Microbiol. Immunol., 50(2), 131–134, 2006

Competitive Study of Monoclonal Antibodies against the HIV-1 Gp41 Core Structure

Osamu Usami[1], Peng Xiao[1], Hong Ling[2], and Toshio Hattori*[,1]

[1]*Division of Infectious and Respiratory Diseases, Internal Medicine, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980–8574, Japan, and* [2]*Department of Microbiology and Parasitology, Harbin Medical University, Harbin, 150086, China*

Received April 18, 2005; in revised form, October 24, 2005. Accepted November 18, 2005

Abstract: **Monoclonal antibodies (MAbs) 50.69, 98.6, and T26 bind specifically to the core structure of the human immunodeficiency virus type 1 (HIV-1) envelope transmembrane glycoprotein (gp41). To clarify the specificity of the anti-core structure MAbs, we performed competitive assays using the MAbs to the H9 human T cell line infected with the IIIB strain of HIV-1 (H9/IIIB). Bound MAb 50.69 inhibited MAb 98.6 binding unidirectionally. The reason for the unidirectional cross competition between MAbs 50.69 and 98.6 is not clear, but these results help to define the antigenic structure of gp41 on the surface of infected cells.**

Key words: **HIV-1, Gp41, 50.69 antibody, 98.6 antibody**

The binding of envelope glycoprotein 120 (gp120) of human immunodeficiency virus type 1 (HIV-1) to cellular receptors on target cells leads to conformational changes of envelope transmembrane glycoprotein (gp41) that permit viral and cellular membrane fusion (12). Recent crystallographic studies have shown that fusion-active gp41 folds into a six-helix α-helical bundle, in which three N-terminal helices (N peptides) form an interior, parallel-coiled-coil trimer, while three C-terminal helices (C peptides) pack in the reverse direction into three hydrophobic grooves on the surface of this coiled coil (2, 9, 16). We have previously reported that the decline of anti-DP107 (aa 553–590) (α-helical N-peptide) antibody is associated with the clinical progression in HIV-1-infected individuals, suggesting that antibodies against the structure may have a protective role (7). To define the epitopes within these regions to which infected humans respond during the course of infection, the specificity of human MAbs to these regions was studied. Using 10 human MAbs identified initially by their reactivity to whole gp41 in HIV-1 virion lysates, Xu et al. previously reported two immunodominant regions of gp41 that define the epitopes within these regions to which infected humans respond during the course of infection (5, 17). The first region of gp41 is in the vicinity of the cysteines between amino acids 598 and 604 (cluster I). The second immunogenic region position is between 644 and 663 (cluster II). Titration of sera from HIV-1-infected patients showed that there was approximately 100-fold more efficient antibody binding to cluster I than to cluster II in patients' sera, confirming the immunodominance of cluster I. Subsequent studies have disclosed that human MAbs against gp41 could recognize the gp41 core structure (14). We called these MAbs anti-core structure MAbs, because the exact antigenic structure of gp41 clarified so far is only the core structure and the native and fusion-active structure of all gp41 molecules has not been clarified yet. MAb 50.69, which is a cluster I MAb, reacts to a N51 (aa 540–590) and C43 (aa 624–666) peptide mixture (N51/C43) (6), but neither to N36 (aa 546–581)/C34 (aa 628–661) nor a single C43 peptide (17). MAb 98.6, which is a cluster II MAb, reacts to N51/C43, N36/C34, and a C43 peptide (14). Both N51/C43 and N36/C34 are known to form a six-helical bundle (2). MAb T26 was reported to bind to the six-helix bundle N36/C34 (1, 3, 4). These studies

*Address correspondence to Dr. Toshio Hattori, Division of Infectious and Respiratory Diseases, Internal Medicine, Graduate School of Medicine, Tohoku University, 1–1, Seiryo-cho, Aoba-ku, Sendai, Miyagi 980–8574, Japan. Fax: +81–22–717–8221. E-mail: thatto@rid.med.tohoku.ac.jp

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry; gp41, envelope transmembrane glycoprotein 41; gp120, envelope glycoprotein 120; gp160, envelope glycoprotein 160; HIV-1, human immunodeficiency virus type 1; H9/IIIB, the H9 human T cell line infected with the IIIB strain of HIV-1; MAbs, monoclonal antibodies; MFI, mean fluorescence intensity; PE-avidin, phycoerythrin-labeled avidin.

suggest that MAb T26 is specific for the core structure because MAb T26 was reported to bind only to the oligomeric form in immunoprecipitation assays. Therefore, these MAbs are expected to bind to different epitopes. These MAbs must bind to the infected cell surface gp41 in the human body, although it is not known whether these MAbs interfere with the bindings of other MAbs. To clarify this issue we did competitive assays among human MAbs 50.69, 98.6 and T26.

One half million H9/IIIB cells, a human T cell line infected with the IIIB strain of HIV-1, were pre-incubated with saturated concentrations of MAbs 50.69, 98.6 or human IgG (Calbiochem, La Jolla, Calif., U.S.A.) for 1 hr at 4 C, followed by washing. The effects of these MAbs on the binding of biotinylated MAbs (B-50.69, B-98.6 and B-T26) to H9/IIIB cells were studied. Biotinylated MAbs 50.69 and 98.6 were provided by Dr. Miroslaw K. Gorny. MAb T26 IgG was purified from culture supernatants of hybridoma cells obtained from Dr. P.L. Earl and biotinylated. Because MAb T26 bound little at 4 C, we did not use MAb T26 as a competing MAb. B-50.69 (2 μg/ml), B-98.6 (2 μg/ml) or B-T26 (32 μg/ml) at saturated concentrations were added to the cells and incubated for 30 min at 4 C for MAb B-50.69 or MAb B-98.6, and for 15 min at 37 C for B-T26. After washing the cells twice, 4 μl phycoerythrin-labeled avidin (PE-avidin) (Serotec, Ltd., Kidlington, Oxford, U.K.) was added and the cells were incubated for 30 min at 4 C followed by fixation. PE-avidin labeled biotinylated MAb was detected by flow cytometry (FACS).

Since we could not detect efficient MAb T26 binding to H9/IIIB at 4 C, we tried to detect the temperature dependency of the MAb T26 binding. MAb T26 bound H9/IIIB and yielded a low mean fluorescence intensity (MFI) at 4 C, and a high MFI at 37 C (Fig. 1). The saturating concentrations were 64 μg/ml at 4 C and 32 μg/ml at 37 C and the saturated MFI at 4 C was 11.5 and significantly lower than the saturated MFI 18.8 at 37 C ($P<0.0167$).

We evaluated the MAb T26 binding at 37 C, and those of MAbs 50.69 and 98.6 at 4 C, and calculated the % inhibition (Fig. 2). MAb 50.69 pre-incubation blocked its own binding as a positive control. MAb 98.6 did not block MAb B-50.69 binding ($P\geqq0.05$). MAb B-98.6 was also examined in a similar condition. MAb 98.6 pre-incubation blocked MAb B-98.6 binding. The MAb 50.69 pre-incubation blocked MAb B-98.6 binding ($P<0.0167$), though the former MAb inhibited the binding more efficiently than the latter did. We were able to observe that MAbs 50.69 and 98.6 bound at 4 C and avoided non-specific binding, although the condition was not physiological (13). We also confirmed that MAb B-T26 binding at 37 C was blocked by bound MAb T26. The MAb 50.69 pre-incubation did not show a significant blocking effect for MAb B-T26 ($P\geqq0.05$). However, pre-incubation of MAb 98.6 blocked its binding very efficiently ($P<0.0167$).

MAb T26 was identified as an anti-gp41 antibody that binds only to oligomers, particularly to trimers, but not to monomers (4). Both MAbs 98.6 and T26 bind to a mixture of N36/C34, but their precise epitopes must be different from each other because MAb 98.6 also binds to monomeric gp41. MAb 98.6 pre-incubation inhibited MAb B-T26 binding to H9/IIIB, indicating the epitopes of MAbs 98.6 and T26 may partially overlap.

On the other hand, MAb 50.69 did not compete with MAb B-T26 at all, indicating that the epitopes of the two MAbs are distant. These data accord with the findings that MAb 50.69 does not bind to a mixture of N36/C34 but MAb T26 does.

MAb 98.6 binds to a unique epitope shared with a mixture of N51/C43, N36/C34 and C43. But MAb 50.69 binds specifically to a mixture of N51/C43 but not C43. It is worthwhile to note that MAb 50.69 pre-incubation inhibited MAb 98.6 binding but the interference was not reciprocal. As shown previously, MAbs 50.69 and 98.6 did not compete in an enzyme-linked immunosorbent assay (ELISA) using viral lysate or recombinant proteins derived from gp41 (17). Earl et al. also performed a competitive assay for anti-gp41 MAbs in ELISA. They also showed that the majority of the anti-gp41 MAbs are conformation dependent and most of determinant I as cluster I MAbs do not compete with determinant II as cluster II MAbs (3). MAb 50.69 was classified as a cluster I antibody, and MAb 98.6 was classified as a cluster II antibody. However, in the present study we performed competitive assays using an infected live cell line and FACS. The difference in the method used may explain the conflicting results. The epitopes of MAbs 50.69 and 98.6, which are expressed on the infected cell surface, possibly have a different conformation from the gp41 peptides previously used in ELISA, while it is known that HIV-1 envelope proteins form oligomers dominantly in viral lysate (6). The envelope proteins on the infected cell surface are heterogeneous with native gp120-gp41 complex, residual gp41 after gp120 shedding, and uncleaved envelope glycoprotein 160 (gp160) precursor, although gp160 derived from viral lysate forms a trimer (10). Atomic force microscopy investigation revealed that monomeric gp120 is dominant on H9/IIIB (8). The unidirectional competition of MAbs 50.69 and 98.6 may be explained by the positional relationship among epitopes on

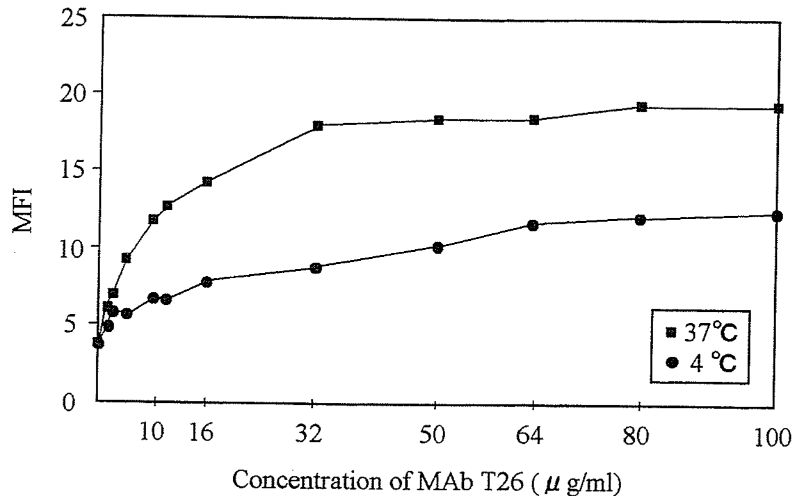

Fig. 1. The temperature dependency of MAb T26 binding. MAb T26 binding to H9/IIIB at 37 C was higher than at 4 C.

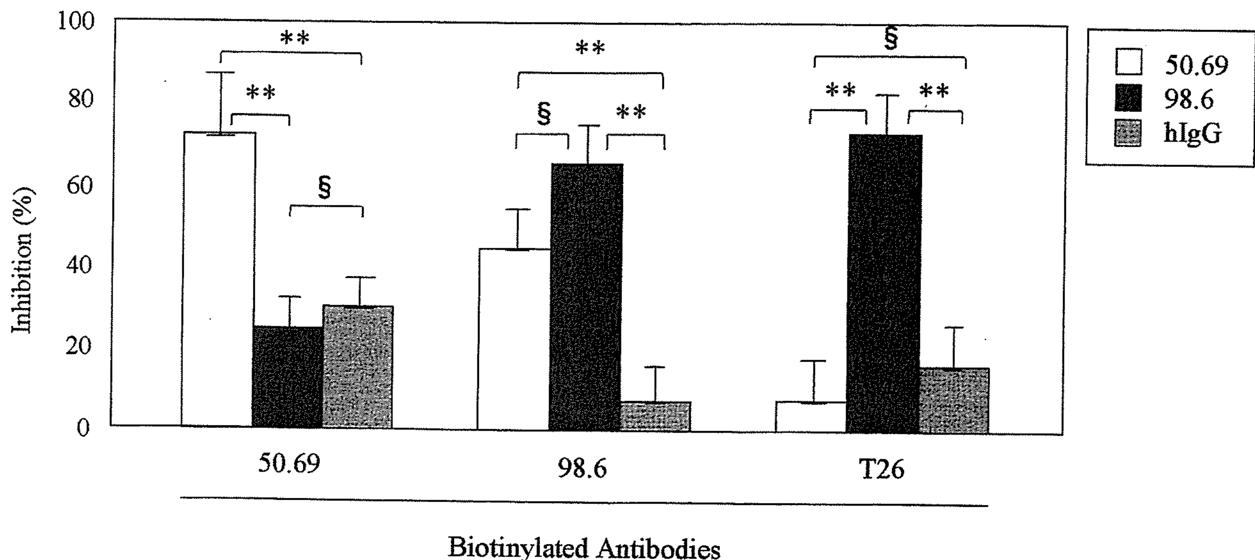

Fig. 2. The decreased binding of biotinylated MAb 50.69, 98.6, or T26 to H9/IIIB cells after preincubation with MAb 50.69 (open bars), 98.6 (filled bars), or hIgG (gray bars). The negative control was calculated as the MFI without preincubation MAb and with biotinylated MAb. The positive control was calculated as the MFI with biotinylated MAb blocked by the same preincubation MAb, the concentration of which was saturated. % inhibition was calculated as [1−(biotinylated MAb MFI−positive control MFI)/(negative control MFI−positive control MFI)]×100 (%). Data are expressed as mean±S.D. Each bar (±S.D.) represents the mean of triplicate determinations (**$P<0.0167$) (§$P\geqq 0.05$). To determine statistically significant differences among the three groups, differences were considered to be statistically significant when $P<0.0167$ by the Bonferroni/Dunn test. Data were analyzed using CellQuest software (Becton Dickinson Biosciences).

monomeric gp41. The size of the epitopes, the induced conformational changes upon binding MAb 50.69, and differences in the affinities of the MAbs also might be responsible for our results.

In this study, we analyzed the binding properties of anti-core structure gp41 human MAbs using infected cells. These analyses will contribute to understanding the structure of gp120-gp41 on the infected cell surface and the complex interactions of humoral antibodies against HIV-1. The unidirectional competition, which has not been able to be observed using gp41-derived peptides so far, suggests that the immune-response against the gp41 core structure varies much among patients and some of them are possibly dominant for certain exclusive epitopes, because patient-derived MAbs are considered to recognize the functional gp41 in the human body. We previously did competition assays between patients' sera and anti-core structure

MAbs, because patients' sera may have a diverse competition with anti-core structure MAbs (15), and found that the patients' sera competed with MAb 50.69 more than MAb 98.6 (unpublished data), suggesting that the antibodies that compete with MAb 50.69 are greater in number than those that compete with MAb 98.6 *in vivo*.

According to the above results the antibodies that bind to the epitope of MAb 50.69 are expected to compete with MAb 98.6. The lower competition of MAb 98.6 with sera suggests that some antibodies that recognize the overlapping epitope of MAb 50.69 do not compete with MAb 98.6.

Our results also indicate that there are few antibodies that have the epitope of MAb 98.6 *in vivo*, and suggest that the antibodies with this epitope may hardly ever be induced, especially among cluster II antibodies. This is assumed to be partly due to the fact that cluster II is a more variable domain than cluster I (11).

The anti-gp41 antibodies in one patient's body are assumed to compose a population including such exclusivity and heterogeneity. Our data not only contribute to analyses of the functional epitopes which are able to induce anti-gp41 antibodies *in vivo*, but also to establishing a method to evaluate the complex anti-gp41 immunity.

This work was supported in part by Scientific Research Expenses for Health and Welfare Programs from the Ministry of Health, Labour and Welfare, Japan and by Grants AI 27742 for CFAR Immunology Core as the source of MAbs. The authors are grateful to Dr. R.C. Gallo for providing H9/IIIB and Dr. Susan Zolla-Pazner and Dr. Miroslaw K. Gorny for providing MAbs 50.69 and 98.6. The authors are also grateful to Dr. Earl for providing MAb T26 and Dr. Carol Weiss for critical reading of the manuscript.

References

1) Broder, C.C., Earl, P.L., Long, D., Abedon, S.T., Moss, B., and Doms, R.W. 1994. Antigenic implications of human immunodeficiency virus type 1 envelope quaternary structure: oligomer-specific and -sensitive monoclonal antibodies. Proc. Natl. Acad. Sci. U.S.A. **91**: 11699–11703.
2) Chan, D.C., Fass, D., Berger, J.M., and Kim, P.S. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89**: 263–273.
3) Earl, P.L., Broder, C.C., Doms, R.W., and Moss, B. 1997. Epitope map of human immunodeficiency virus type 1 gp41 derived from 47 monoclonal antibodies produced by immunization with oligomeric envelope protein. J. Virol. **71**: 2674–2684.
4) Earl, P.L., Broder, C.C., Long, D., Lee, S.A., Peterson, J., Chakrabarti, S., Doms, R.W., and Moss, B. 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. **68**: 3015–3026.
5) Gorny, M.K., Gianakakos, V., Sharpe, S., and Zolla-Pazner, S. 1989. Generation of human monoclonal antibodies to human immunodeficiency virus. Proc. Natl. Acad. Sci. U.S.A. **86**: 1624–1628.
6) Gorny, M.K., VanCott, T.C., Williams, C., Revesz, K., and Zolla-Pazner, S. 2000. Effects of oligomerization on the epitopes of the human immunodeficiency virus type 1 envelope glycoproteins. Virology **267**: 220–228.
7) Hattori, T., Komoda, H., Pahwa, S., Tateyama, M., Zhang, X., Xu, Y., Oguma, S., Tamamura, H., Fujii, N., Fukutake, K., and Uchiyama, T. 1998. Decline of anti-DP107 antibody associated with clinical progression. Aids **12**: 1557–1559.
8) Kuznetsov, Y.G., Victoria, J.G., Robinson, W.E., Jr., and McPherson, A. 2003. Atomic force microscopy investigation of human immunodeficiency virus (HIV) and HIV-infected lymphocytes. J. Virol. **77**: 11896–11909.
9) Lu, M., Blacklow, S.C., and Kim, P.S. 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat. Struct. Biol. **2**: 1075–1082.
10) Pinter, A., Honnen, W.J., Tilley, S.A., Bona, C., Zaghouani, H., Gorny, M.K., and Zolla-Pazner, S. 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J. Virol. **63**: 2674–2679.
11) Robinson, W.E., Jr., Gorny, M.K., Xu, J.Y., Mitchell, W.M., and Zolla-Pazner, S. 1991. Two immunodominant domains of gp41 bind antibodies which enhance human immunodeficiency virus type 1 infection *in vitro*. J. Virol. **65**: 4169–4176.
12) Sattentau, Q.J., and Moore, J.P. 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174**: 407–415.
13) Sattentau, Q.J., Zolla-Pazner, S., and Poignard, P. 1995. Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. Virology **206**: 713–717.
14) Taniguchi, Y., Zolla-Pazner, S., Xu, Y., Zhang, X., Takeda, S., and Hattori, T. 2000. Human monoclonal antibody 98-6 reacts with the fusogenic form of gp41. Virology **273**: 333–340.
15) Usami, O., Xiao, P., Ling, H., Liu, Y., Nakasone, T., and Hattori, T. 2005. Properties of anti-gp41 core structure antibodies, which compete with sera of HIV-1-infected patients. Microbes Infect. **7**: 650–657.
16) Weissenhorn, W., Dessen, A., Harrison, S.C., Skehel, J.J., and Wiley, D.C. 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387**: 426–430.
17) Xu, J.Y., Gorny, M.K., Palker, T., Karwowska, S., and Zolla-Pazner, S. 1991. Epitope mapping of two immunodominant domains of gp41, the transmembrane protein of human immunodeficiency virus type 1, using ten human monoclonal antibodies. J. Virol. **65**: 4832–4838.

Japanese J. Infect. Dis. In press.

YMDD mutations and genotypes of HBV in Northern China

Di Li[1], Hong-Xi Gu[1], Shu-Yun Zhang[2], Zhao-Hua Zhong[1], Min Zhuang[1], Toshio Hattori[3]*

[1]Department of Microbiology, and

[2] Research Center of The Second Affiliated Hospital, Harbin Medical University, Harbin, China

[3]Infectious and Respiratory Disease Department, Tohoku University, Sendai 980-8574 Japan

*Corresponding author: Mailing address for Toshio Hattori, MD

: Infectious and Respiratory Disease Department, Tohoku University, Aobaku, Sendai, Japan. 980-8574

E-mail: Hattori.t@rid.tohoku.ac.jp

SUMMARY: To determine the relationship between YMDD mutations and the genotypes of hepatitis B virus during lamivudine treatment. HBV genotypes were determined by nested PCR with six pairs of HBV genotype-specific primers (A to F) in serum specimens from 142 hepatitis B patients receiving lamivudine antiviral therapy. YMDD mutations were detected by fluorescent hybridization bioprobe PCR and melting curve assay (FH-PCR-MC). Among 142 serum specimens, 13 samples were genotype B (9.2%), 125 samples were genotype C (88%), 4 samples were genotype D (2.8%), and 80 YMDD mutations were found. The YMDD mutation rates were 69.2% and 54.4% in genotype B and in genotype C, respectively. There was no significant difference in the YMDD mutation rate between genotypes B and C. 9 genotype B sera with YMDD mutations were found, including 2 YIDD mutations and 7 YVDD (M+V) mutations. Six-eight genotype C sera with YMDD mutations were found , including 34 mutations I（M+I）and 17 mutations V (M+V). There was a significant difference in the YMDD mutation types between genotype B and C. Our results suggested that the YMDD mutation rate was 56.3% in patients treated with lamivudine for 2-4 years. YIDD was the main mutation type. YMDD mutation rate showed no significant difference between HBV type B and C（$P>0.05$）, while the YMDD mutation types showed a significant difference between HBV type B and C in Northern China（$\chi^2=4.6, P<0.05$）.

INTRODUCTION

Hepatitis B, caused by the hepatitis B Virus (HBV), exists throughout the world and the prevalence is especial high in China. There are approximately 1.2 billion people infected by HBV. Lamivudine is one of the nucleoside medicines. It can improve the condition of chronic hepatitis B patients in terms of the virology, biochemistry and histology. Lamivudine cuts down the HBV DNA levels in patients' sera and can obtain a certain proportion of HBeAg seroconversion (1). Lamivudine has been promoted for hepatitis B treatment, but the patient's condition may deteriorate rapidly after long-term use. The main reason is due to YMDD mutations in the HBV polymerase gene (2). HBV has been classified into eight genotypes (A-H) based on genome sequence divergences. The genotypes of HBV have distinct geographical distributions. Hepatitis B genotypes are associated with virus replication, virus variation, disease prognosis and the choice of drugs (3-7). This study will determine the relationship between the YMDD mutations of hepatitis B virus and HBV genotypes during lamivudine treatment.

MATERIALS AND METHODS

Study population: 142 serum specimens from chronic hepatitis without cirrhosis patients who were hospitalized or being seen in the clinic service in the Infectious Disease Department of the Second Affiliated Hospital of Harbin Medical University from 2003 to 2005. All cases were diagnosed according to the Standard of Chinese Virus Hepatitis in 2000, and cases with liver complications caused by other aetiologies or other types of hepatitis virus were excluded infection. 142 patients include 112 men and 30 women, and their ages ranged from 18 to 78. DNA levels of HBV in serum from all patients were more than 10^3 copies/ml. All cases received lamivudine treatment orally, 100mg everyday for 24-48 months, and did not receive other antiviral therapy

during the study. All the patients gave written informed consent. This study was approved by the Harbin Medical University Committee on Clinical Investigation.

Laboratory findings: HBeAg levels in patients sera was measured with euzymelinked immunosorbent assay (ELISA) using the Diagnostic Kit for Hepatitis B e Antigen (PG Biotechnology, China). The HBV DNA levels were tested by real-time fluorimetry PCR with TaqMan probe using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology, China).

Nucleic acid extraction: HBV DNA amplification for sequence analysis. HBV DNA was extracted from 100 μ l test sera and 100 μ l DNA extract I (PG Biotechnology, China)were oscillated and mixed well, centrifuged at 13,000 rpm for 10 min and the supernatant was discarded. The 25 μ l DNA extract II (PG Biotechnology, China)were added, oscillated and mixed well, and centrifuged at 2000 rpm for 10 sec. Then, it was placed in a dry-bathe for 10 min at 100℃, centrifuged at 13,000 rpm for 10 min, and the supernatant was retained in the stock..

Genotyping of HBV by PCR: HBV genotypes were determined by nested PCR with six pairs of HBV genotype-specific primers (A to F). Vide reference (8).

Detection of YMDD mutation: YMDD was detected by fluorescent hybridization bioprobe PCR and melting curve assay (FH-PCR-MC) using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology, China). YMDD mutation type after lamivudine treatment for 2 years was determined and YMDD mutation type was examined every 6 months.

Statistical analysis: Statistical analyses were performed using Adopt SPSS 10.0 software. The rate comparison was made using the chi-square criterion. A difference with a $P<0.05$ was considered significant. Student' s t test and nonparametric test were used compare differences

between groups, where appropriate.

RESULTS

Analysis of HBV genotypes and clinical data: A simple and precise genotyping system based on nested PCR with six pairs was developed for the determination of genotypes of HBV. All 142 samples were genotyped by nested PCR analysis: the majority was genotype C, which accounted for 88%; genotype B accounted for 9.2%; genotype D accounted for only 2.8%. No other genotypes were detected (genotypes A, E, F). No statistically significant differences were observed in mean age, male-to-female ratio, mean serum DNA levels, HBeAg positive and treatment period with genotype B and that genotype C. Genotype D was not included in the statistical analysis because of the low number of cases (Table. 1).

Table 1. HBV genotype distribution and characteristics of the patients

Genotype	Cases	Positive rate	Men/women	Age (mean±SD)	HBVDNA (10^6mean±SD)	$HBeAg^+$/$HBeAg^-$	Treatment period (month)
Genotype B	13	9.2%	10/3	37.9±11	12.9±21.6	9/4	34.2±6.8
Genotype C	125	88%	99/26	38.4±11.9	11.3±24.5	95/30	34.9±6.4
Genotype D	4	2.8%	3/1	38.8±11.1	11.7±19	3/1	34.8±5.6

YMDD mutation rate and YMDD mutation type in 80 HBV sera: FH-PCR-MC in detection of HBV YMDD mutation has high sensitivity and specificity. It is a convenient and rapid, and may be used in YMDD typing. HBV Wild type and Mutation type were detected in 62 and 80 by FH-PCR-MC, respectively. The YMDD mutation rate was 56.3% in the 142 specimens. Among 80 YMDD mutations, 38 YIDD (M+I) mutations (47.5%), 24 YVDD (M+V) mutations (30%) and 18 YIDD+YVDD mutations (22.5%) were found, respectively. Among 80 YMDD mutations,

YIDD mutation rate was higher than YVDD mutation rate. The mutation type was mainly YIDD. The YMDD mutation rates were 69.2% and 54.4% in genotypes B and C. Although the YMDD mutation rate was higher in patients with genotype B than in those with genotype C, there was no statistically significant difference between genotype B and C（P>0.05）. Genotype D was not included in the statistical analysis because of the low number of cases (Table.2).

Table 2. YMDD mutation rate and YMDD mutation type in 80 HBV sera.

Genotype	n	YIDD	YMDD+YIDD	YVDD	YMDD+YVDD	YIDD+YVDD	YMDD mutation rate
Genotype B	9	2(22.2)	0	6(66.7)	1(11.1)	0	69.2%(9/13)
Genotype C	68	30(44.1)	4(5.9)	15(22.1)	2(2.9)	17(25)	54.4%(68/125)
Genotype D	3	2(66.7)	0	0	0	1(33.3)	75%(3/4)
Total	80	34(42.5)	4(5)	21(26.2)	3(3.8)	18(22.5)	56.3%(80/142)

Relation between HBV YMDD mutation type and genotypes B, C: Among the 68 patients with genotype C, 23 (33.8%) patients were mixed mutation type. Among the 9 patients with genotype B, 1 (11.1%) patient was mixed mutation type. Although the YMDD mixed mutation type rate was higher in patients with genotype C than in those with genotype B, there was no statistically significant difference between genotype B and C（P>0.05）. Among the 36 patients with YIDD (M+I) mutation types, 2 patients were genotype B, 34 patients were genotype C. Among the 24 patients with YVDD (M+V) mutation types, 7 patients were genotype B, 17 patients were genotype C. The YMDD mutation types (YIDD and YVDD) showed significant differences between genotype B and C（χ^2=4.6, P<0.05. Genotype D was not included in the statistical analysis due to the low number of cases (Table.3).

Table 3. Relationship between HBV YMDD mutation type and genotypes B, C

Genotype	n	YIDD (M+ I)	YVDD (M+ V)
Genotype B	9	2 (22.2)	7 (77.8)
Genotype C	51	34 (66.7)	17 (33.3)

DISCUSSION

Lamivudine has been shown to be a potent and nontoxic inhibitor of hepatitis B virus (HBV) replication in chronically infected patients. Long-term lamivudine treatment for chronic hepatitis B virus infection induces the emergence of lamivudine resistant HBV YMDD mutant strains. In the case of YMDD variants, the methionine (M) is substituted with either isoleucine (I), designated as YIDD or valine (V), designated as YVDD (9).

It has been reported that the HBV YMDD mutation rate increases along with the duration of lamivudine therapy. The YMDD mutation rates were 16%-32%, 47%-56% and 69%-75% in the groups of patients administered lamivudine for 1, 2, 3 years, respectively (10). A study from Asia described in that the 1, 2, 3 year YMDD mutation rates were 15%, 38% and 53% after lamivudine therapy (11). The present 142 chronic hepatitis B patients received lamivudine for 2-4 years. The HBV YMDD mutation rate should be 56.3%, based on the previous reports. The mutation type was mainly YIDD. The presence of YIDD motif preceded the exclusive presence of the YVDD motif, we concluded that the YIDD motif could occur as a temporal intermediate (12). It has been reported that YIDD or YVDD motif alone did not shift to the mixed type (YVDD and YIDD) when they were examined 12 months after the detection of mutant virus, in Japan (13). In our study, YMDD mutation type was determined every 6 months for 2 years after lamivudine treatmen. Recently we found 2 patients, who were not included in this study, shifted to the mixed type (YIDD and YVDD) (unpublished observation), indicating the shift can occur in a relatively

long period. During the past few years, many investigators have investigated the forecasting factors of drug resistance and mutations, but without reaching a conclusion. Some factors concerned with YMDD mutations, could raise the curative effect of lamivudine. Recently many investigations have concentrated on the time of the YMDD mutation appearance, the mutation type, the YMDD mutation and ALT level, the DNA level, and the rate of HBeAg seroconversion, but no relationship between the mutations of HBV and the HBV genotypes has been reported. The viral genotype that represents the features of natural infection is the result of evolutionary variation of the virus. The HBV genotype has a certain association with the route of virus infection, genetic mutations, and the progression of chronic hepatitis B and the efficacy of antiviral therapies. The genotypes of HBV also have distinct geographical distributions. Genotypes B and C are were only found in those who originated from eastern Asia and Far East including Taiwan (14). In Japan, the great majority of HBV isolates belong to genotype B or C (15). Genotypes B, C are spread dominantly in China while genotypes A, D relatively rare. Genotype B is prevalent in South China and genotype C in North China (16). The clinical prognosis after infection with HBV varies according to the genotype. In the previous study, clearance of HBeAg occurred earlier and more frequently, and development of cirrhosis was less common in patients with genotype B compared with genotype C (15). Genotype C is associated with chronic liver disease, genotype B mostly associated with acute hepatitis in China (17). The present study analyzed 142 sera of chronic hepatitis B in North China. The results showed that genotype C occupied 88%, and genotype B 9.2% and genotype D 2.8%. Genotype C is predominant in patients with chronic liver disease in North China. A study in Japan reported that among 234 patients with chronic hepatitis B infected with genotype A, B, or C and treated with

lamivudine for more than 1 year, the emergence of mutations was not different among genotypes A, B and C by the Kaplan-Meier method. (18). In Japan, it was reported that the emergence rate of lamivudine resistance was independent of the genotype (A, B, and C), after treatment of patients with chronic hepatitis B with various genotypes with lamivudine,. In contrast, the emergence rate was significantly higher in the Ba ("a" stands for Asia) subgroup of HBV than in Bj ("j" for Japan) subgroup ($P<0.05$) (19). In the Hong Kong study, there was no difference in the antiviral response and the rate of development of YMDD mutations in 82 patients with chronic hepatitis B with genotype B and C after 1 year of lamivudine treatment (20). A report on 87 patients receiving lamivudine in Guiyang, results showed that, though genotype B was dominant in that region, there was a higher mutation rate of drug resistance among genotype C cases (21). In another report, 135 chronic hepatitis B patients received lamivudine for 1 year, and the YMDD mutation rate in genotype C cases was higher than that in genotype B cases in Guangdong (22). But Guiyang and Guangdong province located in Southern parts of China. In the present study, 9 YMDD mutations were found in genotype B, and 68 YMDD mutations were found in genotype C. The YMDD mutation rates were 69.2% and 54.4% in genotype B and in genotype C, respectively in North China（$P>0.05$）. It has been reported that the YIDD mutation occurs more frequently in genotype D, while YVDD is more common in genotype A (23). It is suggested that the YMDD mutation type is related to the genotype. But the present study showed that the YVDD mutation occurred more frequently in genotypes B, the YIDD mutation in genotypes C, the YMDD mutation showed significant differences between the HBV genotypes B and C.（$\chi 2=4.6$, $P<0.05$）. It has been reported that the replication ability of the YVDD mutant strain might be stronger than that of the YIDD mutant strain (24). The mutation types among different genotypes aren't identical, because

the genotypes are associated with virus replication and virus variation. Because of the small number of cases of genotype D, only HBV genotype B and C were examined in this study. Large-scale prospective studies of each genotype should be conducted in the future to confirm these findings.

ACKNOLEDGMENTS

We are grateful to Dr. Kenji Abe for continuous encouragements and Dr. Shu-Lan Lu for clinical samples. This work was supported by Japan Health Science Foundation, No.0109 and Doctor Foundation of Harbin Medical University.

REFERENCES

1. Suzuki, Y., Kumada, H., Ikeda, K., Chayama, K., Arase, Y., Saitoh, S., Tsubota, A., Kobayashi, M., Koike, M., Ogawa, N. and Tanikawa, K. (1999): Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J. Hepatol., 30, 743-748.
2. Kidd-Ljunggren, K., Miyakawa, Y. and Kidd, A.H. (2002): Genetic variability in hepatitis B viruses. J. Gen. Virol., 83, 1267-1280.
3. Arauz-Ruiz, P., Norder, H., Robertson, B.H. and Magnius, L.O. (2002): Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J. Gen. Virol., 83, 2059-2073.
4. Moriyama, M., Taira, M., Matsumura, H., Aoki, H., Mikuni, M., Kaneko, M., Shioda, A., Iwaguchi, K., Arai, S., Ichijima, S., Iwasaki, H., Tanaka, N., Abe, K. and Arakawa, Y. (2003): Genotype analysis, using PCR with type-specific primers, of hepatitis B virus isolates from

patients coinfected with hepatitis delta virus genotype II from Miyako Island, Japan. Intervirology, 46, 114-120.

5. Miyakawa, Y. and Mizokami, M. (2003): Classifying hepatitis B virus genotypes. Intervirology, 46, 329-338.
6. Chu, C.J., Keeffe, E.B., Han, S.H., Perrillo, R.P., Min, A.D., Soldevila-Pico, C., Carey, W., Brown, R.S. Jr., Luketic, V.A., Terrault, N. and Lok, A.S. (2003): Hepatitis B virus genotypes in the United States: results of a nationwide study. Gastroenterology, 125, 444-451.
7. Yuen, M.F., Sablon, E., Yuan, H.J., Wong, D.K., Hui, C.K., Wong, B.C., Chan, A.O. and Lai, C.L. (2003): Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. Hepatology, 37, 562-567.
8. Naito, H., Hayashi, S. and Abe, K. (2001): Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. J. Clin. Microbiol., 39, 362-364.
9. Moskoviz, D.N., Osiowy, C., Giles, E., Tomlinson, G and Heathcote, E.J.(2005): Response to long-term lamivudine treatment (up to 5 years) in patients with severe chronic hepatitis B, role of genotype and drug resistance. J Viral Hepat., 12, 398-404.
10. Atkins, M., Hunt, C.M., Brown, N., Gray, F., Sanathanan, L., Woessner, M., Lai, C.l., Dusheiko, G., Dienstag, J., Wright, T., Bamard, J., Bourne, E. and Condreay, L. (1998): Clinical significance of YMDD mutant hepatitis B virus in a large cohort of lamivudine-treated hepatitis B patients (Abstract). Hepatology, 8, 319.
11. Leung, N.W., Lai, C.L., Chang, T.T., Guan, R., Lee, C.M., Ng, K.Y., Lim, S.G, Wu, P.C.,

Dent, J.C., Edmundson, S., Condreay, L.D. and Chien, R.N; On behalf of the Asia. (2001): Hepatitis Lamivudine Study Group. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology, 33, 1527-1532.

12. Niesters, H.G., Honkoop, P., Haagsma, E.B., de Man, R.A., Schalm, S.W. and Osterhaus, A.D. (1998): Identification of more than one mutation in the hepatitis B virus polymerase gene arising during prolonged lamivudine treatment.J Ifect Dis.,177,1382-1385
13. Akuta, N., Suzuki, F., Kobayashi, M., Matsuda, M., Sato, J., Takagi, K., Tsubota, A., Suzuki, Y., Hosaka, T., Someya, T., Kobayashi, M., Saitoh, S., Arase, Y., Ikeda, K. and Kumada, H. (2003):Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. J Med Virol., 71,504-510.
14. Kao, J.H., Liu, C.J. and Chen, D.S. (2002): Hepatitis Bviral Genotypes and lamivudine resistance. J Hepatol., 36, 303-304
15. Tomokuni, N., Tatsuji M., Tomofumi, N., Hiroki, N., Hiroshi, S., Fukunori, K., Etsuro, O. and Masashi, M. (2003): Difference in Prognosis Between Patients Infected With Hepatitis B Virus With Genotype B and Those With Genotype C in the Okinawa Islands: A Prospective Study. J. of Med Virol, 70, 350–354.
16. Gu, H.X., Xu, Z.L., Liu, J.Y., Zhong, Z.H., Wang, H.Q., Zhang, S.Y., Li, D., Zhang, H.H. and Abe, K. (2004): Epidemiology of HBV genotypes by nested PCR with multi-paired primers. World Chin J Digestol., 12, 1073-1076 (in Chinese).
17. Dong, M. and Zhong, Y.X. (2005):Distridution of hepatitis B virus Genotypes and its chinical significance.Chin J Hepatol., 13, 56-57 (in Chinese).
18. Suzuki, F., Tsubota, A., Arase, Y., Suzuki, Y., Akuta, N., Hosaka, T., Someya, T., Kobayashi, M., Saitoh, S., Ikeda, K., Kobayashi, M., Matsuda, M., Satoh, J., Takagi, K. and Kumada, H. (2003): Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology, 46, 182-189.
19. Akuta, N., Suzuki, F., Kobayashi, M., Tsubota, A., Suzuki, Y., Hosaka, T., Someya, T.,

Kobayashi, M., Saitoh, S., Arase, Y., Ikeda, K. and Kumada, H. (2003):The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J. Hepatol, 38,315-321.

20. Yuen, M.F., Wong, D.K., Sablon, E., Yuan, H.J., Sum, S.M., Hui, C.K., Chan, A.O., Wang, B.C. and Lai, C.L. (2003): Hepatitis B virus genotypes B and C do not affect the antiviral response to lamivudine. Antivir. Ther., 8, 531-534.
21. Wang, G.Y., Zhu, W.J. and Yang, X.L. (2005): YMDD mutation of hepatitis B virus and genotyping in Guiyang region. Chin J Hepatol., 13, 193-195 (in Chinese).
22. Zhou, J.L. and Wu, S.P. (2004): Effects of comparison of lamivudine therapy B virus genotype B and C. Chin J Hepatol., 12, 489-490 (in Chinese).
23. Zollner, B., Petersen, J., Puchhammer-Stockl, E., Kletzmayr, J., Sterneck, M., Fischer, L., Schroter, M., Laufs, R. and Feucht, H.H. (2004): Viral features of lamivudine resistant hepatitis B genotypes A and D. Hepatology, 39, 42-50.
24. Zhang, X., Liu, C., Gong, Q., Zhang, S., Zhang, D., Lu, Z. and Wang, Y. (2003): Evolution of wild type and mutants of the YMDD motif of hepatitis B virus polymerase during lamivudine therapy. J Gastroenterol Hepatol.,18, 1353-1357.

INT J TUBERC LUNG DIS 10(8):906–910
© 2006 The Union

Method for efficient storage and transportation of sputum specimens for molecular testing of tuberculosis

H. Guio,* H. Okayama,† Y. Ashino,* H. Saitoh,* P. Xiao,* M. Miki,† N. Yoshihara,‡ S. Nakanowatari,† T. Hattori *

* Department of Infectious and Respiratory Diseases, Graduate School of Medicine, Tohoku University, Sendai, † Japanese Red Cross Sendai Hospital, Sendai, ‡ Japanese Research Center of Tuberculosis, Tokyo, Japan

SUMMARY

SETTING: The polymerase chain reaction (PCR) is a highly sensitive method for the detection of *Mycobacterium tuberculosis* and is available in most countries, though to a lesser extent in rural areas.

OBJECTIVE: To amplify *M. tuberculosis* DNA sequences of sputum spotted on FTA® cards and compare them with the results of microscopic examination among culture-positive samples.

DESIGN: A total of 102 sputum specimens of TB patients in treatment were spotted on FTA cards and stored at room temperature until DNA analysis. We assessed the IS*6110* region of *M. tuberculosis*. The efficacy of the PCR assay for the direct detection of *M. tuberculosis* was evaluated and compared with the results of cultures (Middlebrook 7H9 broth) and smears of fresh sputum specimens.

RESULTS: We were able to detect 10 fg/µl of mycobacterial DNA even after 6 months in storage. The PCR sensitivity and specificity using the FTA card system were 82% and 96%, while microscopic examination showed 41% and 95%, respectively.

CONCLUSION: The FTA® card system for the storage of bacterial DNA from sputum samples should be considered for the molecular diagnosis of tuberculosis. Samples can easily be obtained from geographically isolated populations and shipped by mail for accurate molecular diagnosis.

KEY WORDS: tuberculosis; sputum; PCR; FTA® card

CURRENT GLOBAL TUBERCULOSIS (TB) control efforts are based on the diagnosis of cases followed by adequate treatment. Difficulties involved in the collection, transport and processing of samples in clinical practice have also been a major issue. An initial error made by microscopic diagnosis will not be known until weeks later, when the clinical signs are more evident (in false negatives). Microscopic examination of sputum acid-fast bacilli (AFB) smear is still the most widely available diagnostic tool for TB. Unfortunately, smear microscopy is neither specific for *Mycobacterium tuberculosis*, nor is it very sensitive. Depending on the number of specimens examined, smear microscopy detects 30–60% of culture-positive TB suspects.[1] In most situations in which TB is diagnosed by AFB microscopy, it should be assumed to be *M. tuberculosis* until proven otherwise.[2] Detection of AFB smear-negative patients, who make up a significant proportion of all TB patients, is even more problematic.[3,4] As *M. tuberculosis* grows very slowly, diagnosis by culture is a long process, requiring 3–8 weeks in solid media and 1–4 weeks in liquid media.[5]

The development of rapid and accurate procedures for the diagnosis of TB has been a long-standing goal for two main reasons—to improve case finding and case management, and to improve disease surveillance.[5] Molecular amplification assays such as polymerase chain reaction (PCR), which can specifically amplify large quantities of DNA from small starting quantities (10–100 mycobacteria/ml), have been shown to be a promising alternative even for developing countries.[6,7] Microscopic examination requires $>10^3$ to 10^4 mycobacteria/ml, and it is necessary for the diagnostic laboratory to either process the specimen shortly after collection or store it at 4°C to inhibit the growth of contaminating micro-organisms. The latter procedure entails additional labour costs for the processing and conservation of specimens, and reductions in sensitivity.[8]

The FTA® card system (Whatman International Ltd, Abingdon, Cambridge, UK) was originally developed for storing blood samples for DNA testing; its matrix binds and lyses cells, resulting in amplifiable DNA being immobilised on the paper sections, which can

Correspondence to: Dr Toshio Hattori, Division of Respiratory and Infectious Diseases, Postgraduate Division, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. Tel: (+81) 22 717-8220. Fax: (+81) 22 717-8221. e-mail: hattori.t@rid.med.tohoku.ac.jp

Article submitted 6 October 2005. Final version accepted 20 March 2006.

be punched out directly for use. The card's size allows storage of several hundred samples at room temperature. The filter is impregnated with a chaotropic agent that denatures infectious agents, and thus, as the samples no longer represent a biohazard, their storage and transport can be managed without burdensome precautions.

In this study, we set out to investigate the sensitivity and specificity of a PCR system for the detection of *M. tuberculosis* in sputum samples spotted on FTA® cards and to evaluate the potential of using this method to overcome the difficulties of transporting and storing sputum samples during the TB diagnosis process.

INDIVIDUALS, MATERIALS AND METHODS

Patient and clinical specimens

A total of 102 sputum specimens from 35 TB patients at the Japanese Red Cross Sendai Hospital were collected prospectively. The clinical diagnosis of TB was established by patient histories and clinical and radiological findings, as recommended by the American Thoracic Society.[9] All the patients provided written informed consent. The study was approved by the Tohoku University Committee on Clinical Investigation and by the Ethics Committee of the Red Cross Hospital.

Evaluation and application of the sputum onto the FTA® cards

Spontaneously produced sputum was the specimen of choice. No patient was assisted by respiratory therapy technicians nor stimulated with hypertonic saline aerosol to produce acceptable sputum. The gross appearance of the sputum was evaluated according to Miller & Jones' classification: M1 (pure mucus), M2 (little purulent content), P1 (purulent sputum less than one third of the volume), P2 (purulent sputum between one third and two thirds of the volume) and P3 (purulent sputum more than two thirds of the volume).[10] Standard precautions, such as the use of gloves and a mask, were taken when manipulating sputum specimens.

To improve the chance of detecting *M. tuberculosis*, we chose the thicker (purulent) particles of the sputum and applied them directly onto a FTA® card using a foam-tipped applicator (Whatman®, Tokyo, Japan) that was squeezed over an area of 2.5 cm in diameter. The card was then allowed to dry for 1 h at room temperature. Heat was not used during the drying period. The cards were then put into storage desiccant packets (Whatman®, Japan) and stored at room temperature until DNA analysis.

Culture and hybridisation

Equal volumes of N-acetyl-L-cysteine/NaOH (4%) were mixed with the specimens for digestion and decontamination. The resulting mixtures were allowed to stand for 15 min at room temperature, then centrifuged at 3000 × g for 15 min. The sediment was re-suspended in 2 ml of phosphate buffered saline (PBS) and 0.5 ml was inoculated into Middlebrook 7H9 broth (Middlebrook, Becton Dickinson, Cockeysville, MD, USA). DNA was extracted in all the culture-positive samples to identify and confirm *M. tuberculosis* using a DNA-DNA calorimetric microdilution plate hybridisation kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan).

Preparation of isolated DNA from FTA® cards

Four discs of 1.2 mm from the spotted area of the filters were cut out using a sterile hole puncher (Harris Micro punch 1.2 mm, Whatman®, Japan) and placed in 1.5 ml PCR tubes. The punch was cleaned by placing the end of the punch in the flame of a Bunsen burner and by sterilisation with 70% ethanol between cuts from different samples. The discs were washed three times for 5 min with 800 µl FTA® purification reagent (Whatman®, Japan), which removes PCR inhibitors and other potential contaminants to ensure the quality of the DNA for downstream analysis. The discs were also rinsed twice with 800 µl TE buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 8.0) (Promega, Tokyo, Japan) for 5 min each time. The discs were then dried at room temperature for 1 h and used directly as templates in the PCR reaction mix in the same tube.

PCR procedure

The primers were synthesised using an Automated Multiplex Oligonucleotide Synthesizer (Roche Diagnostics, Tokyo, Japan) (Table 1). Two different PCR assays were performed. First, as an internal control for monitoring successful DNA extraction, β-globin was amplified by using primers GH21 and PCO3 to yield a 250-bp product.[11] The total reaction volume was 50 µl and the reaction mixture contained each primer (10 pmol each), 2U taq DNA polymerase (Invitrogen Cat N°10342-020), 5 µl 10x PCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 1 µl 10 mM dNTP mixture (Invitrogen Cat N°18427-013), 1.5 µl 50 mM $MgCl_2$ and 38.1 µl distilled water. PCR was performed in an MJ Research PTC-200 DNA Engine Thermal Cycler (MJ Research, Waltham, MA, USA) using the following amplification conditions: 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 30 s and one cycle at 72°C for 10 min. Finally, a 123-bp sequence of insertion element *IS6110* was amplified using T4-T5 primers.[12] This insertion sequence is present in different numbers and locations in the genomes of most clinical isolates of *M. tuber-*

Table 1 PCR primers used in this study

Target	Sequencing	bp	Ref.
β-globin GH21-PCO3	5'-GGA-AAA-TAG-ACC-AAT-AGG-CAG-3' 5'-ACA-CAA-CTG-TGT-TCA-CTA-GC-3'	250	10
M. tuberculosis *IS6110* T4-T5	5'-CCT-GCG-AGC-GTA-GGC-GTC-GG-3' 5'-CTC-GTC-CAG-CGC-CGC-TTC-GG-3'	123	11

culosis, which makes it a useful probe for diagnostic and epidemiological purposes. The final composition of the PCR mix was the same as in the previous case. The PCR conditions were preheating at 94°C for 5 min, then 40 cycles at 94°C for 2 min, 68°C for 2 min, 72°C for 2 min, and then 72°C for 10 min.

Determination of sensitivity

To determine the sensitivity of *M. tuberculosis* detection, 10-fold serial dilutions (1 ng to 10 fg) of H37Rv DNA (kindly provided by Dr I Sugawara, Research Institute of Tuberculosis, Tokyo, Japan) were performed using T4-T5 primers. The final composition and PCR conditions were the same as for amplifying IS*6110*.

All amplification products were detected on 1.5% agarose gel in 1 × TAE buffer stained with ethidium bromide and visualised by ultraviolet transillumination.

Control procedures

A positive control tube containing 0.1 ng H37Rv DNA and a negative control tube containing no DNA were included with each set of reactions. To evaluate cross-contamination during sampling, we performed control punches using unspotted cards.

Statistical methods

The sensitivity and specificity of each pair of primers for the detection of *M. tuberculosis* were calculated on the basis of the study reference standards, with the liquid culture method taken as a gold standard. In addition, the results of individual PCR were employed for the analysis of smear-positive and -negative samples according to the culture results.

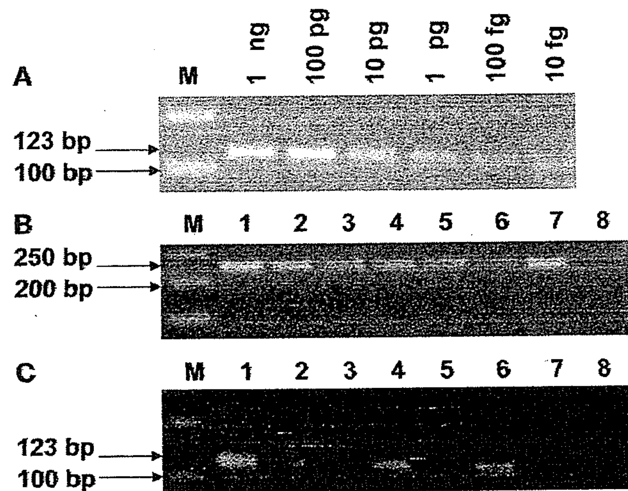

Figure 1 PCR results. **A.** Sensitivity of detection of *M. tuberculosis*: ten-fold serial dilution of H37Rv DNA was amplified. **B.** PCR amplicons from β-globin (250 bp): M: marker; lanes 1–7: results using FTA® cards from TB patients; lane 8: negative control. **C.** PCR amplicons from *M. tuberculosis* (123 bp) stored on FTA® cards: M: marker; lane 1: positive control (H37Rv); lanes 2, 4 and 6: cards from TB patients; lanes 3, 5 and 7: no spotted cards (control punch); lane 8: negative control. In each case, 10 μl samples were electrophoresed through a 1.5% agarose gel and photographed under UV illumination. PCR = polymerase chain reaction; bp = base pairs; TB = tuberculosis; UV = ultraviolet.

RESULTS

Patient characteristics

We evaluated by PCR 102 sputum samples from 35 TB patients (23 male, 12 female), all of whom were receiving anti-tuberculosis treatment for periods ranging from 2 weeks to 1 year. The average age was 51.4 years.

Gold standard

Twenty-two positive culture samples were taken as gold standard. In all of these samples, *M. tuberculosis* was identified by hybridisation assay.

Appearance of the specimens

Eighty-five per cent of smear positives, 68% of culture positives and 81% of PCR positives contained >30% of purulent sputum (P2 or P3 in Miller & Jones' classification[10]).

Sensitivity detection

Four small discs of the FTA® card system were used as templates for the PCR processing. The criterion for using four discs was based on the assumption that the mycobacteria were scanty and heterogeneously distributed during the absorption process; in addition, in a pilot study we determined that using four disks increased the degree of amplification (data not shown). The detection limit of the PCR assay for the amplification of IS*6110* was 10 fg/μl of purified *M. tuberculosis* H37Rv (Figure 1A). The amplification of the 123 bp fragments by PCR using the FTA® card system is depicted in Figure 1C.

Effect of PCR inhibitors

The ability to detect *M. tuberculosis* by PCR can be impaired by the presence of substances inhibitory to Taq DNA polymerase. The β-globin PCR assay generated the expected 250-bp band (Figure 1B) in 90 (89%) of the samples. All the culture-positive samples were also positive in the amplification of β-globin. This finding may suggest that the PCR-negative, culture-positive samples contained low concentrations of TB bacteria rather than PCR inhibitors, which would tend to rule out inhibition as a cause.

PCR and smear results compared with culture

Among the 22 culture-positive samples, 18 (82%) were PCR-positive and 9 (41%) smear-positive (Table 2). The remaining four culture-positive samples were both PCR- and smear-negative. All 9 smear- and culture-positive samples were also positive by PCR. The sensitivity and specificity of PCR were 82% and 96%, compared to 41% and 95%, respectively, for smear examination. Differences were observed on comparing the sensitivity of smear microscopy with that of