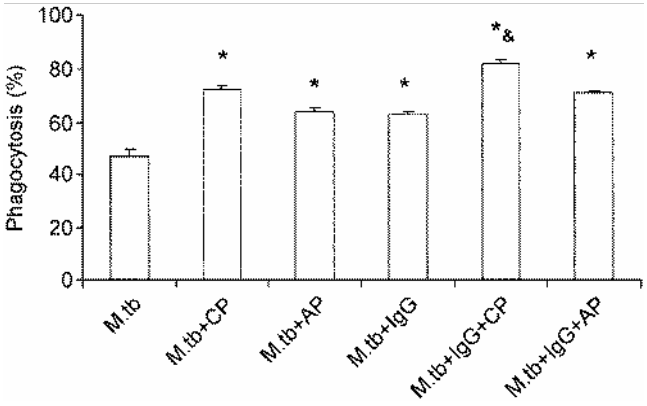

Fig. 5—Effect of anti-*M. tb* IgG antibody on phagocytosis of *M. tuberculosis* by normal human macrophages. Significant increase in phagocytosis was observed in the presence of complement and/or anti-*M. tb* IgG antibody compared to uptake of untreated bacilli. *P< 0.05. Addition of both IgG antibody and complement (CP) increased percentage phagocytosis significantly compared with antibody or complement alone ($^{\&}P$<0.05).

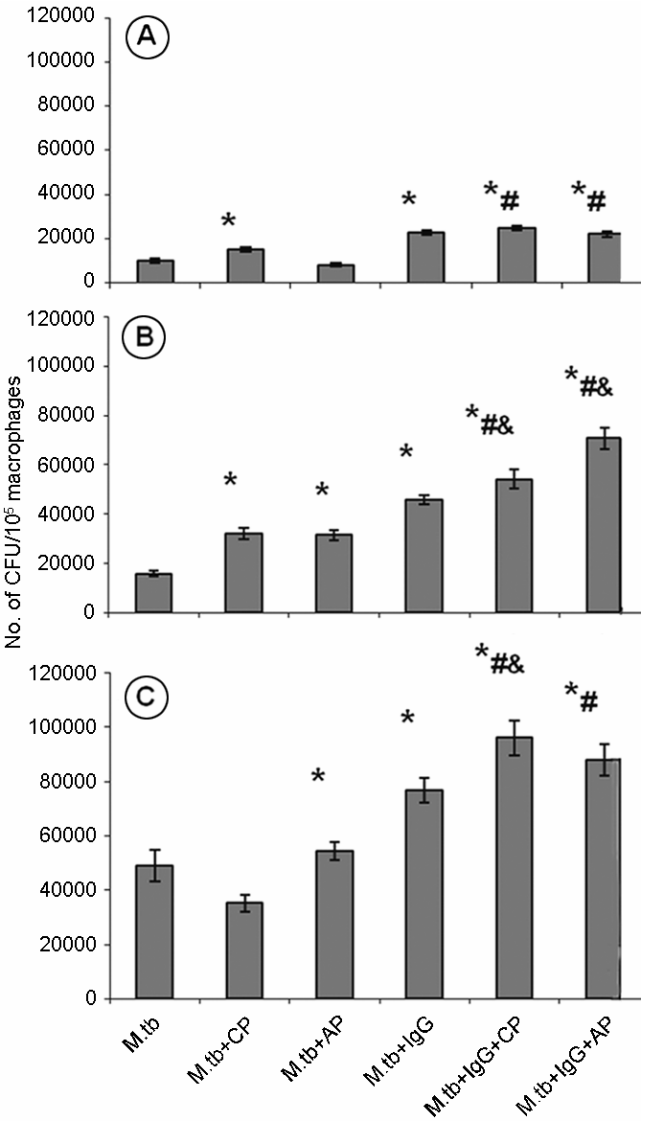

Fig. 6—Effect of anti-*M. tb* IgG antibody on intracellular viability of *M. tuberculosis* measured at (A)-1 h, (B)-24 h and (C)-48 h post-infection of normal human macrophages under various conditions. A significant increase in the viability of bacilli was observed in the macrophages infected with *M. tuberculosis* and treated with complement and/or anti-*M. tb* IgG compared to those infected with *M. tuberculosis* alone (*P< 0.05). $^{\#}P$< 0.05 implies statistically significant augmenting effect of anti-*M. tb* IgG on increase in the viability mediated through complement. Viability was also significantly increased upon addition of both antibody and complement, compared to complement alone ($^{\&}P$<0.05).

($^{\&}P<0.05$, Fig. 6B). At 48 h post-infection, a robust increase in the number of viable bacilli was observed at all conditions, except in the macrophages treated with CP components alone (Fig. 6C). Addition of antibody significantly increased the viability of bacilli alone ($^{\#}P<0.05$) and also in combination with complement (CP components) ($^{\&}P<0.05$; Fig. 6C). Thus, it was interesting to note in this experiment that intracellular growth of *M. tuberculosis* did not increase in the presence of complement alone after 48 h of infection, compared to that observed at 24 h post-infection.

discussion

One of the reasons proposed for the failure of BCG vaccination to protect against post primary forms of TB is that a prior sensitisation with non-tuberculous environmental mycobacteria, which downregulates subsequent response to both BCG and *M. tuberculosis*[24,25].

The presence of moderate amounts of anti-mycobacterial antibodies in BCG unvaccinated and vaccinated individuals in endemic normal subjects is well documented[26,27]. Although a possible therapeutic role for immune sera in the control of tuberculosis has been conferred[28], little attention has been paid to a possible role for antibody in protection from *M. tuberculosis* infection. Mycobacteria coated with specific antibodies were more effectively processed and presented by dendritic cells for stimulation of $CD4^+$ and $CD8^+$ T-cell responses[16]. Mice treated with mAb IgG3 specific for arabinomannan and then challenged with *M. tuberculosis* localised the pathogen within the granuloma centers, suggesting that the mAb conferred protection by enhancing a cellular immune response[14]. Joller *et al.*[17] also reported the protective effects of antibodies mediated through Fc receptors using *Legionella pneumophila* and *Mycobacterium bovis* BCG. These findings indicate that some mAbs to *M. tuberculosis* are able to modify the course of experimental TB infection. Few studies have also documented the effect of antibody on complement activation by mycobacteria[9,11,29].

In the present study, effect of both opsonins, anti-*M. tb* IgG antibody and complement, in the early interaction of *M. tuberculosis* with human macrophages was investigated. It was observed that pre-opsonisation of *M. tuberculosis* with anti-*M. tb* IgG resulted in enhanced activation of C3 (through both pathways) and C4 (through CP), but not factor B. This indicates that binding of both C3 and C4 is augmented by antibodies without influencing factor B.

Initial contact between intracellular microorganisms and phagocytes can be mediated by opsonic[30] and non-opsonic interactions[31]. The former is mediated by either Igs or C3b/C4b complement fractions that interact with Fc receptors and CRs, respectively. These processes bring about phagocytosis of the pathogen by the professional phagocytes. It was observed in the present study that opsonisation with complement and/or antibody increases the phagocytosis of *M. tuberculosis* by macrophages. Addition of anti-*M. tb* antibody alone had a significantly enhancing effect on phagocytosis of mycobacteria by macrophages. This effect was further significantly enhanced in the presence of complement components (especially CP components). Similar observations were reported by Hostetter *et al.*[32], who showed that phagocytic index of macrophages was maximum in immunised and naive sera compared to that of heat-inactivated immune/naïve sera.

The increased phagocytosis may be due to predominant CR-mediated uptake or alternatively the interaction between Fc and CRs as earlier suggested[33]. In addition, there was an increased binding of C3 and C4 by the anti-*M. tb* IgG-opsonised bacilli, which might account for the increased uptake of bacilli through phagocytosis by the macrophages. Use of multiple receptor types for entry of *M. tuberculosis* into macrophages has been described previously and it is hypothesised that *in vivo* this may be the most relevant mechanism of uptake. In the presence of complement, a subset of $CD4^+$ T cells carrying CR1 and CR2 were infected with HIV at a higher efficiency *in vitro*[34]. Also monocyte and macrophage-derived cell lines, which are CR3 positive, become more permissive for HIV infection in the presence of complement[35].

The role of CRs on monocytes in the course of tuberculous infection has been a subject of much controversy. Schlesinger *et al.* reported that in the presence of antibodies against CR1 and CR3, a significant reduction in the adherence of *M. tuberculosis* and *M. leprae* to human MDMs was observed[7]. However, Hu *et al.*[36] observed that CR3-deficient mice did not alter the course of tuberculous infection.

Assessment of intracellular viability of *M. tuberculosis* in the present investigation showed that both complement and antibody had an enhancing effect on the viability of bacilli. However, there was no significant change in the number of CFU at 48 h post-infection when the macrophages were treated with the

bacilli and CP components alone, compared to that observed at 24 h post-infection. This is an interesting finding, which indicates a probable protective role of complement-mediated phagocytosis. Nevertheless, antibody alone or in combination with complement (both CP and AP components) significantly increased the growth of bacilli in the macrophages (Fig. 4). However, it was also earlier reported that entry of *M. tuberculosis* into macrophages through CRs promotes poor phagosome–lysosome fusion[37].

The study has few drawbacks. In the present study, the complement binding was not quantitated, which could have helped to assess the actual levels of complement uptake by the bacilli. In addition, apoptosis and necrosis were not assessed, which could have thrown more light on the macrophage–mycobacteria interaction and intracellular viability aspects.

The observations of this study might corroborate the hypothesis that prior exposure to environmental mycobacteria is one of the reasons for the failure of BCG vaccination in adult forms of TB. The present findings indicate that *M. tuberculosis*-specific antibody, despite enhancing complement activation, might suppress the complement-mediated immune response against tuberculosis, which was evidenced by the increased viability of bacilli in the macrophages. Furthermore, increased phagocytosis mediated through complement and/or antibody might account for the immunopathological response, which could imply that exposure to environmental mycobacteria can induce immunosuppression. Further studies are needed to delineate the exact role of mycobacteria-specific antibody and to decipher the molecular mechanisms involved in the complement and/or Fc receptor–mediated routes of entry of the bacilli into the macrophages.

Therefore, the present study indicates that the role of humoral and innate immunity, at the level of complement system, during the early stages of tuberculosis infection is an essential component to be considered in the development of novel drug therapies against TB and thus devise methods by which the antibody-induced immunosuppression can be obviated.

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RESEARCH COMMUNICATION

Prevalence of Human Papillomavirus and Co-Existent Sexually Transmitted Infections among Female Sex Workers, Men having Sex with Men and Injectable drug abusers from Eastern India

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Abstract

Background: Human papillomavirus (HPV) is a very common sexually transmitted disease affecting both men and women and is responsible for different ano-genital cancers in either sex. Co-existing sexually transmitted infections (STI) including HIV have been considered as important co-factors for carcinogenesis induced by HPV. The purpose of this study was to determine the prevalence of any HPV, HPV 16 and HPV 18 and also concomitant STIs among female sex workers (FSW), men having sex with men (MSM) and injectable drug users (IdU). Material and Method: This cross-sectional study was conducted among 45 FSWs, 26 MSMs and 58 IdUs who attended the STI or de-addiction clinics. Genital scrape samples collected from glans penis and coronal sulcus in males and cervical squamo-columnar junction in females were tested for HPV dNA by PCR using HPV L1 consensus primer. Type specific PCR to detect HPV 16 and 18 was done on the samples positive on consensus PCR. All participants were tested for associated STIs including HIV and hepatitis B and cervical cytology was done on all females. Results: Among the FSWs, HPV was detected in 73.3% and HPV 16 and 18 was detected in 25.7%. Though the HPV prevalence was similarly high among MSMs (69.2%) and IdUs (72.4%), the prevalence of HPV 16 and 18 was much lower in these groups compared to the FSWs. Prevalence of cervico-vaginal infection with Trichomonas vaginalis and syphilis was significantly higher in the HPV positive women compared to the HPV negative women. There was no statistically significant difference in the prevalence of other STIs among HPV positive and negative women and men. Conclusion: HPV infection is highly prevalent among FSW, MSM and IdUs. *Trichomonas vaginalis* infection is more frequent in HPV positive women

Keywords: Human papillomavirus - co-existing STIs - female sex workers - men having sex with men - drug users

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Introduction

Human papillomavirus (HPV) is the most common sexually transmitted viral infection and studies estimate that globally 50-80% of ever sexually active men and women are infected with the virus at least once during their lifetimes (Koutsky, 1997). HPVs have been classified into low and high risk types depending on their oncogenic potentials. Anogenital infections with high-risk HPV types (types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70) predispose men and women to the development of preinvasive and invasive disease in cervix, vulva, anus and penis. Besides HPV, genital tract infections from other organisms like *Trichomonas vaginalis* (TV), *Chlamydia trachomatis and Herpes simplex virus* (HSV) type 2 have also been implicated in cervical cancers in women. Longitudinal studies have observed that TV infection is associated with high relative risk of both preinvasive and invasive cervical cancer (Viikki et al., 2000). Chlamydia, the obligate intracellular bacterium is frequently found in association with benign proliferative, pre-neoplastic and malignant changes in cervical epithelium (Bułhak et al., 2007). There is evidence that chlamydia infection may contribute to neoplastic changes in the transformation zone of uterine cervix independent of high risk HPV infection (Schlott et al., 2005). Although prospective studies based on serology did not find any independent association between HSV and cervical carcinogenesis, there is evidence that HSV 2 as a cofactor can lead to cervical cancer in presence of high risk HPV (Szostek et al., 2009). Human Immunodeficiency virus (HIV) positive women have significantly higher prevalence of genital squamous intraepithelial lesions and of multifocal HPV related diseases (Palefsky et al., 1999). If cervical cancer

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does develop in HIV positive women, it may be more aggressive in nature and less responsive to treatment.

The female sex workers (FSW), the men having sex with men (MSM) and the injectable drug abusers (IDU) registered with the STI and de-addiction clinics are at high risk of contacting various sexually transmitted infections (STI) due to their high risk sexual behaviours. Co-existence of HPV and other sexually transmitted infections can increase their predisposition to different ano-genital cancers. Association between different STIs is frequent, but there are few statistics on the prevalence of co-infections specially in the high risk groups we have included in our study. The present study aims to evaluate the magnitude of HPV infection in FSWs, MSMs and IDUs and to assess the risks of concomitant STIs in the HPV infected sub-populations.

Materials and Methods

The cross-sectional study was conducted on 45 female sex workers (FSW), 26 men having sex with men (MSM) and 58 male injectable drug abusers (IDU) registered with some of the clinics of National AIDS Prevention and Control Organization at West Bengal, a province in eastern India. The patterns of high risk behaviour (FSW, MSM or IDU) with their HIV infection status were obtained from the existing medical records maintained in the respective clinics. All the potential subjects were counselled by the investigators in presence of a trained counsellor of AIDS Control Society about the objectives and methodology of the study. Those who provided written informed consent were enrolled in the study. Ethical clearance was obtained from Institutional Ethics Committee, Medical College, Kolkata.

The subjects were interviewed to fill up a structured questionnaire on socio-demographic characteristics. Their high risk behaviour patterns were reconfirmed during the interview. Scrape samples were collected with Cervex brushes® (Rovers Medical Devices B. V., Netherlands) from ectocervix and endocervix in females and from glans penis and coronal sulcus in males for HPV testing. The brush was swirled and the tip was detached into sterile cold PBS solution. The vials containing the samples were transported to the laboratory maintaining cold chain. An Ayre's spatula was used to obtain cervical scraping for Pap smear from each FSW. Multiple cervical and vaginal swabs were obtained from the FSWs for Chlamydia direct antigen detection test, Candida culture in Saborauds Dextrose Agar and TV culture in Kupferburgs media. Blood samples were collected from all subjects for serological tests like Venereal Disease Research Laboratory test (VDRL), *Treponema pallidum* Haem-agglutination test (TPHA), hepatitis B surface antigen (HBsAg) detection test, HSV 2 IgM and HIV antibody test. Patients whose samples showed VDRL reactivity of ≥1:8 and TPHA positivity were considered to be positive for syphilis.

DNA extraction from genital scrape samples was done by the standard phenol chloroform extraction method. PCR was performed using GP5+/GP6+ L1 consensus primers. PGMY09/11 L1 consensus primers were used to confirm negative cases and detect cases with low levels of HPV. Amplification of β-globin DNA was performed as a positive control for the presence of amplifiable DNA in the specimen. Type-specific PCR using HPV 16, 18 specific primers was done to detect these two genotypes in samples positive on HPV L1 consensus PCR.

Slides obtained for cervical cytology were stained by Papanicolaou method and interpreted using Bethesda 2001 classification (Solomon et al., 2002).

For Chlamydia detection, the Clearview Chlamydia MF Kit, manufactured by Inverness Medical Professional Diagnostics, UK was used. Chlamydial antigen was extracted from the swab by heating at 80 °C with extraction reagent. The extract was added to the absorbent pad in the sample window. The test strip contained a region of immobilized monoclonal anti – chlamydial antibody in the result window. Positive result was denoted by the appearance of a line in the result window.

For candida detection, cream coloured colonies on Saborauds Dextrose Agar were picked up, smears were prepared and stained by Grams method to visualize budding yeast cells.

Wet mount examination for *trichomonas vaginalis* was done on samples from Kupferburgs media daily for seven

Table 1. Socio-demographic Parameters of the Study Groups

	FSW (N=45)	MSM (N=26)	IDU (N=58)
Age Distribution			
<20 years	3 (6.7%)	7 (26.9%)	6 (10.3%)
20-29 years	28 (62.2%)	7 (26.9%)	31 (53.4%)
30-39 years	10 (22.2%)	1 (3.8%)	17 (29.3%)
>40 years	3 (6.7%)	0	3 (5.2%)
Unknown	1 (2.2%)	11 (42.3%)	1 (1.7%)
Religion			
Hindu	28 (62.2%)	23 (88.5%)	48 (82.8%)
Muslim	17(37.8 %)	2 (7.7%)	6 (10.3%)
Others	0	0	2 (3.4%)
Unknown	0	1 (3.8%)	2 (3.4%)
Literacy			
Illiterate	33 (73.3%)	2 (7.7%)	11 (18.9%)
Primary & Middle	5 (11.1%)	9 (34.6%)	19 (32.8%)
High school & above	4 (8.9%)	14 (53.8%)	27 (46.6%)
Unknown	3 (6.7%)	1 (3.8%)	1 (1.7%)
Duration of Risk Behaviour			
<1 year	10 (22.2%)	1 (3.8%)	4 (6.9%)
2-5 years	21 (46.7%)	5 (19.2%)	40 (69%)
6-10 years	4 (8.9 %)	4 (15.4%)	9 (15.5%)
>10 years	9 (20%)	1 (3.8%)	3 (5.2%)
Unknown	1 (2.2%)	15 (57.7%)	2 (3.4%)

Table 2. Prevalence of Any HPV and of HPV Types 16 & 18 in the Study Groups

	FSW (n=45)	MSM (n=26)	IDU (n=58)
Any HPV	35 (73.3%)	18 (69.2%)	42 (72.4%)
HPV 16	7 (15.6%)	0	2 (5.2%)
HPV 18	2 (4.4%)	0	0
Both HPV 16 & 18	2 (5.7%)	0	0

Table 3. Prevalence of Various STIs by Genital HPV Infection Status in the Study Groups

	FSW (N=45)			MSM (N=26)			IDU (N=58)		
	HPV +ve (N=35)	HPV –ve (N=10)	p	HPV +ve (N=18)	HPV –ve (N=8)	p	HPV +ve (N=42)	HPV –ve (N=16)	p
Candida +ve	31 (88.6%)	9 (90.0%)	0.69	Not done	Not done		Not done	Not done	
T Vaginalis +ve	8 (22.9%)	0	0.04	Not done	Not done		Not done	Not done	
Chlamydia +ve	5 (14.3%)	2 (20.0%)	0.8	Not done	Not done		Not done	Not done	
HBsAg +ve	0	0		0	1 (12.5%)	0.31	1 (2.4%)	1 (6.2%)	0.47
HSV 2 +ve	12 (34.3%)	1 (10.0%)	0.13	3 (16.7%)	1 (12.5%)	0.64	1 (2.4%)	1 (6.2%)	0.47
Syphilis +ve	13 (37.1%)	0	0.02	0	2 (7.7%)		0	0	
HIV +ve	6 (17.1%)	1 (10.0%)	0.5	1 (5.6%)	0	0.69	2 (4.8%)	0	0.52

Table 4. Cytology diagnosis by Genital HPV Infection Status in the FSWs (X^2=4.87; p=0.18)

	HPV + ve (n=35)	HPV – ve (n=10)
Negative	15 (42.9%)	7 (70%)
ASCUS	3 (8.6%)	-
LSIL	16 (45.7%)	2 (20%)
HSIL	-	-
Inadequate	1 (2.8%)	1 (10%)

days to detect the motile organisms.

The serological tests were performed using standard kits available and following the manufacturer's instructions. VDRL was performed using kits manufactured by Institute of Serology, India. For TPHA kits manufactured by New Market Laboratories, UK were used. HBsAg was detected by ELISA using kits manufactured by Qualpro Diagnostics, India. HSV 2 detection was by ELISA techniques using kits manufactured by Calbiotech, USA. HIV rapid detection was done by a membrane based flow-through immunoassay for detection of antibodies to HIV 1 and HIV 2 in serum using Retroquick HIV Kit manufactured by Qualpro Diagnostics, India.

Results

The socio-demographic parameters and the duration of risk behaviours of the three study groups are given in Table 1. All the drug abusers were males. The prevalence of HPV (any type) was uniformly high in all the groups; 73.3% in FSWs, 69.2% in MSMs and 72.4% in IDUs. Highest proportion of HPV infected cases were observed in men and women aged 20-29 years. HPV infection was most common in female sex workers who reported to be in the trade for 2-5 years. Significantly higher prevalence of HPV types 16 and 18 was observed among the FSWs (24.4%) compared to the MSMs (0%) and the IDUs (5.2%) (Table 2). The frequencies of concomitant sexually transmitted infections among the HPV infected and non-infected study populations are shown in Table 3. The prevalence of TV (22.9%) and syphilis (37.1%) was significantly higher in the HPV positive FSWs compared to the HPV negative ones (0% and 0% respectively). None of the women with TV infection was HPV 16 or 18 positive. HSV 2 and HIV infections were observed more frequently among the HPV positive women though the difference between HPV positive and negative women was not statistically significant.

No difference in the prevalence of hepatitis B, HSV 2, syphilis and HIV was observed between the HPV positive and negative men belonging to the MSM or IDU groups.

Cytological abnormalities of cervix (ASCUS or worse) were more frequently seen among the HPV positive female sex workers compared to those who were HPV negative (54.3% vs 20%) (Table 4). However, no woman in the study had high grade abnormalities on cytology.

discussion

This is the first study from India to report the prevalence of HPV and other concomitant STIs in women and men belonging to the high risk groups (FSWs, MSMs and IDUs). The high prevalence of HPV among female sex workers and HIV infected women has been reported from India earlier (Sarkar et al., 2008:2011). Our study observed almost similar high prevalence not only in FSWs but also in men having sex with men and in men addicted to injectable drugs of abuse. Although the overall HPV prevalence was comparable among the three groups, the significantly higher prevalence of oncogenic HPV types 16 and 18 in FSWs possibly signifies higher risk of neoplasias among the women compared to the men we studied.

The present study has observed higher prevalence of TV, HSV 2, syphilis and HIV among the HPV infected female sex workers. The results do not provide evidence to substantiate the hypothesis that certain STIs and HPV may act synergistically in cervical carcinogenesis. The concomitant appearance of HPV and other STIs in sexually promiscuous women is best explained by the fact that they are co-variables of sexual behaviour. It seems reasonable to consider that these sexually transmitted infections are commonly found in women with HPV because of their promiscuous sexual behaviour. Even then some of the observations in our study indicate higher susceptibility of the HPV infected women to certain STIs which needs further investigations.

In our study the observed prevalence of *Trichomonas vaginalis* and syphilis infection among the HPV infected women was significantly higher compared to the non-infected women. Though it was not possible to derive from the study whether the higher prevalence would increase the risk of neoplasias, a meta-analysis of 24 studies found a significant positive association between TV infection and cervical neoplasia (including both cervical intraepithelial neoplasia and cervical cancer) (Zhang & Begg , 1994). A Finnish prospective study also observed TV infection to be associated with a high relative risk of both preinvasive

and invasive cervical cancer combined (Standardized Incidence Ratio 6.4) (Viikki et al., 2000).

The frequency of *Candida* and *Chlamydia* infection in the HPV positive women was not higher than their HPV negative counterparts in our study. Till date there is no evidence that fungal infection in the vagina is associated with significant increase in the risk of cervical neoplasias either in presence of absence of HPV (Viikki et al., 2000). Though an in vitro study has implicated *Chlamydia* in cervical carcinogenesis (Schlott et al., 2005), a prospective five-year follow-up study of 530 women with cervical HPV infection failed to demonstrate any influence of coexistent cervical infection with *Chlamydia* on the clinical course of HPV lesions (Yliskoski et al., 1992). The rates of persistence of HPV infection or progression to neoplasias were same in the HPV positive women with co-existing *Chlamydia* infection compared to HPV positive women without Chlamydia infection.

The number of subjects in the study was low since most of the eligible men and women we approached did not volunteer to participate in the study due to the fear of being further stigmatized. Most of the FSWs with abnormal Pap smear refused colposcopy when it was explained to them that colposcopy could lead to cervical biopsy for which they would have to abstain from sex for one week. Still the present study reveals some interesting facts about the very high risk but hard to reach population.

In conclusions, HPV infection is highly prevalent in women and men with high risk sexual behavior. Cervico-vaginal infection with *Trichomonas vaginalis* is more frequent in HPV positive women and the natural history of such concomitant infection needs to be studied further.

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Original Article

Enteric Pathogens in HIV/AIDS from a Tertiary Care Hospital

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ABSTRACT

Background: Patterns of enteric infections in HIV in developing countries may differ in several important ways from developed countries, the knowledge of which can often guide therapy when resource limitations hamper the exact diagnosis of the etiological agent in HIV-associated diarrhea. **Objectives:** The primary objective of this study was to define and compare the microbial etiologies of diarrhea in HIV-1 infected and non infected patients and in HIV infected non diarrheal patients. **Materials and Methods:** This study was conducted between April 2007 and July 2007 at the Department of Microbiology, Maulana Azad Medical College, New Delhi. Stool samples from 50 HIV seropositive cases with diarrhea (study group), 50 HIV seropositive cases without diarrhea (control group I), and 50 HIV seronegative cases with diarrhea (control group II) were examined. After the diagnosis of HIV infection was made, routine parasitological and bacteriological detection was done. An ELISA was used for the detection of Clostridium difficile toxin and Cryptosporidium antigen in stool samples. **Results:** The overall prevalence of enteric parasitosis in the study group was 20% and the bacteria identified were Escherischia coli in 24% of the case, Clostridium difficile in 10% of the cases, Salmonella species and Vibrio cholerae in 4% of the cases, and Shigella species in 2% of the cases. Candida species was identified in 36% of the cases. **Conclusions:** Identification of the etiological agent of diarrhea in a patient with AIDS is very important as it can help in the institution of appropriate therapy and the reduction of morbidity and mortality in these patients.

Keywords: Bacteriology, diarrhea, diagnosis, HIV, parasitology

Introduction

HIV/AIDS (Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome) is a major problem in India with more than 6 million recorded cases by the end of 2005.[1] Patients with HIV are prone to developing a panorama of diseases during their lifetime. AIDS represents the most severe squeal of immunosuppression caused by HIV and is a constellation of diseases reflecting the late manifestation of HIV infection. Immunosuppression caused by HIV infection leads to the development of severe opportunistic infections and otherwise rare tumors. Among them, diarrhea is a significant cause of morbidity observed in a majority of studies.[2,3] In fact, it is the second leading cause of hospital visits in developing nations and makes its place in the top ten worldwide.[4] Reports indicate that diarrhea occurs in 30-60% of patients with AIDS in developed countries and in about 90% of such patients in developing countries.[5] In tropical countries, chronic diarrhea associated with weight loss (slim disease) is often the presenting illness of HIV-1 infected individuals.[6] This diarrhea wasting syndrome in association with a positive HIV-1 serology test is an AIDS-defining criterion, according to the World Health Organization (WHO) classification.[7]

HIV infection and AIDS are fast becoming a major threat in India. Patterns of enteric infections in developing countries like ours, where hygiene is poor and intercurrent infection rates are high, may differ in several important ways from patterns of developed countries. Knowledge of the pattern of the infection can often guide therapy when resource limitations hamper the exact diagnosis of the etiological agent in HIV-associated diarrhea. The primary objectives of this study were to define the microbial etiologies of diarrhea in HIV-1 infected individuals and compare them with those of HIV seronegative diarrheal patients and HIV-1

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infected individuals without diarrhea.

Materials and Methods

Study population and study design

This study was conducted prospectively between April 2007 and July 2007 at the HIV and Diarrhea and Parasitology Laboratory, Department of Microbiology, Maulana Azad Medical College attached to Lok Nayak Hospital, New Delhi, which is a tertiary care hospital with 1500 beds catering to the health needs of people in Delhi and the adjoining states of North India.

After receipt of informed consent from patients and approval from the ethical committee, 150 stool samples were taken as follows:
Fifty stool samples from HIV seropositive cases with diarrhea (study group).
Fifty stool samples from HIV seropositive cases without diarrhea (control group I).
Fifty stool samples from HIV seronegative cases with diarrhea (control group II).

Each patient was interviewed using a questionnaire concerning general demographic characteristics, type of illnesses, and clinical symptoms.

Inclusion criteria

An episode of diarrhea was defined as the occurrence of an average of at least three liquid bowel movements daily for at least 1 week before the visit to the hospital.

Exclusion criteria

Persons who had received antibiotics/antiparasitic treatment for diarrhea within the past 14 days were excluded.

Laboratory studies

Diagnosis of HIV infection and microbiological diagnosis of enteric infection in stool samples were done in all 150 cases.

Diagnosis of HIV infection

The diagnosis of HIV infection was done by following the standard protocol at our voluntary counseling and testing center (VCTC) that employs pre test and post test counseling and obtains informed consent before HIV testing. Three different immunoassays (Micro ELISA, J. Mitra and Co. Pvt. Ltd, India; Retroquic, Qualpro Diagnostics, India; and Capillus, Trinity Biotech, USA) were used following the standard manufacturer's instructions.

Diagnosis of enteric infections

Stool specimens were collected fresh and processed in the Diarrhea and Parasitology Laboratory within 4 hours of collection. Parasite detection was done by direct examination after formalin-ether-concentration of stool specimens as wet saline and iodine mount techniques for the detection of protozoan trophozoites and cysts and helminthic eggs and larva. Additionally, all samples were subjected to Gram stain and a modified acid-fast stain for coccidian oocysts.[8]

A part of the stool sample was processed for the detection of bacterial pathogens using selective and differential media as per standard protocol.[9]

For detection of *Clostridium difficile* toxin in stool samples, the *Clostridium difficile* Toxin A + B Antigen Detection Microwell ELISA (IVD Research Inc., Carlsbad, CA 92008 USA) was used following the manufacturer's instructions.

For detection of the Cryptosporidium antigen in stool samples, the Cryptosporidium Antigen Detection Microwell ELISA (IVD Research Inc., Carlsbad, CA 92010 USA) was used following the manufacturer's instructions.

Results

This prospective study was conducted between April 2007 and July 2007. A total of 150 patients were examined in this study; 50 from HIV seropositive cases with diarrhea (study group), 50 from HIV seropositive cases without diarrhea (control group I), and 50 from HIV seronegative cases with diarrhea (control group II).

The age and gender profile of the study and the control subjects is presented in Table 1. In the study group, the age range of 31-40 years old was the most predominant in overall size (46%). The age range of 21-30 years old and 0-10 years old had the maximum number of cases with 42% and 78% in control groups I and II, respectively. Males outnumbered females in the study group and control group II subjects with a male to female ratio of approximately 1:4 and 3:2, respectively. However, no such gender difference was observed in control group I.

From the study, the overall prevalence of enteric parasitosis in the study group, control group I, and control group II were found to be 20%, 2%, and 18%, respectively. The overall prevalence of enteric parasites in males was 6% (9/150) and 7.3% (11/150) in females. The proportion of protozoan parasites in the study group was 70% as compared with 100% and 66.7% in control groups I and II, respectively. The distribution of different enteric parasites in the study group and control subjects is depicted in Table 2.

The bacteria identified in the stool of HIV seropositive

individuals with diarrhea were *Escherischia coli* in 12 (24%) cases, *Clostridium difficile* in 5 (10%) cases, Salmonella species in 2 (4%) cases, *Vibrio cholerae* in 2 (4%) cases, and Shigella species in 1 (2%) case. In HIV seropositive individuals without diarrhea, *Escherischia coli* again was the most predominant bacteria identified in stool samples (10%) followed by *Clostridium difficile* in 4% cases. In HIV seronegative individuals with diarrhea, whereas *Clostridium difficile* was not seen, *Escherischia coli* was found in 22% of the cases. In addition, *Vibrio cholerae* was isolated in 26% of the cases with 69.2% and 30.8% belonging to Ogawa and Inaba subtypes, respectively [Table 3].

As far as fungal pathogens are concerned, the Candida species was identified in 36%, 12%, and 2% of the cases in the study group, control group I, and control group II subjects, respectively.

Discussion

Although HIV/AIDS and water-borne infections, exemplified by diarrhea, are the leading causes of morbidity and mortality in developing countries, their association has received only cursory attention. Identification of the etiological agent of diarrhea in a patient with AIDS is very important as it can help in the institution of appropriate therapy and the reduction of morbidity and mortality in these patients. This study was therefore conducted to ascertain the scope and frequency of potential enteric bacterial pathogens isolated from

Table 1: Age and gender distribution of the study and control subjects

Age groups (years)	HIV seropositive with diarrhea study group (n=50)				HIV seropositive without diarrhea control group I (n=50)				HIV seronegative with diarrhea control group II (n=50)			
	Males	Females	Total	%	Males	Females	Total	%	Males	Females	Total	%
0-10	2	2	4	8	1	1	2	4	23	16	39	78
11-20	0	0	0	0	4	1	5	10	2	3	5	10
21-30	10	6	16	32	5	16	21	42	1	1	2	4
31-40	12	11	23	46	11	6	17	34	3	0	3	6
41-50	3	1	4	8	2	1	3	6	0	0	0	0
51+	2	1	3	6	2	0	2	4	1	0	1	2
Total	29	21	50	100	25	25	50	100	30	20	50	100

Table 2: Prevalence of specific intestinal parasites among the study and control subjects

Parasites detected	HIV seropositive with diarrhea study group (n=50) frequency (%)	HIV seropositives without diarrhea control group I (n=50) frequency (%)	HIV seronegative with diarrhea control group II (n=50) frequency (%)
Ascaris lumbricoides	2 (20)	0	2 (22.2)
Hookworm	0	0	1 (11.1)
Hymenolopsis nana	0	0	0
Entamoeba histotytica	2 (20)	0	1 (11.1)
Giardia lamblia	2 (20)	0	5 (55.5)
Blastocystis hominis	0	0	0
Cryptosporidium parvum	2 (20)	1 (100)	0
Isospora belli	1 (10)	0	0
Cyclospora cayetanensis	0 (0)	0	0
Strongyloides stercoralis	1 (10)	0	0
Trichuris trichuria	0	0	0
Total	10	1	9

Table 3: Enteric microbial pathogens other than parasites identified in stool samples of study and control subjects

Pathogen other than parasite	HIVseropositive with diarrhea study group (n=50) number (%)	HIV seropositive without diarrhea control group I (n=50) number (%)	HIV seronegative with diarrhea control group II (n=50) number (%)
Candida species	18 (45)	6 (46.2)	1 (4)
Salmonella species	2 (5)	0	0
Shigella species	1 (2.5)	0	0
Vibrio cholerae			
Ogawa	2 (5)	0	9 (36)
Inaba	0	0	4 (16)
Clostridium difficile	5 (12.5)	2 (15.4)	0
Enteropathogenic *Escherishia coli*	8 (20)	3 (23.1)	9 (36)
Enteroinvasive *Escherishia coli*	4 (10)	2 (15.4)	2 (8)
Total	40	13	25

stool samples of HIV-positive and -negative individuals with and without diarrhea. The etiology for such diarrhea could be parasitic, bacterial, fungal, enteric viruses, or HIV itself may contribute to diarrhea. In addition to microbes, other factors such as medication, immune deregulation, autonomic dysfunction, and nutritional supplementation play a substantial role in diarrhea of patients with HIV/AIDS.[5] Shigellosis, Campylobacter infections, and Cryptosporidiosis occur relatively more frequently in HIV-1 infected persons than in persons without HIV-1 infection. Some agents produce diarrhea almost exclusively in HIV-1 infected persons (e.g., *Mycobacterium avium* complex, Cytomegalovirus, and HIV-1enteropathy). Others cause more severe, more prolonged, or more often recurrent diarrhea in the presence of HIV-1 infection (e.g., Cryptosporidiosis species, Isospora species, Salmonella species, Astrovirus, Adenovirus, Calcivirus and perhaps Microsporidium species, *Cyclospora cayetanensis*, Shigella species, and Campylobacter species). Some agents apparently have unaltered courses but occur commonly in HIV-1 infected persons (e.g., *Clostridium difficile*).[10] Among persons with HIV infection, 40-80% of diarrheal illnesses have an identifiable cause and a bacterial etiology is common.[11,12] Diarrheal illnesses due to parasites in patients infected with HIV is on the rise. Many studies have highlighted the emergence of important protozoan parasites and helminths as a major cause of morbidity and mortality in patients with AIDS.[2,13,14] In resource limited countries such as India, enteric infections remain common in the general population and for those people infected with HIV, with geographic differences in the reported prevalence of individual pathogens reflecting differences in pathogen prevalence, standards of hygiene, and diagnostic methods used.[2] There are many reports regarding the frequency of various pathogens causing diarrhea from different parts of India.[2,15] Some studies also demonstrated regional variability of pathogens,[16] as well as changing trends of etiology in the same population.[11] With the myriad of etiologies and sometimes-altered natural history of enteric infections in HIV-1 infected persons with diarrhea, it remains uncertain how well clinical manifestations or risk factors predict microbial etiology or are able to guide the empirical choice of therapy for an individual patient.

In our study 31-40 years old, 21-30 years old, and 0-10 years old were the most predominant age groups in overall size affected with 46%, 42%, and 78% of the cases, respectively. Previous studies have also indicated HIV prevalence to be most common in the 21-30 year old age group[17,18] and in the non HIV infected group, diarrhea is more commonly seen in children than in adults in developing countries. Our study shows a male preponderance in HIV infected patients with diarrhea similar to previous studies;[20] however, others have reported more females than males in this population.[17]

We found an overall prevalence of enteric parasitosis in the study group, control group I, and control group II to be 20%, 2%, and 18%, respectively. A previous study reported that a potential enteric pathogen was isolated from all (100%) of the HIV-positive individuals with diarrhea and 68 (52.3%) without diarrhea.[17] Another study reported a prevalence of enteric parasitosis of 36% and 14% in patients with HIV with and without diarrhea, respectively,[18] whereas others have reported a higher percentage of enteric parasitosis in Indian patients with HIV with diarrhea.[19] Studies in Africa have reported a prevalence of intestinal parasitosis varying from 19 to 33%.[21,22] A recent study conducted in Chennai reported 13% enteric parasitosis among HIV-positive individuals and normal individuals without diarrhea.[23] In our study, the overall prevalence of enteric parasites in males was 6% (9/150) and 7.3% (11/150) in females. A previous study from Nepal reported 56% and 44% enteric parasitosis in male and female groups of patients with HIV/AIDS, respectively.[24]

Our study shows that the proportion of protozoan parasites in the study group was 70% as compared with 100% and 66.7% in control groups I and II, respectively. Previous authors have also reported a higher prevalence of protozoa as compared with helminthes in enteric parasitosis[18,22,24] in HIV/AIDS. In HIV-positive patients with diarrhea, out of 10 cases of enteric parasitosis, we isolated helminthes in 3 cases (*Ascaris lumbricoids* in 2 and *Strongyloides stercoralis* in 1) and protozoa in 7 cases (*Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in 2 each and Isospora belli in 1). In HIV-positive patients without diarrhea, we isolated only *Cryptosporidium parvum* in 1 case. In HIV-negative individuals with diarrhea, out of 9 cases of enteric parasitosis, helminthes were found in 3 cases (*Ascaris lumbricoids* in 2 and *Ancylostoma duodenale* in 1) and protozoa were detected in 6 cases (*Entamoeba histolytica* in 1 and *Giardia lamblia* in 5). These findings suggest that patients with HIV/AIDS had more intestinal parasite infections than the control groups and the only parasites clearly more prevalent in this population were *Cryptosporidium parvum*, *Isospora belli,* and *strongyloides stercoralis*. A recent study from North India reported that of the emerging parasites, *Isospora belli* was most frequently detected followed by *Cryptosporidium; Blastocystis hominis* and *E. histolytica* were the most frequent conventional pathogen followed by *Giardia lamblia*.[25] Few other Indian studies have also reported *Isospora belli* followed by *Cryptosporidium parvum* as the most common pathogen among enteric parasites in HIV associated diarrhea.[19,23] The high rate of infection with *Isospora belli* poses a threat to HIV-positive patients. Diagnosed cases of *Isospora belli* were considerably fewer

in this study, which could possibly be either due to more sensitive detection methods used in studies reporting a higher prevalence or a reflection of low prevalence in this study population. The actual rate of this infection in immunocompetent individuals and patients with AIDS is likely to be underestimated due to asymptomatic shedding of oocysts and treatment with trimethoprim - sulphamethoxazole for other infections in AIDS cases, which may confer some protection against this parasite. Other studies have reported *Cryptosporidium parvum* as the most common pathogen among enteric parasites in the HIV/AIDS population.[12,24,26] HIV opportunistic infections, cryptosporidiosis inclusive, tend to vary from one locality to another and from one country to the another depending on the level of contamination in the water, food, and contact with animals, which are important factors in the dissemination of the parasite. Finally, the small unfilterable size (3-5 Lm) of oocysts, their resistance to chlorine disinfections, and low infective dose are the major infective potential of *Cryptosporidium parvum*. No *Microsporidia* and *Cyclospora* were detected in our study similar to a few other studies,[25] though other studies from India have reported a low prevalence of cyclosporidiasis[15,18,23] and microsporidiosis.[19,23] Thorough investigations on a large number of patients are required to know the exact role of these pathogens in HIV-related diarrhea in India. Our findings tend to support the view that the more 'common' parasites (*Ascaris lumbricoides*, *Ancylostoma duodenale*, *Giardia lamblia*, and *Entamoeba histolytica*) are not opportunistic in patients with AIDS and identification of these common parasites in up to 60% of patients with AIDS and the controls is a reflection of poor environmental hygiene. Until recently, more frequently-associated parasites with diarrhea were: *Giardia lamblia*, *Entamoeba histolytica*, *Balantidium coli,* etc. Since the onset of the AIDS epidemic, the number of parasitic pathogens recognized and the frequency with which they are encountered in clinical practice have increased. These parasites can cause self-limiting diarrhea of short duration in healthy individuals, but in the immunocompromised host, including patients with AIDS, diarrhea is usually chronic and, sometimes, life-threatening. The high proportion of patients with AIDS who had diarrhea in the absence of identified parasite infections strongly indicates the existence of other diarrheaogenic agents or mechanisms. The detection of these will require more comprehensive and better controlled studies.

On bacterial culture of stool, the common organisms associated with diarrhea in HIV-infected individuals in our study were *Escherichia coli* (30%), *Clostridium difficile* (12.5%), Salmonella species (5%), *Vibrio cholerae* (5%), and Shigella species (2.5%). Whereas only *Escherichia coli* (38.5%) and *Clostridium difficile* (15.4%) were found in the stool of HIV-infected individuals without diarrhea, the stool of HIV seronegative individuals with diarrhea showed *Vibrio cholerae* and *Escherichia coli* in 52% and 44% of the total bacterial isolates in this group. Previous studies in India have also reported the isolation of similar organisms with slightly variable frequencies in the stool of such patients;[18,23] however, in one of these studies, no bacterial pathogens other than *Escherichia coli* and Klebsiella species were isolated from stool specimens of HIV-positive individuals without diarrhea and normal individuals without diarrhea.[23] Another study from Thailand found that the isolation rates of bacterial enteropathogens causing diarrhea in AIDS patients with diarrhea (APD) (18%, 62/350) were considerably lower than those in non AIDS patients with diarrhea (NAPD) (43%, 152/350) (P<0.05).[27]

As far as fungal pathogens are concerned, we isolated the *Candida* species in 18 cases (36%), 6 cases (12%), and 1 case (2%) of the 50 cases each in the study group, control group I, and control group II, respectively. In a previous study in India, the *Candida* species was identified in 2.6% of the cases consisting of one case in acute diarrhea, one in control group and two cases in chronic diarrhea.

Conclusion

Enteric parasitosis might be one of the major health problems among patients infected with HIV, particularly those with AIDS. Studies from various parts of the world show contrasting prevalence rates with marked geographical variations.[28,29] This emphasizes the need for thorough investigations of these patients to identify pathogens for proper management. Opportunistic enteric pathogens, for which there is no effective treatment, the emergence of new opportunistic infections, and the enlarging pattern of drug resistance continues to be a challenging task. However, better understanding of HIV-1-induced mucosal immunosuppression, sound clinical management, careful diagnostic evaluation, the development of newer antimicrobial agents, and judicious patient management should help to meet this challenge and may help to reduce morbidity and untimely mortality for patients with HIV/AIDS in India.

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Syphilis a risk factor for HIV

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Abstract

Sexually transmitted diseases (STD) that cause inflammatory or ulcerative lesions of genital tract act as important cofactor in increasing the risk of transmission of human immunodeficiency virus (HIV) through sexual contact. When syphilis is present there is about 2-5 fold increased rate of acquiring HIV infection. The incidence of HIV can be reduced by preventing and treating the syphilis and other agents causing sexually transmitted infections (STIs). The current prospective study was conducted over a period of three years(2005-2007).A total of 500 blood samples were collected from patients having history of genital ulcers (study group) and 250 persons with history of non ulcerative lesions (control group) referred from skin and STD department of Guru Nanak Dev Hospital in the Integrated Counseling and Testing Centre (ICTC) attached to Microbiology department, Government Medical College Amritsar. Blood samples were collected after obtaining their written consent after pretest counseling. Serum samples were screened for the presence of HIV-1, HIV-2 antibodies and Syphilis by E/R/S and venereal diseases Research laboratory (VDRL) test respectively. VDRL reactive sera were confirmed by Treponema Pallidum Hemagglutination Assay(TPHA). Out of the 500 patients screened 75% were males and 25% were females. Out of these 20(4%) persons were sero reactive for HIV-1infection and none for HIV-2 infection. Thirty three (6.6%) persons were reactive for Syphilis. Co-infection of HIV and syphilis was 45% in HIV sero reactive patients. Out of 250 control group patients 0.8% were sero reactive for HIV-I.HIV sero prevalence rate was significantly higher amongst genital ulcer disease (GUD) patients as compared to patients with history of having non ulcerative lesions .($p<0.001$) .It is concluded that HIV prevalence was significantly higher in patients having GUD

INTRODUCTION

Syphilis is still a common Sexually Transmitted Disease (STD) in many areas of the world, despite the availability of effective therapy.[1]It causes ulcerative lesions of genital tract and act as an important cofactor in increasing the risk of transmission of HIV through sexual contact.[2] As per National AIDS Control Organization (NACO) 340 million new cases of curative Sexually Transmitted Infections (STI) occurs every year in the world. Out of which 75 to 85% cases are present in developing countries. Out of these new cases 12 million are of syphilis, 62 million are of gonorrhea, 90 million are of Chlamydia and 176 million are of trichomoniasis.[3] Genital Ulcer Disease (GUD) and non ulcerative STDs promote HIV transmission. But the majority of HIV infections occur through the mucosal disruption either on the penis or the cervix.[4] The overall relative risk and population attributable fraction for GUD in facilitating HIV transmission are consistently higher than for other STIs and STI syndromes.[5] The strong association between syphilis and HIV sero prevalence suggests that acceleration of direct linkages between STI testing and HIV counseling and testing would be useful in enhancing the control of STI and HIV in India. The present study was under taken to know the sero prevalence of HIV and syphilis infection among patients presenting with GUD and their correlation.

MATERIAL AND METHOD

The current prospective study was conducted over a period of three years(2005-2007).A total of 500 blood samples were collected from patients (16yrs-50yrs) having history of genital ulcers(study group) and 250 patients without any history of genital ulcers as a control group referred from skin and STD department of Guru Nanak Dev Hospital in the Integrated Counseling and Testing Centre (ICTC) attached to Microbiology department, Government Medical College Amritsar. The study protocol was approved by an institutional ethical committee prior to the investigation .Patients complete history, including demographic characters and risk behavior for HIV infection, were recorded. Pretest counseling was given and informed consent was taken before testing. Fresh serum was subjected to Enzyme Linked Immuno Sorbent Assay (ELISA) test (J Mitra and Co. Pvt. Ltd.) for detection of HIV-I and 2 antibodies. ELISA positive sera were then subjected to another 2 E/R/S [Retroquic(Qualpro diagnostic) and Tridot (J. Mitra and Co. Pvt. Ltd.)] test according to manufacturers instructions and NACO guidelines.[6] Serum samples were also screened for Syphilis by venereal diseases Research laboratory (VDRL) test and then confirmed by Treponema Pallidum Hemagglutination Assay(TPHA)[7]

RESULTS AND DISCUSSION

Out of 500 patients (Study group)screened 375 (75%) were heterosexual males and 125(25%) were heterosexual females. The 459(91.8%) persons were seen in the reproductive age group (16yrs-50yrs). Out of 500 persons 260(52%) belonged to rural area and 240(48%) persons belonged to urban area (Table I). Out of 500 sera 20(4%) were reactive and 480(96%) were non reactive for HIV I antibodies.

None of them were reactive for HIV- 2 antibodies. The age and sex wise distribution of HIV sero reactive and nonreactive persons is shown in table II. Out of 500 persons 33 (6.6%) were reactive for VDRL test and 467 (93.4%) were non reactive for VDRL test. Out of 33 sero reactive persons for syphilis 24 (72.7%) were males and 9 (27.3%) were females. Out of 20 HIV sero positive persons 9 (45%) were co-infected with Syphilis (Table III).

Out of 250 control group patients 140 were males and 110 were females in the reproductive age group. Out of which only one male (0.7%) and one female (0.9%) was sero reactive for HIV-I. HIV sero prevalence rate were significantly higher amongst GUD patients as compared to patients with history of having non ulcerative lesions($p<0.001$).(Table IV).

Table 1: showing Rural / Urban Distribution

RURAL /URBAN	MALE	%AGE	FEMALE	%AGE
RURAL	230	61.3	30	24
URBAN	145	38.7	95	76
TOTAL	375	100	125	100

Table 2: Age and sex wise distribution of HIV Seropositive Cases

AGE IN YEARS	MALE	%AGE	FEMALE	%AGE
16-30	2	10	4	20
31-45	7	35	5	25
46-60	2	10	-	
TOTAL	11	55	9	45

Table 3: Correlation of HIV and Syphilis in Gud patients

Reactivity	HIV	Percentage	Syphilis	Percentage
HIV (alone)	11	55	-	-
SYPHILIS (alone)	-	-	24	72.7
COINFECTION	9	45	9	27.3
TOTAL	20	100	33	100

Table 4: Comparison of HIV Seropositivity in Study Group and Control Group

No. of cases	Study group	Percentage	No. of cases	Control group	Percentage
Male (n=375)	11	2.9	Male (n=140)	1	0.7
Female (n=125)	9	7.2	Female (n=110)	1	0.9
Total (500)	20	4	Total (250)	2	0.8

The venereal transmission of HIV can occur in high risk group persons. In the current study male outnumbered female patients in the ratio of 3:1 in having GUD where as other worker reported it to be 12:1 up to 15:1.[8]This might be due to the fact that males practice high risk behavior and acquire more STIs than females. Among the 500 serum samples screened for HIV 4% were found to be sero reactive for anti HIV antibodies. The incidence is almost the same (4.9%) as reported by Kamali A et al[9] where as other workers reported it to be 2.3%, 30%, 75%[10-12]. HIV positive individual having other STIs are more likely to transmit HIV to others by shedding or releasing HIV cells in both ulcerative and inflammatory genital secretions[13]. In the present study 2.9% males and 7.2% females were sero positive for HIV infection where as other workers reported it to be 4.11% and 5.17% respectively[14].In the control group 0.8% were sero reactive for HIV where as other workers reported it to be 1.7% .[15]

In the present study rural and urban cases were nearly equal in number with slight preponderance of rural cases i.e 52% where as Girgila et al[16] in their study observed that 64.94% cases belong to rural area. This may be due to the fact that being an agriculture state, large number of patient reporting to our hospital belonged to rural area. Though HIV infection is still low in this population the high prevalence of STIs indicates that potential is there for an explosive spread of HIV/AIDS epidemic.

In present study 6.6% persons were reactive for VDRL test where as other workers reported it to be 14% and 22% respectively.[12,11]

Out of these 72.7% were males and 27.3% were females where as other workers reported it to be 12.9% and 12.6% respectively[9].The prevalence of T. palladium was significantly higher among men (p=0.001), similar findings were reported by Moodly P et al[12] (p=0.03). The high prevalence rate of syphilis in males may be due to extramarital relations from where it spreads to females. These data strongly indicate that some GUD patients are engaged in high risk sexual behavior. In our study the incidence of co- infection of syphilis in HIV sero reactive persons were 45% where as other workers reported it to be 31% .[17] It is well known fact that the presence of ulcerative STD increases the transmission of HIV many folds.

CONCLUSION

It is concluded that HIV prevalence was significantly higher in patients having GUD like syphilis.

Thus screening for HIV antibody prevalence in GUD is likely to help in understanding the reality of spread of HIV infection and indicate that positive syphilis serology is an unbiased criterion for identifying individuals at increased risk of HIV infection.

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CASE REPORT

Immune reconstitution inflammatory syndrome in an HIV seropositive leprosy patient

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Introduction

The natural course of leprosy co-infected with human immunodeficiency virus (HIV) is not clearly understood. This is important because of the high and increasing prevalence of HIV infection in many leprosy endemic populations.[1–3] The introduction of highly active anti-retroviral therapy (HAART) has led to the emergence of a new clinical syndrome known as immune reconstitution inflammatory syndrome (IRIS).[4] This syndrome has been described in association with diseases such as herpes zoster, *Mycobacterium tuberculosis, M. avium*, cytomegalovirus and sarcoidosis.[5,6] Recent reports have described IRIS in four patients with leprosy. We report an HIV positive borderline lepromatous leprosy patient developing IRIS following institution of HAART.

Case report

A 32-year-old male reported with numbness over upper and lower limbs in a glove and stocking pattern for the last 2 years. Over the last 6 months, he had had intermittent fever associated with multiple crops of red raised painful evanescent lesions on the face and the limbs. He also developed spontaneous blistering, slippage of footwear and difficulty in doing fine work with his hands. He had progressive weight loss over the past 2 years without persistent diarrhoea, cough or other systemic complaints. He denied high risk behaviour towards the acquisition of HIV infection.

The patient was febrile with multiple discrete submandibular, axillary and inguinal non-tender and mobile lymph nodes. Cutaneous examination revealed marked seborrhoea over face and chest with diffuse infiltration of the forehead and ears. Multiple erythematous tender

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papules and nodules varying in size from 0·4 to 2 cm were present over the forehead, cheek, chin, earlobes and extensor aspect of upper and lower limbs. Multiple lesions of molluscum contagiosium were present on the face. Hypopigmented atrophic scars over the extensor aspect of bilateral upper and lower limbs were also seen.

There was atrophy of the bilateral thenar and hypothenar eminence along with glove and stocking pattern of anaesthesia. Right greater auricular, bilateral ulnar, radial cutaneous, lateral popliteal and posterior tibial nerves were thickened and non-tender. Systemic examination revealed no abnormality.

Haematological examination revealed microcytic hypochromic anaemia (Hb 7·6 g%), neutrophilic leucocytosis (TLC 12,500/mm^3) and raised erythrocyte sedimentation rate (81 mm in the first hour). Slit skin smear examination for *M. leprae* revealed a bacteriological index of 4 along with a morphological index of 40%. Histological examination of a biopsy taken from the lesion on the right cheek demonstrated diffuse infiltrate of foamy macrophages, plasma cells and lymphocytes along with concentric perineural infiltration, validating the clinical diagnosis of borderline lepromatous leprosy. On Fite–Faraco staining, acid-fast bacilli were seen both singly and in clumps within Schwann cells and macrophages. Histological examination of a biopsy taken from a painful nodule on the arm demonstrated neutrophilic abscesses superimposed on a diffuse infiltrate of foamy macrophages and lymphocytes, along with evidence of leucocytoclastic vasculitis, confirming a diagnosis of erythema nodosum leprosum (ENL). ELISA for HIV by Retroquick, Capillus and Immunocomb was reactive, with a CD4 count of 108/mm^3. Stool examination for *Cryptosporidium* and *Isospora*, and sputum examination for *Pneumocystis jiroveci* and *M. tuberculosis* were negative.

The patient was started on rifampicin (600 mg monthly), minocycline (100 mg daily), ofloxacin (400 mg daily) and clofazimine (100 mg thrice daily). Standard WHO MDT regimen including dapsone was not prescribed, as the patient had moderate anaemia with a haemoglobin of 7·6 g%, which is a relative contraindication for the drug. A combination of trimethoprim (80 mg) and sulphamethoxazole (400 mg) twice a day for *Pneumocystis jiroveci* pneumonia prophylaxis was also started. ENL lesions and fever subsided within 4 weeks. Ofloxacin and minocycline were discontinued after 4 weeks and the patient was continued on rifampicin and clofazamine. After 3 months of anti-leprosy treatment (ALT), HAART comprising zidovudine (600 mg), lamivudine (300 mg) and nevirapine (400 mg) daily was started. One month following HAART, the patient developed erythema, edema and pain over pre-existing lesions, eruption of multiple new lesions, bilateral posterior tibial neuritis and foot drop (Fig. 1). He was diagnosed to have type 1 leprosy reaction and was treated with oral prednisolone (40 mg daily), which was subsequently tapered. The neuritis subsided with no improvement in dorsiflexion at ankle joints. Currently the patient is continuing HAART, ALT and physiotherapy. His current CD4 count is 224/mm^3.

Discussion

HIV infection has been shown to be strongly associated with development of active tuberculosis and diseases caused by other mycobacteria, but its association with leprosy is much less clear.[1] An association between HIV and leprosy has been described from Zambia and Tanzania, while reports from Ethiopia and other African countries have not found such a correlation.[7–11] A study conducted on 4025 leprosy patients in India described only five HIV

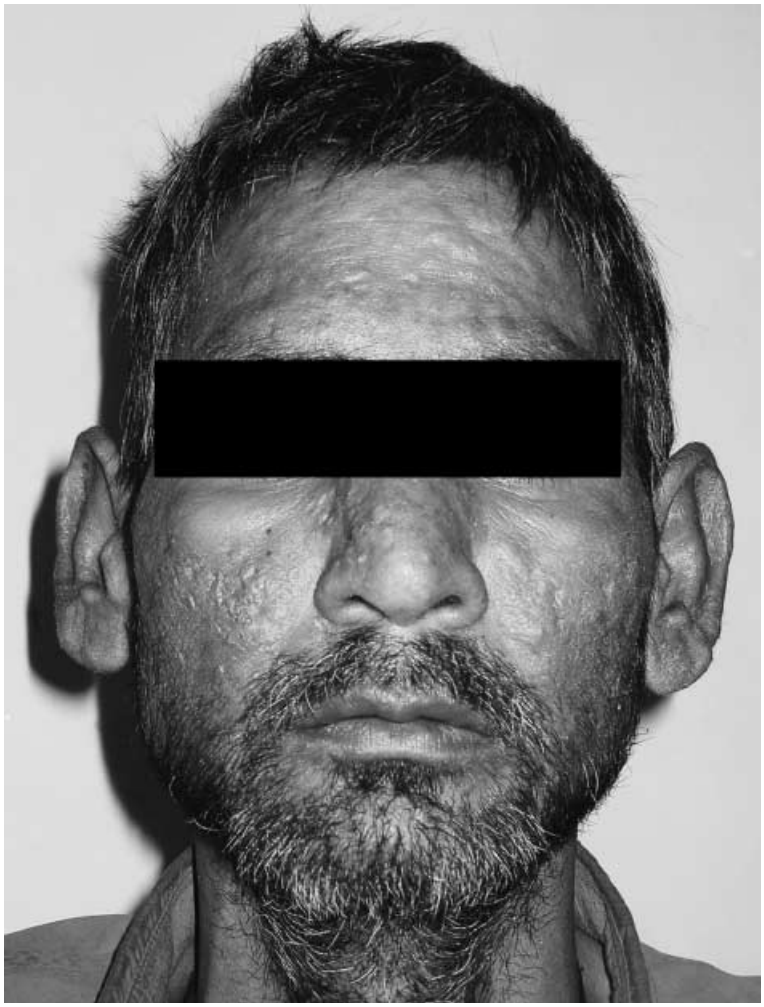

Figure 1. Marked seborrhoea and diffuse infiltration over the face and bilateral ears along with necrosis over the left eyebrow and both the cheeks.

coinfected patients.[1] This may be due to the fact that HIV positive patients in the tropics do not live long enough to develop infections such as leprosy, which has a prolonged incubation period.[1]

The course of *M. avium* and *M. tuberculosis* in HIV infected individuals tends to be more rapid and fulminant, since HIV infection compromises the cell-mediated immunity.[12] The cellular immune response is of particular importance in the clinical manifestation of infection with *M. leprae*. It is expected that when an HIV positive individual becomes infected with *M. leprae*, the lepromatous disease would predominate.[12] Despite the expected association of lepromatous leprosy with the underlying immunosupressed state, studies conducted in Agra (India) found that HIV infection is commonly associated with the tuberculoid form of leprosy and none of these patients progressed to a more severe form of disease.[1]

Many studies have attempted to show the HIV infection as a risk factor for various complications in leprosy.[8] Gebre *et al.* suggested a positive association of HIV co-infection with erythema nodosum leprosum, recurrent reversal reaction and increased risk of seroconversion and mortality but no association with neuritis.[8] In contrast, Vreeburg *et al.* reported fulminant neuritis in a relatively short span of time with co-infection.[11] Further, in a recent case report, four out of five leprosy patients who were coinfected with HIV developed type I leprosy reaction following initiation of HAART therapy.[13]

Our patient presented with ENL and developed neuritis as a part of IRIS. IRIS most commonly occurs in HIV positive patients, at an advanced stage of disease when their CD4 count is less than 200/mm^3.[2] Clinical signs of inflammation appear in opportunistic infections, when HAART triggers a generalized activation during transition phase of viral load suppression and the CD4 lymphocyte count increases.[5] The syndrome has since been described in association with a number of different infectious conditions, including herpes zoster (41 cases), *M. tuberculosis* (37 cases), *M. avium* complex (32 cases) and cytomegalovirus (22 cases).[5] In some cases, IRIS appears in the absence of opportunistic pathogens and manifests itself as an autoimmune or granulomatous disease, of which sarcoidosis is the most frequent (10 cases). The criteria for diagnosis include: (1) patients with full blown AIDS; (2) a significant increase in CD4 lymphocyte counts following antiretroviral therapy; (3) reconstitution of the immune system, accompanied by the detection of a latent infection (leprosy in the present case) and (4) symptoms not consistent with the expected progression of a previously diagnosed opportunistic infection, the expression of a newly acquired infection, or the manifestation of undesirable effects of the HAART.[5,6]

IRIS with leprosy was first reported by Lawn *et al.* in 2003, and recently Couppie *et al.* have reported three patients, of whom two developed a severe ulcerating form of type 1 lepra reaction, responding to corticosteroids.[4,5] The present case with BL leprosy developed IRIS syndrome in the form of type I lepra reaction manifesting as erythematous edematous lesions and polyneuritis with bilateral foot drop, following the onset of HAART. His CD4 count increased from 108 to 224/mm^3 after 3 months of HAART, thus fulfilling the criteria for the IRIS phenomenon.

Systemic corticosteroids are indicated in severe leprosy reaction with neuritis, as in the present case. In HIV positive patients, use of high dose corticosteroids is associated with the development of accelerated Kaposi's sarcoma, avascular necrosis of bone, activation of latent cytomegalovirus and *M. avium*.[14] Therefore these patients should be closely monitored.

As access to HAART is increasing in leprosy endemic countries, the number of IRIS cases due to *M. leprae* is likely to increase in future. Therefore, in India, where the number of HIV positive cases has already crossed the 5 million mark, it is important to recognize leprosy as an IRIS associated condition.

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Original Article

Microbiology Section

Skin and Mucocutaneous Manifestations: Useful Clinical Predictors of HIV/AIDS

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ABSTRACT

Background and Aims: The HIV infection is associated with several dermatological conditions which may be the first pointer towards the existence of HIV. These may present with unusual and atypical manifestations in the course of the HIV infection. Keeping this in mind, the seroprevalence of HIV in these persons and the spectrum of the skin and the mucocutaneous lesions in the HIV positive patients was studied.

Methods: The current prospective study was conducted over a period of 3 years (2006-2008). A total of 604 persons who had any kind of skin and mucocutaneous infections were screened for the HIV infection as per the NACO guidelines after recording their clinical and epidemiological profiles.

Results: Out of the 604 patients who were screened, 90(14.90%) were seropositive for the HIV-I antibodies and none was positive for the HIV-2 antibodies. Seventy three point thirty three percent 73.33 of the seropositive patients were in the age group of 15-40 years, with a male-female ratio of 1:1.05. The heterosexual route was the most common mode of transmission (86.6%).A wide range of infectious and noninfectious lesions were observed. In the HIV seropositive patients, oral candidiasis (32.22%) was the most common infectious disease which was observed, followed by herpes zoster (13.33%), genital warts (7.77%) and genital herpes (6.66%). The most common noninfectious manifestation was seborrhoic dermatitis (8.88%), followed by pruritic papular eruptions (7.77%).

Conclusion: As there is a high prevalence of the HIV infection in patients who have skin and mucocutaneous disorders, the doctors, during the investigation of these patients, must have a high level of suspicion for the HIV infection in their mind. An early detection of HIV optimizes the chemoprophylaxis for many opportunistic mucocutaneous disorders.

Key Words: Skin and mucocutaneous lesions, HIV, Heterosexual route

INTRODUCTION

Ever since its recognition in 1981, each year, around 2.7 million people become infected with HIV and 2 million die of AIDS in the world [1]. As per the NACO guidelines, the number of people who live with HIV/AIDS in India are 2.31 million [2]. The HIV infection is associated with several dermatological conditions which may be the first pointer towards the existence of HIV [3]. A wide range of infectious and noninfectious skin lesions develop during the course of the disease and their frequency patterns and the associated factors have been shown to vary from region to region [4]. In the developing countries, the CD4 count, the viral load, etc., are used for the assessment of the HIV disease. A lack of these facilities or their high costs necessitate a greater dependence on clinical markers. The cutaneous manifestations can serve as a dependable marker of the HIV disease. The present study was undertaken to determine the seroprevalence of HIV and the spectrum of the skin and the mucocutaneous lesions in HIV positive patients.

MATERIALS AND METHODS

A total of 604 blood samples were collected over a period of 3years (2006-2008) at the Integrated Counseling and Testing Centre (ICTC) which is attached to the Department of Microbiology, from patients who had cutaneous and mucocutaneous manifestations, who were referred from the Skin and STD Department of Guru Nanak Dev Hospital which is attached to the Government Medical College, Amritsar, Punjab, India. The patients' complete history which included the presenting complaints, the clinical diagnosis, the demographic characters and the risk behaviour for the HIV infection, were recorded. A pretest counseling was given and an informed consent was taken before the testing. The fresh sera were subjected to the Enzyme Linked Immuno Sorbent Assay (ELISA) test (J Mitra and Co. Pvt. Ltd.) for the detection of the HIV-I and the HIV-2 antibodies. The ELISA positive sera were then subjected to another 2 E/R/S [Retroquic(Qualpro diagnostic) and Tridot (J. Mitra and Co. Pvt. Ltd.)] test according to the manufacturer's instructions and the NACO guidelines [5]. A post test counseling was given. The clinical diagnosis was supplemented with laboratory procedures like microscopy (the KOH preparation) and the Venereal Disease Research Laboratory test (VDRL) wherever they were applicable.

The study protocol was approved by the institutional ethical committee prior to the investigation.

OBSERVATIONS

Out of the 604 patients who were screened, 90(14.90%) were seropositive for the HIV-I antibodies and none was positive for the HIV-2 antibodies. The age and the sex wise distribution, along with their sociodemographic profile, is shown in [Table/Fig-1]. The male:female ratio was 1:1.05. The most common infectious mucocutaneous lesions in the HIV/AIDS patients were oral candidiasis (32.22%), Herpes zooster (13.33%), genital warts (7.77%) and

Variables		No. of positive	Percentage
Age in years	0-14	4	4.45
Typhi	15-20	3	3.33
	21-40	63	70
	>40	20	22.22
Sex	Males	44	48.89
	Females	46	51.11
Geographical distribution	Rural	63	70
	Urban	27	30
Educational status	Illiterate	55	61.11
	Up to 8th standard	20	22.22
	10th standard above	15	16.67
Occupation	Housewives	46	51.11
	Unskilled workers	29	32.22
	Truck drivers	9	10
	Students	6	6.67
Marital status	Married	73	81.12
	Unmarried	8	8.88
	Widow/separated	9	10
Probable mode of transmission	Heterosexual	78	86.67
	Perinatal	5	5.56
	Blood and blood products	4	4.44
	Not specified	3	3.33

[Table/Fig-1]: Sociodemographic profile in seroreactive persons

Infectious disorders	No.positive (%)	Non infectious disorders	No.positive (%)
Oral Candidiasis	29 (32.22)	Seborrhoeic dermatitis	8 (8.88)
Herpes zoaster	12 (13.33)	Pruritic papular eruptions	7 (7.77)
Genital warts	07 (7.77)	Drug rash	2 (2.22)
Genital Herpes	6 (6.66)	Generalized pigmentation	2 (2.22)
Gingivitis	05 (5.55)		
Apathus stomatitis	4 (4.44)		
Leucorrhoea (T. vaginalis infection)	4 (4.44)		
Oral Herpes simplex type –I infection	3 (3.33)		
GDD in males	3 (3.33)		
Dermatophytosis	3 (3.33)		
Scabies	3 (3.33)		
Primary Chancre	2 (2.22)		
Staphylococcal infection	2 (2.22)		

[Table/Fig-2]: Common Skin and Mucocutaneous manifestations in HIV seroreactive persons

genital herpes (6.66%). The most common noninfectious manifestation was seborrhoic dermatitis (8.88%), followed by pruritic papular eruptions (7.77%) [Table/Fig-2]. In 10% of the HIV seropositive persons, more than one lesion was present. In the HIV seronegative patients, the signs and the symptoms of the infectious and the noninfectious skin and mucocutaneous lesions were less severe and only one type of lesion was present.

DISCUSSION

In the present study, 90 patients (14.90%) were seropositive for the HIV antibodies. The seropositivity was reported to be 3.24% in another study which was done in the same institution [6] ($p<0.01$), but in another institution, it was reported to be 44.55% [7]. The higher prevalence of the HIV infection could be because the HIV seropositivity as a whole, had gone up and because the skin manifestations were the most common presentations in the HIV-I infection [8]. They also varied from region to region. In the current study, 73.33% patients were in the reproductive age group (15-40 years), whereas 88.55% patients were documented by NACO [9]. The recent reports about HIV/AIDS in India, mention that most of the infections were seen in the age of 15-44 years, as this was the sexually active age group [10]. In the current study, the male-female ratio was 1:1.05, which showed a slight preponderance of females over males, whereas other workers had observed a 2.2:1 male –female ratio [11]. This shows that the epidemic is increasing steadily among women and among the rural young housewives with a low level of education .In the present study, the major mode of the infection was the heterosexual route (86.6%), which almost collaborated with the data from another study (88.7%) [11].

Due to immunosuppression, the HIV seropositive persons have multiple and widespread cutaneous and mucocutaneous lesions, whereas in immunocompetent patients, the lesions are localized and are mostly of the single type. The co-infection of HIV with Candida may be an important exogenous factor that may influence the severity and the rate of the disease progression in HIV infected individuals [12]. In the present study, oral candidiasis (32.22%) was the most common mucocutaneous manifestation which was seen in the HIV positive persons, which collaborated with the findings of other workers (35.33%) [13]. It was reported to be 61% in another study which was done in the same institution [14] ($p<0.001$), whereas other workers had reported it to be 45% and 11.50% respectively [15,11]. Oropharyngeal candidosis has been reported to occur in from 50-95% of all the HIV positive persons at some point during their progression to full-blown AIDS [16]. A comparative study on the carrier state of Candida and its speciation in the oral flora among healthy individuals, in persons with Diabetes mellitus and in HIV positive individuals was done by other workers and they found a higher carriage rate(54%) in the HIV individuals as compared to that in the other two groups [17]. The ulcerative and the non ulcerative genital diseases in HIV hold importance, as they share a common mode of transmission with HIV. In the current study, the incidence of genital herpes was 6.66%, whereas other workers reported it to be 5.5% [18]. In the present study, the other various genital lesions were leucorrhoea which was caused by the T. vaginalis infection (4. 44%), the Genital Discharge Disease (GDD) in males (3.33%) and primary chancre (2.22%), whereas other workers reported them to be 4% (leucorrhoea)3, 2% (GDD)3 and 7.17% (primary chancre) [19]. respectively. Several studies have shown that the T. vaginalis infection was associated with an increased risk of the HIV infectivity and transmission. T. vaginalis may amplify the HIV-1 transmission by increasing both the susceptibility in an HIV-1 negative person and the infectiousness in an HIV-1 positive patient [20]. Syphilis afflicts up to 25% of the HIV-positive individuals, and it can pres-

ent in the primary stage as a chancre, in the secondary stage with mucocutaneous features and in the tertiary stage with neurologic and cardiac involvement [21]. In this study, genital warts were present in 7.7% patients, which corroborated with the findings of other workers (7.1%) [22], where as other workers reported them to be present in 6%. patients [23]. However, we did not come across any abnormal clinical presentations of these STDs or any other mucocutaneous disorders in these HIV infected cases. The incidences of these mucocutaneous disorders were quite high among our HIV positive patients as compared to that in the HIV negative patients. In the current study,13.33% had a recurrent Herpes zoster infection with narcotizing ulcers in a multidermatomal involvement, which was similar to the findings of the studies of other workers (19.44%) [16]. Herpes zooster can occur early in the course of the HIV disease and it generally precedes the other skin manifestations of the HIV disease. In the patients with HIV, it can present with necrotizing ulcers in a multidermatomal pattern, it can last longer than the usual 2-3 weeks, and it can heal, leaving prominent scars [21]. The next manifestation in the present study was seborrheic dermatitis(8.88%). Almost similar findings were reported by other workers(8.5%) [11]. Seborrhoic dermatitis is one of the common noninfectious skin conditions in India, with a prevalence rate of 8% to 21% in HIV positive patients [8]. This is an entity which is characterized by erythema and scaling of the central part of the face, which involves the nasolabial folds and the eyebrows, as well as the scalp [24].

It is found in up to 40% of the seropositive patients 24 and in up to 80% of the patients with AIDS as compared to its incidence in 3% of the seronegative population [25].

In the present study, the incidence of the pruritic papular eruptions was 7.77%,whereas other workers reported it to be 32.23% [26]. A papular pruritic eruption is a unique dermatosis which is associated with the advanced HIV infection, which is characterized by sterile papules, nodules, or pustules with a hyperpigmented, urticarial appearance and pruritis [27]. The next common manifestations were gingivitis and apathus stomatitis (4.44%) each, whereas other workers reported it to be 82.9%, 17.33% and 3% respectively [22,13,3]. Severe periodontal diseases have been associated with the alterations in the host immune system, which can predispose to gingivitis and the development of periodontitis. Moreover, the relevance of the immune system in the protection of the periodontal tissues has been documented and the impairment of this system could aggravate the periodontal status [28] Previous studies have shown that the microbiology of gingivitis and periodontitis in the HIV patients may differ significantly in comparison to these periodontal pathologies in immunecompetent individuals [29,30].

The incidences of the oral Herpes simplex type-I infection, dermatophytosis and scabies were 3.33% each in our study, whereas they were reported to be 5.7% (oral Herpes simplex type-I infection) [21], 8% (dermatophytosis)15 and 4% (scabies) [23] by other workers. Two patients of scabies had a severe crusted form of scabies on the palms and soles, along with dystrophy of the nail plates of the toes. The crusted scabies could be considered as an opportunistic infection of AIDS, as it was related to the cutaneous immune response,6 while in immunocompetent patients, this form of scabies was not normally seen.

In our study, we observed a generalized pigmentation, a drug rash and a Staphylococcal skin infection (a 2.22% incidence for each), whereas other workers reported the incidences to be 35.9% (generalized pigmentation) [21,17]. 70% (drug rash)26 and 1.3% (Staphylococcal skin infection) [15]. The Staphylococcal skin infection was the most common cutaneous bacterial infection in the HIV patients. This infection could also present in other disorders also, like in diabetic patients. Severe cutaneous disorders occur frequently as the HIV infection advances and the immune function deteriorates. They affect between 80 and 90% of the HIV-infected patients and they occur at any time during the course of the infection [21]. The skin lesions or the combinations of the skin conditions are so unique that the diagnosis of the HIV infection or AIDS can often be suspected from the skin examination alone [31].

CONCLUSION

It can be concluded that the skin and the mucocutaneous manifestations are useful clinical predictors of the HIV infection. These may present with unusual and atypical manifestations in the course of the HIV infection. So, a high level of suspicion for the HIV infection has to be kept in mind by the doctors during the investigations. An early detection of HIV optimizes the chemoprophylaxis for many opportunistic mucocutaneous infections.

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To

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Dr. Antonio Do Rego Bagh,
Alto Santacruz, Bambolim Complex Post Office,
Goa 403202.

SUB : Use of RETRO CHECK – HIV – WB, RETRO QUIC HIV Rapid Diagnostic Kit in the blood banks in the country – Regarding.

Sir,

Please refer to your letter dated 20.10.2003 regarding the above subject. Based on the evaluation carried out by NIB, Noida, RETRO CHECK – HIV – WB, RETRO QUIC HIV Rapid Diagnostic Kit manufactured by M/s Qualpro Diagnostics, Goa may be used in the blood banks in the country.

Yours faithfully

(R. NARAYANASWAMY)
DY. DRUGS CONTROLLER (I)

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