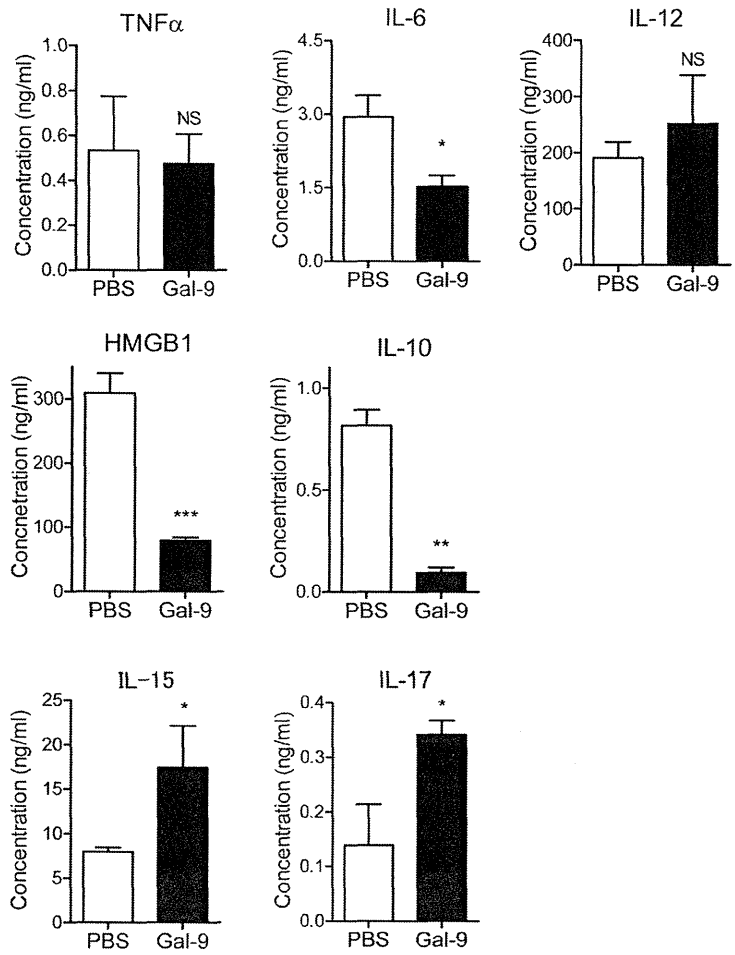

Figure 3 Effects of galectin (Gal)-9 on plasma cytokine levels in cecal ligation and puncture (CLP) mice. Plasma was prepared at 24 h after a single intravenous (i.v.) Gal-9 injection (i.v., 30 μg/mouse) immediately after CLP, and plasma cytokines were assessed by ELISA analysis; n = 8 to 12; NS, not significant; $^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001). HMGB1, high mobility group box 1.

of pro-inflammatory cytokines (TNF-α, IL-12 and IFNγ) during early time points (1 to 6 hours) following LPS-induced inflammation [24].

Effects of Gal-9 on cytokine production from spleen cells

The next set of experiments was performed to compare the levels of cytokine production from spleen cells from PBS- and Gal-9-treated CLP mice. Spleen cells were obtained at 24 hours after CLP and cultured without any stimulation for 48 hours. Spleen cells from Gal-9-treated CLP mice released decreased levels of early and late proinflammatory cytokines such as TNFα, IL-6, IL-12 and HMGB1 (Figure 4). The level of IL-10 was also decreased in Gal-9-treated CLP mice (Figure 4). Although IL-10 is an immunosuppressive cytokine, neutralization of IL-10 leads to improved survival of septic mice by restoring the downregulation of the IL-18 receptor on NK cells and IFN-γ production [28,29].

In contrast, an increase in IL-17 production was observed from spleen cells from Gal-9-treated CLP mice (Figure 4), which is unexpected because Gal-9 reduces IL-17-producing Th17 cells by inducing apoptosis and downregulating the differentiation of Th17 cells [17,18]. IL-17 is a proinflammatory cytokine produced during acute, delayed and autoimmune inflammation [30]. In addition to Th17 cells, other T cells such as CD8 T cells (Tc17) [31,32], natural killer T (NKT) cells [33,34] and γδT cells [35,36] could be the source of IL-17. In autoimmune diseases, it is important to reduce pro-inflammatory IL-17 levels to improve disease severity [17]. In contrast, IL-17 plays a critical role in protecting mice from sepsis-mediated lethality [37,38].

Furthermore, Gal-9 treatment resulted in an increase in IL-15 production compared to PBS-treated mice (Figure 4). IL-15 is an anti-apoptotic cytokine that prevents apoptosis by inducing apoptosis inhibitors

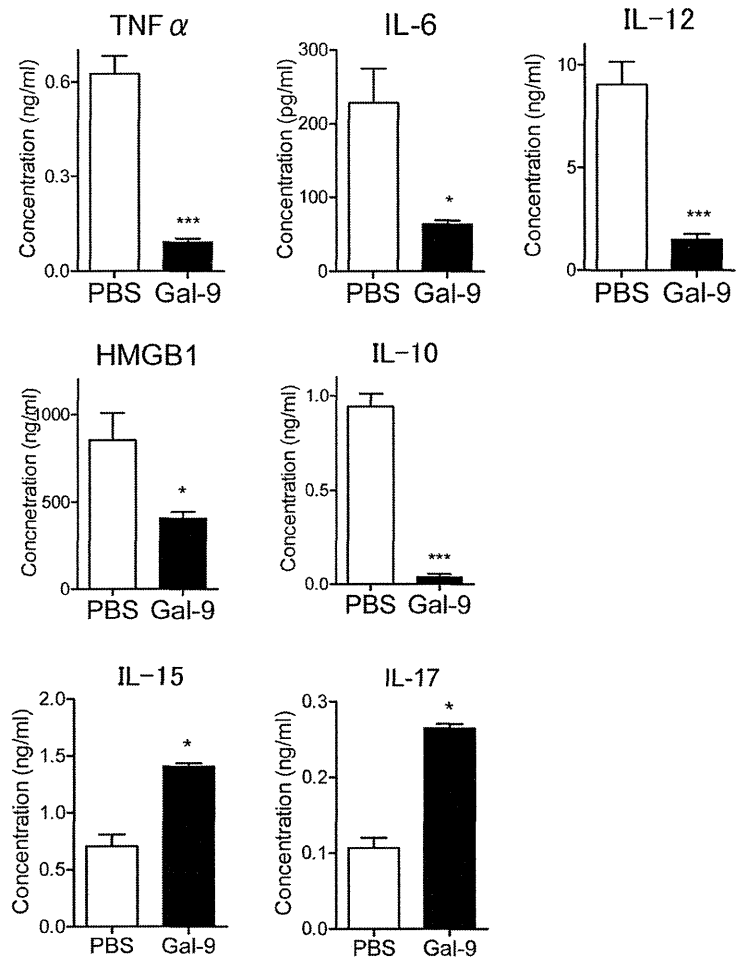

Figure 4 Effects of galectin (Gal)-9 on cytokine production from spleen cells. Spleen cells were obtained at 24 hours after cecal ligation and puncture and PBS or Gal-9 injection. These cells were then cultured without any stimulation for 48 hours, and an ELISA assay was used for quantification of cytokine levels released by the spleen cells; n = 6 to 8; NS, not significant; $^{*}P$ <0.05; $^{***}P$ <0.001.

such as Bcl-2 [39]. Inoue *et al.* [40] recently showed that IL-15 prevents apoptosis, reverses innate and adaptive immune dysfunction and improves survival in a sepsis model. Therefore, it seems reasonable that there is upregulation of IL-17 and IL-15 in Gal-9-treated CLP mice that are resistant to CLP-induced lethality.

The level of IL-10 was suppressed in Gal-9-treated CLP mice (Figure 4). Although IL-10 is an immunosuppressive cytokine, neutralization of IL-10 leads to improved survival by restoring the downregulation of the IL-18 receptor on NK cells and IFN-γ production in septic mice [28,29].

Based on the present results, it is thus suggested that it is important to increase IL-15 and IL-17 production and to decrease IL-10 and HMGB1 production simultaneously to protect mice from CLP-induced lethality. Furthermore, as we have previously proposed [41], Gal-9 should be regarded as a homeostasis-maintaining factor to keep an adequate immune response, not just as an immunosuppressive factor or immunostimulatory factor.

Effects of Gal-9 on spleen T cells

As noted above, there are several types of T cells: CD4 T cells ($CD3^+$ $CD4^+$), CD8 T cells ($CD3^+$ $CD8^+$), γδT cells ($CD3^+$ $GL\text{-}3^+$) and NKT cells ($CD3^+$ $NK1.1^+$). Spleen cells were obtained 24 hours after CLP. Flow cytometric analysis was performed to clarify which types of T cells are expanded by Gal-9 in CLP mice. The frequencies of CD4 and CD8 T cells were not changed by Gal-9 (Figure 5A). We also assessed the frequency of Tim-3 expression on those T cells, because Tim-3 is a ligand of Gal-9 [16]. Approximately 6% of CD4 T cells expressed Tim-3 on their surface, and Gal-9 reduced the frequency of $Tim\text{-}3^+$ CD4 T cells, likely Th1 and Th17 cells (Figure 5B), in agreement with our previous data that

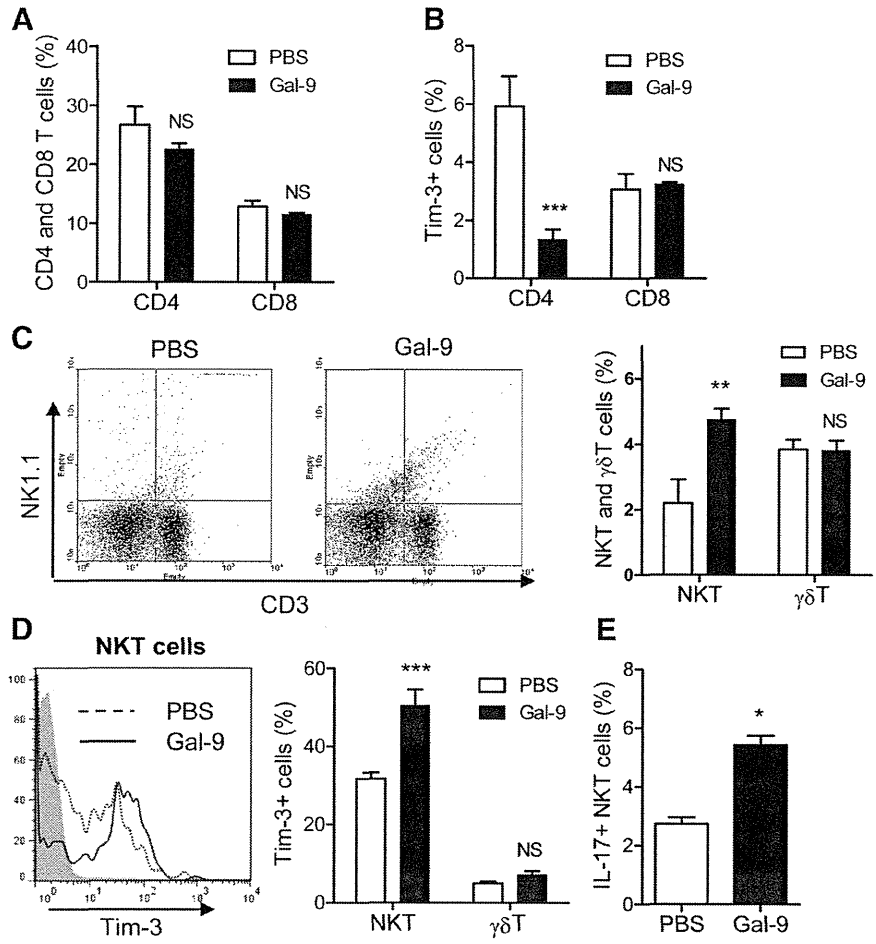

Figure 5 Effects of galectin (Gal)-9 on subpopulations of spleen T cells. Spleen cells were obtained from PBS- or Gal-9-treated cecal ligation and puncture (CLP) mice at 24 hours after a single intravenous injection. Flow cytometric analysis was performed to clarify which types of T cells are expanded by Gal-9 in the spleens of CLP mice. **(A)** The frequencies of CD4 and CD8 T cells. Spleen cells were stained with CD3, CD4, CD8 and Tim-3, and fluorescence-activated cell sorting (FACS) analysis was performed; n = 5 for each group (NS, not significant). **(B)** The frequency of Tim-3+ CD4 and CD8 T cells. Spleen cells were stained with CD3, CD4, CD8 and Tim-3, and FACS analysis was performed; n = 5 for each group (NS, not significant; $^{***}P$ <0.001). **(C)** The frequencies of natural killer T (NKT) cells and γδT cells. Spleen cells were stained with CD3, GL-3 and NK1.1, and FACS analysis was performed; n = 5 for each group (NS, not significant; $^{**}P$ <0.001). **(D)** Expansion of Tim-3+ NKT cells but not γδT cells. Spleen cells were stained with CD3, GL-3, NK1.1 and Tim-3, and FACS analysis was performed; n = 5 for each group (NS, not significant; $^{***}P$ <0.001). **(E)** Gal-9 treatment resulted in an increase of intracellular IL-17+ NKT cells. Spleen cells were stained with CD3, NK1.1 and intracellular IL-17, and FACS analysis was performed; n = 5 for each group ($^{*}P$ <0.05).

show that Gal-9 induces the cell death of both Th1 and Th17 cells through a Gal-9/Tim-3 interaction [16-18]. In contrast, Gal-9 weakly but not significantly increased Tim-3$^+$ CD8 T cells in the spleen of Gal-9-treated mice (Figure 5B), suggesting that Gal-9 does not induce cell death of Tim-3$^+$ CD8 T cells, at least under these conditions. Indeed, we have shown that Gal-9 potentiates CD8 T cell-mediated antitumor immunity via Gal-9-Tim-3 interactions between dendritic cells (DCs) and CD8 T cells [41].

Moreover, Gal-9 increased the frequency of CD3$^+$ NK1.1$^+$ cells, most likely NKT cells (Figure 5C). One third of NK1.1$^+$ NKT cells expressed Tim-3, and Gal-9 significantly increased the frequency of Tim-3-expressing NK1.1$^+$ NKT cells (Figure 5D). In contrast, Gal-9 did not increase the frequency of γδT cells (Figure 5C) or the Tim-3 expression of those cells (Figure 5D). We further found that Gal-9 treatment resulted in an increase in the frequency of intracellular IL-17$^+$ NKT cells in the spleens of CLP mice (Figure 5E). These findings suggest that Gal-9 does not induce cell death of Tim-3-expressing NK1.1$^+$ NKT cells, but enhances activation of those cells to produce IL-17. It is not surprising, because we had similar findings in our previous

work, showing that Gal-9 promotes the activation of DCs to produce a small amount of TNF-α [42].

From the present experiments, NKT cells are proposed to be one of the main cell-types responsible for the Gal-9-induced prolongation of survival, by releasing IL-17 in CLP mice. Regarding the role of NKT cells in sepsis, there are competing opinions: one is that NKT cells are involved in harmful outcomes in sepsis as reviewed by Leung and Harris [43], and another is that those cells exhibit a protective function in sepsis [44,45]. Thus, it becomes important to explain such a discrepancy. NKT cells are broadly categorized into at least two groups: Type I NKT cells and Type II NKT cells [46]. Furthermore, immune response in sepsis consists of an initial hyper-reactive phase and a latent phase. The initial hyper-reactive phase is characterized by the large release of pro-inflammatory cytokines from macrophages et cetera, and the latent anti-inflammatory or immunosuppressed phase is characterized by hypotensive shock, inability to clear the infection, increased susceptibility to nosocomial infections, and possible multiple organ failure and death [47]. Our previous and present experiments suggest that Gal-9 decreases pro-inflammatory cytokine levels at the initial hyper-reactive phase [24]. In contrast, Gal-9 decreases HMGB1 but increases IL-10 and IL-17. Gal-9 also expands Tim-3$^+$ NK1.1$^+$ NKT cells and IL-17$^+$ NKT cells. Therefore, we cannot exclude the possibility that Tim-3 expressing NK1.1$^+$ NKT cells produce IL-17 to protect sepsis through the Gal-9/Tim-3 pathway. Thus, the hypothesis may be proposed that Gal-9 contributes to downregulate the early inflammatory response during sepsis but also to

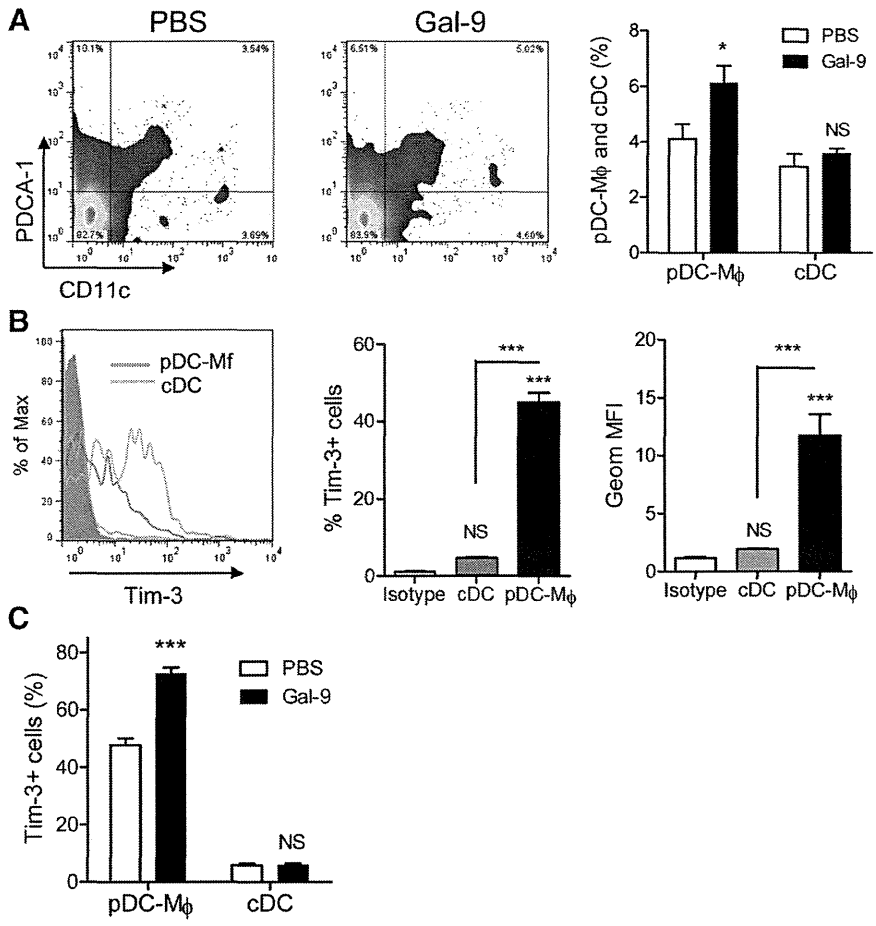

Figure 6 Effects of galectin (Gal)-9 on splenic macrophages and dendritic cells (DCs). Spleen cells were obtained from PBS- or Gal-9 treated cecal ligation and puncture (CLP) mice at 24 hours after a single intravenous injection and stained with F4/80, PDCA-1, CD11c and Tim-3. **(A)** An increased frequency of plasmacytoid (p)DC-like macrophages (F4/80+ PDCA-1+ CD11c+) but not cDCs (F4/80- PDCA-1- CD11c+) was observed; n = 5 for each group (NS, not significant; $^*P$ <0.05). **(B)** High Tim-3 expression on pDC-like macrophages was observed; n = 5 for each group (NS, not significant; $^{***}P$ <0.001). Blue line, pDC-like macrophages; orange line, cDCs; red line, PDCA-1+ CD11c- cells. **(C)** Increased Tim-3-expressing pDC-like macrophages in Gal-9-treated CLP mice; n = 5 for each group (NS, not significant; $^{***}P$ <0.001).

upregulate the delayed inflammatory response; further studies are required to clarified this.

Effects of Gal-9 on spleen macrophages and DCs

Because innate immune cells such as macrophages and DCs may play a critical role in prolonging the survival of septic mice by activating/regulating T cells [7,48,49], we performed the experiments to clarify whether Gal-9 has an effect on the frequency of macrophages or DCs. Gal-9 markedly increased the frequency of plasmacytoid (p)DC-like macrophages (F4/80$^+$ PDCA-1$^+$ CD11c$^+$), although Gal-9 failed to increase the frequency of cDCs (F4/80$^-$ PDCA-1$^-$ CD11c$^+$) (Figure 6A).

Next, we assessed the Tim-3 expression on macrophages and DCs in the spleens of CLP mice. Intriguingly, half of the pDC-like macrophages expressed Tim-3, although cDCs did not significantly express this molecule (Figure 6B). Moreover, we compared the Tim-3 expression on splenic macrophages and DCs between the Gal-9- and PBS-treated CLP mice. Gal-9 significantly increased the frequency of Tim-3-expressing pDC-like macrophages (Figure 6C). In contrast, the frequency of Tim-3$^+$ cDCs was low (Figure 6B), and Gal-9 failed to increase the frequency of Tim-3-expressing cDCs in CLP mice (Figure 6C). HMGB1 is a macrophage-derived late proinflammatory cytokine, and TLR4 and CD14 are required for the release of HMGB1 [50]. pDC-like macrophages suppress LPS-induced release of early proinflammatory cytokines from macrophages by suppressing TLR4 and CD14 expression on macrophages [21,22]. Thus, it was suggested that immunosuppressive pDC-like macrophages may mediate decreased release of early and late proinflammatory cytokines from macrophages that also, at least in part, may be involved in the prolongation of survival seen in these experiments. Of course, further studies are required to ascertain whether Tim-3$^+$ NKT cells and pDC-like macrophages are indeed involved in the survival prolongation induced by Gal-9.

Furthermore, the possibility that Gal-9 blocks the PD-1/PD-L1 pathway cannot be excluded, as blocking PD-1 improves survival in CLP mice [10-12]. Therefore, further studies are required to clarify whether Gal-9 affects PD-1 and PD-L1 expression on macrophages and T cells and whether Gal-9 blocks the PD-1/PD-L1 pathway.

Conclusions

Gal-9 exhibits therapeutic effects on CLP-induced polymicrobial sepsis, potentially by expanding NKT cells and pDC-like macrophages, and by modulating the production of early and late pro-inflammatory cytokines.

Key messages

- Delayed Gal-9 treatment prolongs the survival of polymicrobial sepsis in mice induced by CLP.
- Gal-9 decreases TNFα, IL-6, IL-10 and HMGB1 levels but increases IL-15 and IL-17 levels.
- Gal-9 expands NKT cells and pDC-like macrophages, although it decreases the frequency of Tim-3$^+$ CD4 T cells.
- Many NKT cells and pDC-like macrophages express Tim-3, and Gal-9 increases the frequency of Tim-3$^+$ NKT cell and pDC-like macrophages.

Abbreviations

CFU: Colony-forming unit; CLP: Cecal ligation and puncture; DC: Dendritic cell; ELISA: Enzyme-linked immunosorbent assay; FACS: Fluorescence-activated cell sorting; FCS: Fetal calf serum; Gal-9: Galectin-9; HMGB1: High mobility group box 1; IFN: Interferon; IL: Interleukin; i.v.: Intravenous; L: Ligand; LPS: Lipopolysaccharide; NKT: Natural killer T; PBS: Phosphate-buffered saline; PD-1: Programmed death-1; pDC: Plasmacytoid dendritic cell; PF: Peritoneal fluids; RBC: Red blood cells; RPMI: Roswell Park Memorial Institute medium; SEM: Standard error of the mean; TG: Transgenic; ThGal-9: Surface Gal-9-expressing Th cells; Tim-3: T cell immunoglobulin motif 3; TNF: Tumor necrosis factor; Tregs: Regulatory T cells; WT: Wild-type.

Competing interests

Drs Niki and Hirashima are board members of GalPharma Co., Ltd. Although there are patents and products related to this manuscript in development, this does not alter our adherence to all of the Journal's policies on sharing data and materials as detailed in the guide for authors. The patent for stable-form Gal-9 is issued in Japan (4792390), the USA (8,268,324), EPC (1736541), Canada (2,561,696) and Korea (10–1222281) and is applied for in China (200580010446.1). The other authors declare that they do not have any competing interests.

Authors' contributions

AM, MH, TM and TH designed the experiments and helped to draft the manuscript. TK and AM carried out the FACS analysis and ELISA for spleen cell culture supernatants and drafted the manuscript. JH and MS performed the *in vivo* survival test, ELISA for plasma cytokine levels and bacterial colony formation experiments using peritoneal fluids. JT, HM and HY carried out the ELISA of culture supernatants of spleen cells. TN prepared recombinant galectin-9 for the present study and helped with the FACS analysis.
All authors read and approved the final manuscript.

Authors' information

Akihiro Matsukawa and Mitsuomi Hirashima are co-senior authors.

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Short Communication

Applicability of In-House Loop-Mediated Isothermal Amplification for Rapid Identification of *Mycobacterium tuberculosis* Complex Grown on Solid Media

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SUMMARY: A simple, rapid, and low-cost identification method is required in tuberculosis high-burden countries. We report the applicability of in-house loop-mediated isothermal amplification (LAMP) targeting 16S ribosomal RNA for the rapid identification of *Mycobacterium tuberculosis* complex grown on Lowenstein-Jensen media. Eighty acid-fast staining-positive clinical isolates were selected and used to evaluate the LAMP assay in comparison with polymerase chain reaction and conventional culture-based tests. The LAMP assay identified 60 *M. tuberculosis* isolates from 80 clinical isolates using simple heat-extracted DNA directly from the colony suspension. The results were in complete agreement with those obtained using the other methods, and the utility of the direct LAMP assay from a colony was demonstrated. The LAMP assay appears to be a practical and low-cost method that can be used for the rapid identification of *M. tuberculosis* isolates and suitable for endemic low-resource settings.

Tuberculosis (TB) is still the most common deadly infectious disease worldwide. In Thailand, almost 130,000 people suffer from active TB and approximately 11,000 deaths occur annually. The nation ranks 18th on the list of 22 "TB high-burden countries" in the world (1). Although most mycobacterial infections are still caused by *Mycobacterium tuberculosis* complex (MTC), non-tuberculous mycobacteria (NTM) have been documented to cause a number of human pulmonary infections in developed and developing countries (2-3). The increasing incidence of TB and NTM infections caused by the acquired immunodeficiency syndrome (AIDS) epidemic has resulted in the need for rapid and accurate identification of isolates grown on media so that appropriate treatment can be prescribed.

The culture and identification of MTC is still the gold standard for diagnosing TB, although MTC can be directly identified from clinical specimens using genetic methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) because these methods cannot differentiate between live and dead TB bacilli. However, conventional identification methods depending on biochemical assays and the phenotypic examination of colony formation on solid media are labor-intensive and time-consuming owing to the slow growth rate of MTC (4). In addition, the results of biochemical tests are sometimes inconclusive because of their low sensitivity and reproducibility. Thus, a rapid means of identifying MTC is essential for enhancing diagnostic services in mycobacteria laboratories and for improving the management of patients. PCR-based methods for the detection of MTC grown on Lowenstein-Jensen (L-J) media have been reported and proposed as an alternative method (5). Till date, the use of traditional nucleic acid amplification appears to be restricted to the laboratory setting, equipment, and technical expertise. The immunochromatography test (ICT) is commercially available and is widely used for the rapid confirmation of *M. tuberculosis* cultures (6-7). Although ICT is an easier method for the detection of MTC from culture samples, adequate growth with prolonged incubation is necessary to avoid false negative results (8). In recent years, several new molecular techniques, including LAMP, have been developed and used for *M. tuberculosis* detection (9-12). Compared with modern molecular methods, which are relatively complex and economically unsuitable for laboratories with limited resources, LAMP is a low-cost

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molecular assay that combines specificity and sensitivity. The advantages of the LAMP technique include its simplicity, i.e., isothermal amplification at a constant temperature of 60°C-65°C without any need for sophisticated equipments such as a thermal cycler, and the direct visual inspection of gene amplification in the reaction tube as opposed to analysis by gel electrophoresis (13-17). The positivity of the reaction can be easily detected by the naked eye as a color change or the observation of a white precipitate (15,17). Because of its simplicity and cost effectiveness, LAMP is a promising molecular technique that could be readily applied to the rapid detection and identification of *M. tuberculosis* in resource-limited settings.

This study aimed to assess the performance and applicability of in-house LAMP, known as TB-LAMP, to the rapid and accurate identification of MTC grown on L-J medium, which is used commonly in conventional mycobacterial culture. Sputum samples were collected, decontaminated by N-acetyl-L-cysteine-NaOH treatment, inoculated onto L-J slants, and examined for growth or contamination at 37°C (4). The bacteria that grew on L-J medium were examined to determine their colony morphology, growth rate, pigmentation, and Ziehl-Neelsen staining results. DNA was extracted from colonies recovered from L-J media that were positive for acid-fast bacilli (AFB) using a simple boiling method. In brief, putative small colonies were picked and suspended in a microcentrifuge tube containing 100 μl distilled water. DNA was extracted by boiling the cell suspension using a dry heat block at 80°C for 10 min. After brief spinning at 2,000 × g for 5 s, an aliquot of cell lysate was added directly to the LAMP reaction mixture as described in previous studies (12,18). In brief, LAMP for MTC was performed in a 20-μl reaction mixture, which contained 2.0 μl of 10 × LAMP buffer [200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM NH_4SO_4, and 1% Triton X-100], 14 mM dNTPs, 0.8 M betaine, 300 mM $MgSO_4$, 30 pmol FIP and BIP primers, 20 pmol of FL and BL primers, 5 pmol F3 and B3 primers, 8 U *Bst* DNA polymerase (New England Biolabs, Inc., Ipswich, Md., USA), 1 μl Fluorescent Detection Reagent (FDR; Eiken Chemical Co., LTD., Tokyo, Japan) and 7 μl cell lysate. The set of 6 primers used in this study was described in our previous studies (12,18). After incubation at 65°C for 1 h in a small heat block, DNA amplification was detected in LAMP reactions as a color change from orange to green using the naked eye (Fig. 1). The results were compared with those of multiplex PCR analysis, which could differentiate between MTC and NTM in a single tube, as well as classical biochemical tests, which comprised niacin accumulation, nitrate reduction, catalase production, and susceptibility to paranitrobenzoic acid (PNB) when heavy growth of each isolate was achieved (4).

The specificity and sensitivity of TB-LAMP were intensively examined in our previous study of direct detection from clinical specimens (12) and liquid culture (18). TB-LAMP was specific to MTC and could directly detect as few as 9 tubercle bacilli in sputum samples. However, the direct detection of MTC from clinical specimen cannot differentiate between living and dead TB bacilli, while liquid culture examination is not sustainable in many developing countries because of its

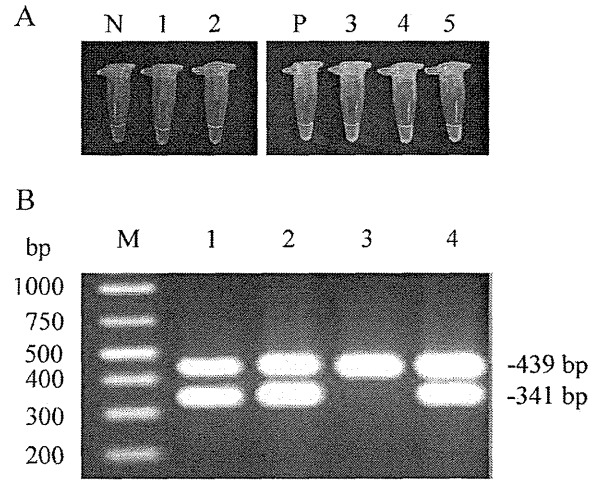

Fig. 1. Identification of *M. tuberculosis* complex isolates by TB-LAMP and multiplex PCR.
DNA extracted by heat lysis from mycobacterial clinical isolates positive for acid-fast bacilli was used for TB-LAMP and multiplex PCR reactions. *M. tuberculosis* complex and non-tuberculous mycobacteria were identified by visual observation of the color change in LAMP and by agarose gel electrophoresis in multiplex PCR. A. Visual appearance of LAMP results showing N: negative control, 1-2: non-tuberculous mycobacterial isolates, P: positive control, and 3-5: *M. tuberculosis* complex isolates. B. Multiplex PCR results analyzed by agarose gel electrophoresis. A 439-bp DNA band specific for *Mycobacterium spp.* reported to contain NTM in the samples. PCR positive for 439 bp specific for *Mycobacterium spp.* and 341 bp specific for MTC reported as containing MTC in the samples. Lane M: DNA marker; Lane, 1, 2 and 4: PCR product from *M. tuberculosis* complex isolates and Lane 3: PCR product from non-tuberculous mycobacterial isolates.

cost, although it is recommended in many settings. Solid culture examination is still considered essential, and it has been used in many developing countries. Thus, we examined the feasibility of TB-LAMP for the detection of MTC in early culture-positive samples. Using TB-LAMP, 60 clinical isolates, which were presumed to be MTC on the basis of visual observations of the colonies, were rapidly identified as MTC by TB-LAMP. As expected, the remaining 20 isolates, which were presumed to be NTM, yielded negative results with TB-LAMP. The TB-LAMP results were in complete agreement with the multiplex PCR results (Fig. 1). In addition, all the isolates with the biochemical characteristics of MTC and susceptibility to PNB exhibited positive results by TB-LAMP (Table 1). There were no false-positive identification results using TB-LAMP among all 20 NTM isolates (Table 1). These concordant identification results confirmed the specificity of TB-LAMP for MTC, and the simple DNA extraction from the tiny colony on L-J medium at the beginning of culture growth made identification by this method much quicker than that by the combination of biochemical tests and susceptibility to PNB. In addition, the overall procedure of the TB-LAMP assay allowed *M. tuberculosis* identification to be completed in less than 2 h without any requirements of expensive or complex instruments. The LAMP system uses a simple aluminum heat block that can be powered by a handy battery, therefore, it can be used in difficult settings that experience power interrup-

Table 1. Concordance of identification results by TB-LAMP with conventional methods

	M. tuberculosis complex*	Non-tuberculous mycobacteria
TB-LAMP		
Positive	60 (100%)	0 (0%)
Negative	0 (0%)	20 (100%)

*Isolates were identified by conventional methods, multiplex PCR, biochemical tests, and PNB susceptibility.

tions. This study clearly demonstrated the utility of TB-LAMP for the rapid identification of MTC grown on L-J medium. Conventional identification has a long delay because of the need for heavy growth and labor-intensive procedures; however, it could be substituted with this alternative nucleic acid isothermal amplification method. The preparatory steps required to extract DNA for the LAMP reaction in a simple heating method also reduced the infection risk and the cost of the test. Conventional nucleic acid amplification tests are not widely used in developing countries because of contamination by carry-over products. An advantage of LAMP is that the results can be observed without any further analysis unlike conventional nucleic acid amplification tests. Naked-eye observation of the color change in the reaction mixture without opening the lid of the reaction tube reduces the risk of cross-contamination.

TB is the most common mycobacterial infection in developing countries; therefore, this simple and economic identification method based on TB-LAMP could be suitable for use in any laboratories that perform mycobacterial culture.

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Conflict of interest None to declare.

Ethics approval Not applicable as this study used clinical isolates.

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Simple Multiplex PCR Assay for Identification of Beijing Family *Mycobacterium tuberculosis* Isolates with a Lineage-Specific Mutation in *Rv0679c*

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The Beijing genotype of *Mycobacterium tuberculosis* is known to be a worldwide epidemic clade. It is suggested to be a possibly resistant clone against BCG vaccination and is also suggested to be highly pathogenic and prone to becoming drug resistant. Thus, monitoring the prevalence of this lineage seems to be important for the proper control of tuberculosis. The Rv0679c protein of *M. tuberculosis* has been predicted to be one of the outer membrane proteins and is suggested to contribute to host cell invasion. Here, we conducted a sequence analysis of the *Rv0679c* gene using clinical isolates and found that a single nucleotide polymorphism, C to G at position 426, can be observed only in the isolates that are identified as members of the Beijing genotype family. Here, we developed a simple multiplex PCR assay to detect this point mutation and applied it to 619 clinical isolates. The method successfully distinguished Beijing lineage clones from non-Beijing strains with 100% accuracy. This simple, quick, and cost-effective multiplex PCR assay can be used for a survey or for monitoring the prevalence of Beijing genotype *M. tuberculosis* strains.

The *Mycobacterium tuberculosis* Beijing genotype, first identified by van Soolingen et al. (1), is known to be a worldwide epidemic clade (2–4). Its possible resistance to BCG vaccination, in addition to its tendency to have a multidrug-resistant (MDR) phenotype, might give a selective advantage to the wide geographic distribution of the Beijing genotype strains (3, 5–7). Although some of the Beijing genotype strains show hypervirulence in animal infection models (7–9), neither the virulence factor nor the phenotypically specific factor of this lineage has been elucidated. The origin of the Beijing lineage is thought to be east Asia, where the prevalence of this clade is from around 40% to >90% (1, 3, 4, 10–13). However, in some other global areas, i.e., countries in the former Soviet Union and South Africa, the prevalence of the Beijing lineage has increased markedly in a short period, and some increases were suggested to be related to MDR (4, 11, 14). In those areas, higher clonality of the circulating strains was suggested, and most were categorized as being in the modern or typical Beijing clade, which is defined as a strain having one or two IS*6110* insertions in the noise transfer function (NTF) chromosomal region (11, 15). On the other hand, a higher variety of strains can be observed in east Asian countries. Especially in Japan and Korea, the majority of the strains belong to another cluster called the ancient or atypical Beijing clade (12, 16). Details regarding the higher pathogenicity of the Beijing lineage are controversial. Some studies have suggested that the modern Beijing clade is more prone to be pathogenic, tends to be drug resistant, and is likely able to escape from BCG vaccination (4, 8, 11, 14); however, some of the ancient Beijing clones were also shown to have higher pathogenicity (17) or a tendency toward acquiring drug resistance (16).

Since Beijing lineage prevalence has a great impact on the tuberculosis (TB) control program, several methods to distinguish this clade have been developed. First, van Soolingen et al. (1) identified this clade by its specific IS*6110* restriction fragment length polymorphism (RFLP) signatures. Soon after, these strains were shown to have a specific spoligotype pattern lacking spacer numbers 1 to 34, and this has been proposed as the definition of the clade (18, 19), since IS*6110* RFLP genotyping is time-consuming, and comparing results between laboratories is difficult. The deletion of spacers observed in the Beijing spoligotype is caused by the insertion of IS*6110* in the direct repeat (DR) region (18). Since this typical spoligotype pattern has become a specific marker of the Beijing genotype, some PCR methods to detect this specific deletion, named region of difference 207 (RD207), have been developed (20–22). In addition to RD207, another deleted region named RD105 was also shown to be a good marker for discrimination of the Beijing genotype, although this deletion is common for all the east Asian lineages, including the non-Beijing strains (10, 23); however, most of these published detection methods require expensive real-time PCR equipment and high-cost reagents (24). The conventional PCR assay targeting RD207 still seems to be at a disadvantage, since it relies on an unstable inser-

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tion sequence that is likely to be a target of homologous recombination.

Instead of unstable repetitive structures, single nucleotide polymorphisms (SNPs) were recently considered to be a robust target for defining the accurate position of a strain on the phylogenetic tree, since horizontal gene transfer or gene recombination between different strains is rare in the *M. tuberculosis* complex (MTC) (12, 24, 25). Filliol et al. (26) drew phylogenetic trees of the MTC using several typing methods and showed that the dendrogram drawn with SNPs most accurately reflected the true evolution of the MTC. Some of those SNPs are suggested to be specific to the Beijing or east Asian lineages. In a search for membrane proteins that are suitable for vaccine antigens and/or are targets for the specific detection of the MTC, we found a candidate protein encoded by the *Rv0679c* gene. This protein was expressed on the cell surface as a lipoarabinomannan-associated protein (27, 28), and the coding sequence has an SNP that seems to be specific to the Beijing clade. In this study, we confirmed the lineage specificity of this SNP and developed a simple and low-cost multiplex PCR assay to distinguish the Beijing lineage strains.

MATERIALS AND METHODS

Preparation of genomic DNA from *M. tuberculosis* isolates. *M. tuberculosis* was isolated from the sputa or other clinical specimens of patients by conventional procedures using *N*-acetyl-L-cysteine (NALC)–NaOH. A total of 619 isolates obtained in Japan ($n = 145$), Bangladesh ($n = 122$), Nepal ($n = 110$), Myanmar ($n = 198$), and China (Heilongjiang Province, $n = 44$) were used in this study. Some of these isolates were the same as those in previous studies, and the details are described elsewhere (13, 29–31). Colonies grown on egg-based medium (either Ogawa or Löwenstein-Jensen medium) were resuspended in distilled water and boiled for 20 min, and the supernatant was used in the Bangladeshi and Myanmar samples. In the Japanese and Nepalese samples, colonies were suspended in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA (Tris-EDTA [TE] buffer [pH 8]), and 0.5 ml chloroform; 0.5 g glass beads of 0.17-mm diameter was added; and they were disrupted with a bead beater (MicroSmash; Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation at 10,000 × *g* for 5 min, DNA in the supernatant was precipitated by ethanol, and the precipitated genomic DNA was resuspended in TE buffer for further use. In China, bacteria grown in a BACTEC *Mycobacterium* growth indicator tube (MGIT) (Becton, Dickinson and Company, Franklin Lakes, NJ) were used, and DNA was extracted by lysozymes and the phenol-chloroform method (13). All the DNA samples extracted in each country were brought to Japan, and the following steps were carried out in the Hokkaido University Research Center for Zoonosis Control. To determine the specificity of the method, DNAs extracted from five reference MTC strains (i.e., *M. tuberculosis* H37Rv, *Mycobacterium africanum* ATCC 25420, *Mycobacterium orygis* Z0001, *Mycobacterium microti* TC 89, and *Mycobacterium bovis* BCG Tokyo 172) and 30 nontuberculous mycobacterial (NTM) species, including *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii*, were used.

Gene sequencing and comparison. A subset of 197 *M. tuberculosis* samples, 68 from Japan, 92 from Bangladesh, and 37 from Nepal, were chosen from the total 619 clinical isolates, and the *Rv0679c* gene fragment was amplified by PCR. The PCR mixture contained GoTaq PCR buffer (Promega Co., Madison, WI), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.3 μM each primers og0001 and og0002 (Table 1), 0.5 M betaine, 1 ng genomic DNA from *M. tuberculosis*, and 0.5 units of GoTaq polymerase. Amplification was carried out by applying 35 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s, polymerase reaction mixture at 72°C for 40 s, and a final extension at 72°C for 5 min. The amplified DNA fragment was subjected to sequence analysis with BigDye Terminator v3.1 (Life Technologies Co., Carlsbad, CA) reagents by a sequencer, the 3130 genetic analyzer (Life Technologies Co.), according to the manufacturer's protocol. The *Rv0679c* sequence was also compared with those of 80 whole-genome sequenced MTC strains registered in the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) or TB (http://genome.tbdb.org/annotation/genome/tbdb/MultiHome.html) (32) databases by the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Genotyping. The spoligotype of *M. tuberculosis* clinical isolates was determined as described previously (33). Briefly, the DR region was amplified with a primer pair, and the PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes against the international spoligotyping database (SpolDB4) (3).

The detection of an RD105 deletion was performed by multiplex PCR in Beijing clones and by conventional PCR in east Asian strains other than those of the Beijing type, since the deletion pattern is different between those two groups (10). The reaction mixture consisted of GoTaq PCR buffer (Promega), 0.2 mM each dNTP, 0.3 μM (each) two or three primers (Table 1), 0.5 M betaine, 1 μl extracted DNA sample, and 0.5 units of GoTaq polymerase. The target was amplified by 35 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 40 s, with a final extension at 72°C for 5 min. RD207 deletion was detected by two PCR assays described by Warren et al. (22), and TbD1 was detected by PCR using the Huard et al. (25) protocol (Table 1). The amplified DNA fragment was subjected to agarose gel electrophoresis with ethidium bromide (EtBr) to see the size of the band under a UV transilluminator.

The multilocus sequence type (MLST) was determined with 9 SNPs, which were described by Filliol et al. (26) and were selected for Beijing subtyping by Iwamoto et al. (16). Each locus was amplified with a primer pair (Table 1), and the product was subjected to sequencing. SNPs were detected by comparing the sequences with those of H37Rv (34). The sequence type (ST) was identified according to Filliol et al. (26).

Beijing lineage identification by multiplex PCR. Multiplex PCR for the identification of the Beijing lineage was performed under the following conditions. The PCR mixture, in a final volume of 15 μl, contained 1× PCR buffer (1.5 mM Mg; TaKaRa Bio, Inc., Shiga, Japan), 0.5 μl dNTP solution mix (10 mM each dNTP; New England BioLabs, Inc., Ipswich, MA), 0.5 μl each of Fw and R1 primers, 0.2 μl R2 primer (primer solutions in 10 μM; Table 1), 1.5 μl of 5 M betaine, 0.45 μl of 25 mM $MgCl_2$ (to make a final Mg concentration of 2.25 mM), 1 ng of sample DNA, and 0.5 units of TaKaRa Hot Start *Taq* polymerase (TaKaRa). Amplification was carried out with the first denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 66°C for 10 s, extension at 72°C for 15 s, and the final extension at 72°C for 3 min. The amplicon was subjected to electrophoresis in a 2% agarose gel that included EtBr. DNA samples extracted from the isolate BCG Tokyo 172 and a well-characterized clinical isolate (Beijing OM-9) were used as controls for the non-Beijing and Beijing banding patterns, respectively. Sensitivity was determined with serially diluted genomic DNA obtained from these BCG and Beijing control strains. A specificity study was performed with genomic DNA samples (2 ng/μl each) from the MTC and NTM strains described above.

RESULTS

Spoligotyping and MLST. A total of 619 clinical isolates were subjected to spoligotyping, and 393 were identified as being in the Beijing lineage and 226 as a non-Beijing group (Table 2). The non-Beijing group consisted of a variety of strains belonging to the following lineages: east African-Indian (EAI), central Asian (CAS), Latin American Mediterranean (LAM), Haarlem, S, T, X, and non-Beijing east Asian (3). Ninety-four of the Beijing isolates were subjected to MLST analysis and were subtyped into 8 sequence-type classes, namely, ST26, ST3, STK, ST25, ST19, ST10, ST22, and ST8, which are listed in evolutional order from ancient to modern Beijing types (16, 26).

TABLE 1 Primers used in the study

Target	Primer name	Nucleotide sequence	Purpose	Reference
Rv0679c	og0001	CCGGGAACTAGGAATGGTAA	Sequencing	This study
	og0002	AGCAACCTCGCAATCTGAC	Sequencing	This study
	ON-1002 (Fw)	GTCACTGAACGTGGCCGGCTC	Multiplex PCR for Beijing type identification	This study
	ON-1258 (R1)[a]	TCGGTCACCGTTTTTGTAGGTGACCGTC	Multiplex PCR for Beijing type identification	This study
	ON-1127 (R2)	AGCAACCTCGCAATCTGACC	Multiplex PCR for Beijing type identification	This study
RD105	RD105-F (−239~−218)	GGAAAGCAACATACACACCACG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-R	AGGCCGCATAGTCACGGTCG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-M (+304~323)	TCCTGGGTGCCGAACAAGTG	Multiplex PCR for east Asian type determination[b]	This study
	RD105EA-F (−80~−60)	TCGGACCCGATGGCTTCGGTG	PCR for east Asian type determination[c]	This study
	RD105EA-R (61~42)	TGATCACGGTTCGCCCGCAG	PCR for east Asian type determination[c]	This study
RD207	RD207-1F (Warren)	TTCAACCATCGCCGCCTCTAC	PCR for Beijing type identification (set 1)	22
	RD207-1R (Warren)	CACCCTCTACTCTGCGCTTTG	PCR for Beijing type identification (set 1)	22
	RD207-2F (Warren)	ACCGAGCTGATCAAACCCG	PCR for Beijing type identification (set 2)	22
	RD207-2R (Warren)	ATGGCACGGCCGACCTGAATGAACC	PCR for Beijing type identification (set 2)	22
TbD1	TbD1F	CGTTCAACCCCAAACAGGTA	PCR for ancestral *M. tuberculosis* determination	25
	TbD1R	AATCGAACTCGTGGAACACC	PCR for ancestral *M. tuberculosis* determination	25
797736[d]	Beijing ST-1F	GACGGCCGAATCTGACACTG	MLST for Beijing lineage	This study
	Beijing ST-1R	CCATTCCGGGTGGTCACTG	MLST for Beijing lineage	This study
909164[d]	Beijing ST-2F	CGTCGAGCTCCCACTTCTTG	MLST for Beijing lineage	This study
	Beijing ST-2R	TCGTCGAAGTGGACGAGGAC	MLST for Beijing lineage	This study
1477596[d]	Beijing ST-3F	GTCGACAGCGCCAGAAAATG	MLST for Beijing lineage	This study
	Beijing ST-3R	GCTCCTATGCCACCCAGCAC	MLST for Beijing lineage	This study
1692067[d]	Beijing ST-5F	GATTGGCAACTGGCAACAGG	MLST for Beijing lineage	This study
	Beijing ST-5R	TGGCCGTTTCAGATAGCACAC	MLST for Beijing lineage	This study
1892015[d]	Beijing ST-6F	GCTGCACATCATGGGTTGG	MLST for Beijing lineage	This study
	Beijing ST-6R	GTATCGAGGCCGACGAAAGG	MLST for Beijing lineage	This study
2376133[d]	Beijing ST-7F	TCTTGCGACCCGATGTGAAC	MLST for Beijing lineage	This study
	Beijing ST-7R	GAGCGCAACATGGGTGAGTC	MLST for Beijing lineage	This study
2532614[d]	Beijing ST-8F	CCCTTTTCTGCTCGGACACG	MLST for Beijing lineage	This study
	Beijing ST-8R	GATCGACCTTCGTGCACTGG	MLST for Beijing lineage	This study
2825579[d]	Beijing ST-9F	CCTTGGAGCGCAACAAGATG	MLST for Beijing lineage	This study
	Beijing ST-9R	CTGGCCGGACGATTTTGAAG	MLST for Beijing lineage	This study
4137829[d]	Beijing ST-10F	CGTCGCTGCAATTGTCTGG	MLST for Beijing lineage	This study
	Beijing ST-10R	GGACGCAGTCGCAACAGTTC	MLST for Beijing lineage	This study

[a] Beijing-type specific mutation-detection primer. Underlined 2-base sequences at the 5′ end are not complementary sequences.
[b] This assay was used for Beijing genotype strains.
[c] This assay was used for non-Beijing genotype strains.
[d] This SNP nucleotide position on the *H37Rv* genome is according to references 26 and 34.

Sequence analysis of the *Rv0679c* gene of *M. tuberculosis* isolates. Nucleotide sequences of the full-length *Rv0679c* gene obtained from 197 clinical *M. tuberculosis* isolates collected in Japan, Bangladesh, and Nepal were compared with the *Rv0679c* sequence in *M. tuberculosis* H37Rv (34). Only a single nucleotide difference of cytosine to guanine at position 426, which leads to an amino acid change at codon 142 from Asn (AAC) to Lys (AAG), was detected in 87 isolates, all of which were identified as being in the Beijing lineage by spoligotyping and, supportively, by RD207 PCR (22) (data not shown). One Bangladeshi isolate showed a mixed peak of C and G at position 426 and was revealed as a mixed culture of Beijing and another strain by RD105 and RD207 detection PCR (Table 2). None of the non-Beijing isolates had the mutation, and vice versa. In public databases, 14 strains reported from several countries were revealed to have this mutation, and all were confirmed as being in the Beijing lineage by checking for the RD207 deletion *in silico* (18). None of the other 66 MTC strains, which were determined to be non-Beijing, had this mutation. The 498-bp *Rv0679c* sequence was well conserved among the MTC strains, and the following three strains in the database showed alterations: *M. tuberculosis* strains C and T17 and *Mycobacterium canettii* CIPT 140010059.

TABLE 2 *Rv0679c* multiplex PCR results compared with other typing results in 619 *M. tuberculosis* clinical isolates

Isolate origin	Spoligotype family[a]	RD207, RD105, or other typing methods[b]	Sequence type[c]	*Rv0679c* M-PCR type[d]	No. of isolates
Beijing or Beijing-like					393
Japan	Beijing	ND	26	Beijing	10
	Beijing	ND	3	Beijing	24
	Beijing	ND	STK	Beijing	13
	Beijing-like	RD207$^+$	STK	Beijing	1
	Beijing	ND	25	Beijing	3
	Beijing	ND	19	Beijing	9
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	4
	Beijing	ND	ND	Beijing	23
Bangladesh	Beijing	ND	26	Beijing	3
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	2
	Beijing	ND	8	Beijing	1
	Beijing	ND	ND	Beijing	29
	Beijing-like	RD105$^+$, RD207$^+$	ND	Beijing	1
Nepal	Beijing	ND	ND	Beijing	64
Myanmar	Beijing	ND	ND	Beijing	141
	Beijing-like	RD105$^+$, RD207$^+$	ND	Beijing	1
China (Heilongjiang)	Beijing	ND	ND	Beijing	40
Non-Beijing or undesignated/new[a]					216
Japan	Undesignated/new[e]	RD105$^+$, RD207$^-$	ND	Non-Beijing	29
	Others[f]	ND	ND	Non-Beijing	16
Bangladesh	—[g]	ND	ND	Non-Beijing	73
Nepal	—[h]	ND	ND	Non-Beijing	45
Myanmar	—[i]	ND	ND	Non-Beijing	51
China (Heilongjiang)	Undesignated/new	ND	ND	Non-Beijing	2
Mixed clone samples					6
Bangladesh	Undesignated/new	Mixed peak in sequence[j] RD105$^+$, RD207$^+$	ND	Beijing	1
Myanmar	Undesignated/new	RD105$^+$, RD207$^+$	ND	Beijing	2
	EAI2_NTB	RD105$^+$	ND	Beijing	1
	EAI5	RD105$^+$	ND	Beijing	1
China (Heilongjiang)	Undesignated/new	RD105$^+$	ND	Beijing	1
New spoligotype lacking spacers 1–34[k]					4
Japan	New	RD105$^+$, RD207^{+k}	ND	Beijing	1
Nepal	New	RD105$^-$, TbD1^{+k}	ND	Non-Beijing	1
Myanmar	New	RD105$^+$, RD207$^+$	ND	Beijing	1
China (Heilongjiang)	New	RD105$^+$, RD207$^+$	ND	Beijing	1

[a] Spoligotype labeling is according to SpolDB4 (3).
[b] A positive superscript indicates that a deletion was detected; a minus superscript indicates that the RD was not deleted or the region was intact. ND, not determined.
[c] Sequence type is according to reference 26.
[d] M-PCR, multiplex PCR.
[e] East Asian lineage.
[f] Including the clades LAM1, LAM9, T1, T2, T3, T3-Osaka, and new (other than the east Asian lineage).
[g] Including the clades EAI1_SOM, EAI2-MANILA, EAI3_IND, EAI5, EAI6_BGD1, EAI7_BGD2, EAI unidentified, CAS, CAS1-DHLHI, CAS2, LAM9, T1, T4, H1, H3, X1, X2, and undesignated/new.
[h] Including the clades EAI3_IND, EAI5, CAS, CAS1-DHLHI, LAM1, LAM5, T1, T2, T3, H3, S, and undesignated/new.
[i] Including the clades EAI2_MANILA, EAI2_NTB, EAI5, EAI6_BGD1, EAI7_BGD2, CAS1-DHLHI, LAM9, T1, T3, X2, S, and undesignated/new.
[j] Overlapped peak of C and G was observed at nucleic acid position 426.
[k] Details are described in Table 3.

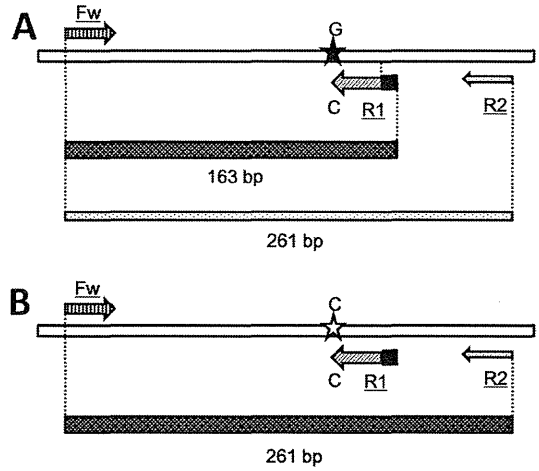

FIG 1 PCR primers and products of *Rv0679c*-targeting multiplex PCR for Beijing lineage discrimination. (A) In the Beijing sample, the 163-bp product is amplified more dominantly than is the 261-bp product. (B) In the non-Beijing sample, 163-bp product is not amplified because of the mismatch of the 3′ end of R1. Fw, forward primer; R1, reverse primer 1 (Beijing lineage specific); R2, reverse primer 2. Two-base noncomplement nucleotides at the 5′ end are shown by black squares.

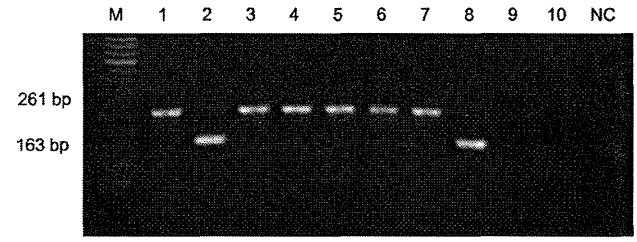

FIG 2 Electrophoresis results of the multiplex PCR products. Lane M, 50-bp ladder DNA size marker; lane 1, *M. bovis* BCG Tokyo 172 (non-Beijing lineage control) strain; lane 2, *M. tuberculosis* OM-9 strain (Beijing lineage control); lane 3, *M. tuberculosis* H37Rv; lane 4, *M. africanum* ATCC 25420; lanes 5–8, *M. tuberculosis* clinical isolates (lane 5, non-Beijing east Asian; lane 6, EAI; lane 7, LAM9; lane 8, Beijing); lane 9, *M. avium* strain JATA51-1; lane 10, *M. kansasii* JATA21-1; lane NC, negative control.

In strain C, the C185T SNP was observed, and in T17, a cytosine was inserted at position 92. In *M. canettii* CIPT 140010059, two SNPs and a codon insertion, ACC at position 154, were observed.

Beijing lineage identification by multiplex PCR. Multiplex PCR was developed targeting the Beijing-specific SNP on *Rv0679c*, employing a primer with the mutated nucleic acid at the 3′ end of the sequence (primer R1; Fig. 1 and Table 1); the optimal reaction conditions were determined as described in Materials and Methods. With this system, a bright band of 163 bp was observed as an amplified product of the primers Fw and R1 in the Beijing genotype samples (Fig. 1A and 2). An additional band of 261 bp, which is the product of primers Fw and R2, can be seen depending on the conditions, although it is always significantly thinner than the 163-bp band because of the low R2-primer concentration (see Materials and Methods). In contrast, only the 261-bp band is observed in a non-Beijing genotype sample (Fig. 1B and 2). Since the sequences of the primers are specific to the MTC, no amplification occurs in the absence of MTC genomic DNA (Fig. 2, data for *M. avium* and *M. kansasii*). A total of 619 clinical isolates obtained in the five Asian countries of Japan, Bangladesh, Nepal, Myanmar, and China were subjected to this Beijing lineage-identifying multiplex PCR, and the results were compared with their spoligotypes. All the isolates determined as having a Beijing or Beijing-like genotype by the SpolDB4 (n = 393) were determined to be in the Beijing lineage by the multiplex PCR (Table 2). On the other hand, no samples that included only non-Beijing genotype DNA (n = 216) were identified as being in the Beijing lineage. Twenty-nine non-Beijing east Asian lineage strains, which were suggested by a characteristic spoligotype having spacer 34 and were defined by RD105 detection, were determined to be non-Beijing by the multiplex PCR. Six isolates that showed a discrepancy between their spoligotype and the multiplex PCR result were further determined by RD207 or RD105 detection PCR and were revealed to be a mixture of Beijing and other subtype strains (mixed clone samples, Table 2). Four samples from different countries had confusing spoligotypes that lacked spacers 1 to 34 and additionally lacked some of the spacers from 35 to 43. These samples could also be identified correctly (Tables 2 and 3). The minimum detection limits were 100 and 1,000 cells per reaction in the Beijing genotype and BCG strains, respectively (data not shown).

DISCUSSION

In this study, we demonstrated that the SNP of C to G at position 426 in the *Rv0679c* gene is specific to the Beijing genotype strains. We developed a new multiplex PCR using this SNP to identify Beijing lineage isolates. This PCR assay successfully distinguished Beijing genotype strains from others, including the non-Beijing east Asian strains, with 100% accuracy. The Beijing lineage genotype is usually identified by spoligotyping, specific patterns of IS*6110* RFLP, or the detection of RD207, which is led by an insertion of IS*6110* in the DR region. However, spoligotyping is well known to show gene conversions, and strains having no genetic relationship sometimes show the same spoligotype (3, 26). Fenner et al. (35) reported pseudo-Beijing strains that had a typical Beijing spoligotype even though they actually belonged to the CAS family. This type of confusion seems to occur especially in areas that have a higher prevalence of principal genetic group 1 (PGG1) lineages, including the EAI, CAS, and east Asian lineages, since PGG1 strains usually possess spacers 35 and 36, which are lacking in PGG2 and PGG3 strains (3, 36). In other areas, mixed infections of more than two strains sometimes disrupt correct spoligotyping by showing mixed spacer patterns. The Manu1-SIT100 and Manu2-SIT54 types, which lack the spacers 34 or 33 and 34, respectively, are known to be producible by the mixture of Beijing family and T1 strains (3, 37). In this study, we found that some samples showed discrepant results between *Rv0679c* multiplex PCR and spoligotyping that determined a strain to be of the Beijing genotype by multiplex PCR, despite having another spoligotype. Using RD105 and RD207 detection methods, all of these samples were confirmed to be a mixture of Beijing and another strain. This type of mixed culture is sometimes observed in countries with a higher TB burden, where a coinfection of more than two strains is not rare (22). Some of the spoligopatterns of those samples showed faint positive spacers, suggesting the mixed presence of other strains. Even clear and correct spoligotypes can sometimes lead to misjudgments. In the current study, some samples showed only one to several spacers to be positive in the Beijing spacer area,

TABLE 3 Typing result comparison in clinical isolates having confusing spoligotype patterns

Sample (identification)	Spoligotype pattern	Spoligotype family[a]	*Rv0679c* M-PCR type	Detection type				Final typing result
				RD207 set 1[b]	RD207 set 2[b]	RD105	TbD1	
Japan (O-05-44)	□□□■	New	Beijing	−	+[c]	+	−	Beijing
Nepal (no. 51)	□□□■■□□□□	New	Non-Beijing	−	−	−	+	Ancestral[d]
Myanmar (no. 95)	□□□■■■■■	New	Beijing	+	+	+	ND	Beijing
China (2460)	□□□■■□□□□□□□	New[e]	Beijing	+	+	+	ND	Beijing

[a] These patterns were not found in the SpolDB4 list.
[b] PCR sets 1 and 2 in reference 22.
[c] A faint correctly sized band and an additional band of a different size were observed.
[d] Ancestral type of *M. tuberculosis* strain possessing TbD1 region (25).
[e] The spoligotype pattern of this sample has been reported in reference 13.

namely, from spacers 35 to 43. Most were identified as being of the Beijing genotype by multiplex PCR, while one was judged to be a non-Beijing strain. All Beijing genotype-positive results were confirmed by RD105 and RD207 PCRs, and the non-Beijing isolate was revealed as an "ancestral type," which involves EAI but not the Beijing lineage, by TbD1 detection (Table 3) (25). These examples support the high specificity and applicability of this SNP-targeting PCR. The disadvantages of IS*6110* RFLP and RD207 detection have already been described above. RD207-detection PCR did not work as expected in the sample that lost spacers 1 to 42 (Japan O-05-44; Table 3), suggesting that some additional reconstruction had occurred at the IS*6110* insertion site of the DR region. SNPs in MTC genomes can provide robust lineage information, whereas repetitive elements, such as direct repeats in the DR region, the mycobacterial interspersed repetitive unit (MIRU) tandem repeats (38), or IS*6110*, are prone to alteration. One hundred percent concordance of the PCR results with the genetically confirmed Beijing type is not surprising because of the rigidity of the SNPs in the MTC (25, 26). Of the 393 Beijing family isolates, 94 were subtyped by MLST and consisted of 8 STs covering a wide range of the Beijing family, from ancient to modern types (Table 2). This suggested that a specific mutation in *Rv0679c* seemed to have occurred in the Beijing lineage at the same time as the RD207 deletion event.

Rv0679c is an MTC-specific gene, as shown by Cifuentes et al. (27), and no significantly similar sequence was detected by an NCBI BLASTn search in the GenBank database. Thus, this multiplex PCR assay can be used for the identification of the MTC, as well as for the differentiation of Beijing and non-Beijing lineages (Fig. 2). The Beijing mutation detection primer (R1; Fig. 1 and Table 1) was designed to have two additional noncomplement bases at the 5′ end to block the second amplification by the PCR product that produces the 261-bp fragment with an outer R2 primer. Additionally, the higher concentration and melting temperature of the R1 primer compared to those of the outer R2 primer increase the Fw-R1 product more than the Fw-R2 product. With these techniques, the Beijing band (163 bp) can be shown to be significantly brighter than the non-Beijing band (261 bp) when the sample is derived from Beijing lineage *M. tuberculosis* strains (Fig. 2). The relatively higher annealing temperature of 66°C gave good contrast of those two bands and prevented nonspecific amplifications. Modified *Taq* or other polymerases that have 3′-to-5′ exonuclease activity should be avoided, since those enzymes can trim the mutated nucleotide at the R1 primer end. It is recommended to check the PCR conditions using positive controls for Beijing and non-Beijing types (i.e., BCG) every time (Fig. 2). The detection limit of 100 to 1,000 copies per reaction might be relatively high; however, it can be improved by about 10 times by increasing the PCR cycle number to 40, although the necessity of identifying the MTC lineage in direct clinical specimens seems to be low.

In papers featuring SNPs as epidemiological markers, synonymous mutations are usually selected to avoid the effect of evolutional pressure (26). However, both SNPs for the differentiation of PGG1, PGG2, and PGG3 were nonsynonymous mutations in *katG* and *gyrA* (36), and so far, they have provided robust differentiation results. In the MTC, nonsynonymous mutations on functional genes can be observed in a relatively higher frequency than in other bacteria because of extremely reduced purifying selection pressure (39). Thus, nonsynonymous mutations can be preserved unless they are significantly disadvantageous. Indeed, 100% of the Beijing family strains in the current study could be identified with this nonsynonymous mutation, suggesting that it at least has no adverse effect on those strains. The function of the Rv0679c protein is still unclear, although its expression on the cell surface has been confirmed (27, 28). Cifuentes et al. (27) reported that the surface-localized Rv0679c protein contributed to the *M. tuberculosis* invasion of host cells and proposed the protein as a vaccine candidate. The substituted amino acid at position 142 was located in the C-terminus region of the protein, which was included in the "high-activity binding peptide" to target cells (27). Thus, this highly conserved nonsynonymous SNP, which results in an amino acid substitution with different characteristics (Asn → Lys), might have some biological meaning in explaining Beijing lineage pathogenicity. Since BCG vaccine strains, as well as other non-Beijing strains, have Rv0679c-Asn142, this substitution might affect the antigenicity of the Beijing bacterial surface and might contribute to the possible evasion of BCG-derived immunity. Further investigation of the association of the Rv0679c Asn142Lys substitution with Beijing strain outer membrane characteristics and antigenicity is ongoing.

In conclusion, a simple, robust, and low-cost multiplex PCR assay for the detection of Beijing lineage *M. tuberculosis* strains was successfully developed using a Beijing-specific SNP on *Rv0679c*. This PCR assay can be used in local laboratories to monitor the prevalence of the Beijing genotype, and this is strongly recommended to control this possibly highly pathogenic and drug resistance-prone sublineage.

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HIV-1 Drug-Resistance Surveillance among Treatment-Experienced and -Naïve Patients after the Implementation of Antiretroviral Therapy in Ghana

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Abstract

Background: Limited HIV-1 drug-resistance surveillance has been carried out in Ghana since the implementation of antiretroviral therapy (ART). This study sought to provide data on the profile of HIV-1 drug resistance in ART-experienced and newly diagnosed individuals in Ghana.

Methods: Samples were collected from 101 HIV-1-infected patients (32 ART-experienced cases with virological failure and 69 newly diagnosed ART-naïve cases, including 11 children), in Koforidua, Eastern region of Ghana, from February 2009 to January 2010. The *pol* gene sequences were analyzed by in-house HIV-1 drug-resistance testing.

Results: The most prevalent HIV-1 subtype was CRF02_AG (66.3%, 67/101) followed by unique recombinant forms (25.7%, 26/101). Among 31 ART-experienced adults, 22 (71.0%) possessed at least one drug-resistance mutation, and 14 (45.2%) had two-class-resistance to nucleoside and non-nucleoside reverse-transcriptase inhibitors used in their first ART regimen. Importantly, the number of accumulated mutations clearly correlated with the duration of ART. The most prevalent mutation was lamivudine-resistance M184V ($n=12$, 38.7%) followed by efavirenz/nevirapine-resistance K103N ($n=9$, 29.0%), and zidovudine/stavudine-resistance T215Y/F ($n=6$, 19.4%). Within the viral protease, the major nelfinavir-resistance mutation L90M was found in one case. No transmitted HIV-1 drug-resistance mutation was found in 59 ART-naïve adults, but K103N and G190S mutations were observed in one ART-naïve child.

Conclusions: Despite expanding accessibility to ART in Eastern Ghana, the prevalence of transmitted HIV-1 drug resistance presently appears to be low. As ART provision with limited options is scaled up nationwide in Ghana, careful monitoring of transmitted HIV-1 drug resistance is necessary.

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Introduction

The number of people worldwide living with HIV/AIDS in 2010, according to the latest report from the United Nations Programme on HIV/AIDS, was estimated to be 34.0 million [1]. Although the highest prevalence of HIV/AIDS remains in sub-Saharan Africa, current massive and rapid scaling up of antiretroviral therapy (ART) has resulted in the decline of the epidemic in this region [1]. Indeed, HIV prevalence in Ghana gradually declined from a peak of 3.6% in 2003 to 2.1% in 2011 due to the National AIDS Control Programme implementing a strategy for achieving universal access to ART. The program has been continuously expanding since 2003, and the coverage of ART in 2011 was estimated to be 26.6% (59,007/221,884) and 63.6% (8,057/12,661) for total HIV-infected individuals and for HIV-positive pregnant women, respectively [2].

The first-line regimen of ART recommended in Ghana is the combination of two nucleoside reverse-transcriptase inhibitors (NRTIs) and a non-nucleoside reverse-transcriptase inhibitor (NNRTI) [3]. Specifically, the two NRTIs selected are lamivudine (3TC) and either zidovudine (AZT) or stavudine (d4T), then either nevirapine (NVP) or efavirenz (EFV) as the NNRTI [3]. For the

second-line regimen in Ghana, two protease inhibitors (PIs) are available, nelfinavir (NFV) or lopinavir/ritonavir (LPV/r), either of which is recommended to use with two NRTIs, abacavir (ABC) and either tenofovir (TDF) or didanosine (ddI) [3].

Drug-resistant HIV variants selected during ART have the potential to be transmitted to others. Indeed, drug-resistant HIV has been widely described in ART-naïve individuals. For example, a recent systematic review revealed that the overall prevalence of drug-resistant HIV-1 transmission reached 12.9% in North America, 10.9% in Europe, 6.3% in Latin America, 4.7% in Africa, and 4.2% in Asia [4]. Thus, the higher prevalence of drug-resistant HIV-1 transmission has been reported in higher ART coverage areas, mostly in developed countries. It is important to note that, along with ART scale-up, the prevalence of transmitted HIV-1 drug-resistance increased from 2.8% before 2001 to 5.3% after 2003 in African countries [4]. As the transmission of drug-resistant HIV may seriously affect the efficacy of first-line ART, surveillance to monitor the prevalence of transmitted HIV drug-resistance has become an important issue in African countries. The prevalence of transmitted HIV-1 drug resistance in Ghana was reported in two studies. One was conducted in 2003 in the Greater-Accra Region of Ghana [5], and the other one was conducted between 2002 and 2004 in Accra and two sites of the Eastern region, Agomanya and Atua [6]. Both studies reported no case of drug-resistant HIV-1 transmission [5,6]. As at December 2009, the national response had established programs for the provision of ART in hospitals and health centers in several districts in the ten regions of Ghana [7]. However, since ART was expanded in Ghana, the situation of transmitted HIV-1 drug-resistance has not been reported.

To clarify the prevalence, pattern, and spectrum of HIV-1 drug resistance in the era of scaled up ART in Ghana, particularly in ART-experienced patients and transmission to new individuals, we surveyed HIV-1 drug resistance among ART-experienced and -naïve patients enrolled between 2009 and 2010 in Koforidua, the capital of the Eastern region, Ghana. Concomitantly, we analyzed HIV-1 subtypes in detail to further understand the epidemiology of HIV-1 infections in Ghana.

Methods

Patients

HIV-infected patients who visited the Koforidua Regional Hospital (KRH) from February 2009 to January 2010 were enrolled in the study. KRH is the main HIV/AIDS clinic in the capital of the Eastern region of Ghana. This hospital is responsible for HIV prevention and intervention programs in the area and provides free ART with care and support to HIV-infected patients. The Institutional Review Board of the Noguchi Memorial Institute for Medical Research granted ethical approval for this study. All patients or their caregivers gave written consent to participate in the study.

$CD4^+$ T-cell Count and Plasma HIV-1 Viral Load Monitoring

For an indication of immune status, $CD4^+$ T-cells were measured using a FACSCount flow cytometer (Becton Dickinson, San Jose, California, USA). Plasma HIV-1 viral loads (pVLs) were quantified using an in-house real-time reverse-transcription and polymerase chain reaction (RT-PCR) assay as previously reported [8]. ART-experienced patients with pVL>150 copies/mL were considered as virological failures.

HIV-1 Drug-resistance Genotyping

HIV-1 drug-resistance genotyping was performed as previously reported with some modifications [9]. In brief, viral RNA was extracted from 200 μL of plasma samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). RT-PCR was performed with QIAGEN one-step RT-PCR kit (Qiagen), and nested PCR was subsequently performed using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, USA). Specific primers known as DRPRO5, DRPRO2L, DRPRO1M, and DRPRO6 were used for the protease (PR) region (424 bp, positions 2,168 to 2,591 in the reference HXB2 sequence), and DRRT1L, DRRT4L, DRRT7L, and DRRT6L primers for the reverse transcriptase (RT) region (838 bp, positions 2,510 to 3,347) [9]. Details of the primers used in the study are shown in Table 1. Nucleotide sequencing was performed using ABI 3730 auto-sequencer followed by editing with SeqScape software v2.5 (Applied Biosystems). HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. In addition, transmitted HIV-1 drug-resistance mutations were defined using the mutation list proposed by Bennett et al. [11].

HIV-1 Subtyping

HIV-1 subtyping was performed using the *pol* gene sequences (1,095 bp, positions 2253 to 3347). Phylogenetic tree was constructed with the references of subtypes A-D, F-H, J, K, and all circulating recombinant forms (CRFs) 01 to 51, except 30, 41, and 50, obtained from the HIV Sequence Database at the Los Alamos National Laboratory. In addition, HIV-1 sub-subtype A3 (DDI579, DDJ360, and DDJ369) and A4 (97CD_KCC2, 97CD_KTB13, and 02CD_KTB035) isolates were added to the phylogenetic tree analysis, as these sub-subtypes have been reported as circulating in several African countries [12,13]. Multiple sequences were aligned using the MUSCLE program, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v5.05 [14]. Phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap replicates. In similarity plotting and boot-scanning analyses, nine HIV-1 subtypes, A-D, F-H, J, and K, and three CRFs, CRF02_AG, CRF06_cpx, and CRF09_cpx, were used as references. Similarity plotting and boot-scanning were performed using SimPlot software v3.5.1 with window and step sizes of 250 and 20 nucleotides, respectively [15]. One HIV-1 isolate identified with an unknown mosaic pattern both in similarity plotting and boot-scanning analyses was considered as a unique recombinant form (URF).

Statistical Analysis

The Fisher's exact test and the Mann-Whitney U-test were used in SYSTAT software v10.2 (SYSTAT Software, Chicago, USA) for analysis of statistical significance between categorical variables and quantitative valuables, respectively. All tests were two-sided and the level of significance was set at $P<0.05$.

Accession Numbers

Nucleotide sequences have been registered as #AB751399 to AB751499 in the DNA databank of Japan.