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EXPERIMENTAL ARTICLES

RD7 Genotyping of *Mycobacterium tuberculosis* Strains Isolated from Patients with Lung Tuberculosis in Various Areas of Kazakhstan

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Abstract—A three-primer PCR assay has been designed for detecting possible deletions in the RD7 chromosomal region of the *Mycobacterium tuberculosis* complex. The assay gives rise to amplicons of different sizes depending on the presence or absence of deletions. The PCR assay was applied to 176 isolates from lung tuberculosis cases collected in various areas of Kazakhstan in the summer of 2004. Prior to assay, the isolates were characterized by culture and biochemical tests. The RD7 genotyping showed neither polymorphism nor deletions in the RD7 genome region. Some strains were additionally characterized by a PCR-RFLP analysis of the *gyrB* and *hsp64* genes. The RFLP patterns corresponded to *M. tuberculosis.* The results of the study were consistent with certain previous studies, which indicates the population stability of RD7 in *M. tuberculosis* strains. Species identification of the isolates showed that *M. tuberculosis* sensu stricto was the principal causative agent of human lung tuberculosis in Kazakhstan.

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The *Mycobacterium tuberculosis* complex (MTBc) is a group of microorganisms where up to seven species are recognized, including the following: *M. tuberculosis*, *M. bovis* (including BCG vaccine strain), *M. africanum*, *M. microti*, *M. canettii*, *M. caprae*, and *M. pinnipedii* [8]. This group is genetically conservative. The genomes of its members are similar by more than 99.9%, and the sequences of ribosomal RNA genes and many housekeeping genes are perfectly identical. The main source of genetic polymorphism in MTBc is mobile genetic elements (IS elements and prophages), various repetitive sequences (DR and MIRU), and deletions [17].

Deletions in MTBc genomes were first found through a comparison of chromosomal DNAs of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. The genome of the vaccine strain was demonstrated to lack large segments referred to as "regions of difference" (RDs) [16, 23]. Further studies showed that the total number of deletions in MTBc genomes can reach into the hundreds, as well as that they can occur in all MTBc species, including *M. tuberculosis.* Other designations of deletions were proposed in addition to RDs, e.g., RvDs and TbDs. Presently, these deletions are collectively referred to as "large-sequence polymorphisms" (LSPs) [5]. Study of the prevalence of deleted regions among MTBc species has led to suggestions of their role in MTBc evolution and a new understanding of the phylogenetic relationships between MTBc species. According to modern notions, *M. tuberculosis* is the direct descendant of an ancestor, whereas strains infecting animals were produced by evolutional adaptation to nonprimate mammals, accompanied by the loss of certain genome regions [6, 18]. The application of some deletion loci as molecular markers allowed the determination of criteria of phylogenetic and taxonomical relationships among MTBc species [5] and development of procedures for isolate identification and differentiation [12, 13, 22]. The new criteria were extensively used for the characterization of regional mycobacterial populations [4, 7, 11, 15, 21, 24]. These studies are still few. Most of them cover small populations in certain regions of the globe, but the results of deletion-based genotyping are becoming preferable for strain identification in comparison with phenotypic traits. In particular, *M. africanum* type II was reclassified as *M. tuberculosis* [25].

Deletions in RD7, RD8, and RD10 form a phylogenetic branch in the evolutionary tree of MTBc, whose members, with the exception of *M. africanum*, subtype Ia, are animal parasites. Therefore, RD7 is a convenient marker that not only allows obligate human tuberculosis agents (*M. tuberculosis*, *M. canettii*, and *M. africanum* subtype Ib) to be discriminated from mycobacterial species infecting mainly animals, but also makes them able to attack humans. The RD7 deletion ranks among the longest at 12.7 kb. Two RDs are in the immediate vicinity of RD7, i.e., RD2 [10] and RD713, whose deletion overlaps RD7 [19], which suggests the genetic instability of this chromosome region.

Countries of the former Soviet Union are a gap in the map of MTBc deletion marker prevalence, although they greatly contribute to tuberculosis morbidity with regard to their areas, populations, and rates of tuberculosis incidence. Kazakhstan is a trouble spot in this context. From 1991 to 2001, the rates of morbidity and mortality of tuberculosis in Kazakhstan increased to more than double [2]. The epidemiological indices have steadied in recent years, but they are still high. According to WHO reports, the rate of tuberculosis incidence in Kazakhstan in 2005 was 144 per 100000 (http://www.who.int/countries/kaz/en).

We performed RD7 genotyping of 176 tuberculosis-causing strains isolated from lung tuberculosis patients in various regions of Kazakhstan.

MATERIALS AND METHODS

Clinical mycobacterium isolates were obtained during the summer of 2004 from sputum of lung tuberculosis inpatients of tuberculosis dispensaries in Almaty (23 isolates), Atyrau (47 isolates), Karaganda and Temirtau (46 isolates each), the Kurmangazy District (55 isolates), and in penitentiaries of Almaty (6 isolates).

Sputum samples were inoculated to MGIT test tubes and incubated in a BACTEC 960 system.

Test tubes showing bacterial growth after 4-day incubation were subjected to further analyses, including examination with a Ziehl–Nielsen stain, subculturing on a solid Levenstein–Jensen medium to confirm bacterial growth, subculturing on a Levenstein–Jensen solid medium with 1000 μg/ml sodium salicylate to differentiate MTBc from nontuberculosis mycobacteria, and inoculation to dishes with blood agar to prove the absence of contaminating microorganisms.

Biochemical tests for thermostable catalase (by the Sato and Satace method modified by N. M. Makarevich), peroxidase (by the modified Bogen method), and nitrate reductase; the niacin test; and tests for growth with sodium salicylate (1 mg/ml) and thiophene-2 carboxylic acid hydrazide (TCH) at concentrations of 2 and 5 μg/ml were performed according to conventional protocols [1, 3].

Drug sensitivity was tested by the absolute concentration method on solid Levenstein–Jensen media [1, 3].

Polymerase chain reaction was carried out with slant agar scrapings where mycobacterium growth was detected. DNA was isolated by lysis of bacterial suspension in buffer of the following composition: 20 mM Tris HCl (pH 8.2), 2 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20, 100 μg/ml proteinase K at 56°C for 1.5 h followed by heating at 95°C for 10 min.

The RD7 region was analyzed by PCR with primers Mt3, Mtb592, and Mtt640 purposely designed for the present study. The gyrB region was amplified with primers MTUBf (TCGGACGCGTATGCGATATC) and MTUBr (ACATACAGTTCGGACTTGCG) [14]. The hsp65 region was amplified with Tbl1 (ACCAACGATGGTGTGTCCCAT) and Tbl2 (CTTGTCGAACCGCATACCCT) [9]. The reaction mixture for PCR had the following composition: 10 mM Tris HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 5% formamide, 0.5% Tween 20, 8% sucrose, 0.2 mM Cresol Red, 200 μM each dNTP, 0.5 μM each primer, and 1 U of Taq polymerase. The reaction volume was 30 μl, of which the sample constituted 5 μl. Amplificates were resolved by electrophoresis in 1.5% agarose gel with ethidium bromide.

The restriction analysis of amplicons obtained with MTUBf/MTUBr primers was performed in the volume 20 μl, of which the PCR mixture constituted 8 μl and the appropriate restriction buffer constituted 2 μl. The reaction was carried out with 1–2 units of RsaI restriction endonuclease (Fermentas, Lithuania). The reaction mixture was incubated at 37°C for 2 h. The restriction analysis of amplicons obtained with Tbl1/Tbl2 was performed in the volume 30 μl, of which the PCR mixture constituted 10 μl and restriction buffer TangoY constituted 3 μl, with the addition of 1–2 units of restriction endonuclease *HhaI* (Fermentas). The reaction mixture was incubated at 37°C overnight.

Restriction fragments were resolved by electrophoresis in 3% NuSieve^R (3 : 1) agarose (FMC Bioproducts) gel with ethidium bromide.

After electrophoresis, gel slabs were photographed under UV illumination with a Canon A300 digital camera equipped with an orange filter or with a Deltatekh gel documentation system (Russia).

RESULTS AND DISCUSSION

Tuberculosis mycobacterium isolates were obtained from lung tuberculosis patients in various regions of Kazakhstan (Fig. 1). Tentative identification was performed according to morphological, culture, and biochemical traits. Assessment of morphological and culture traits included growth rates on solidified media, including those with various supplements; pigment production; colony morphology; acid resistance; and temperature optimum (Table 1). Most isolates had the phenotype typical of *M. tuberculosis.* Two phenotypic traits showed variability, i.e., the colony morphology (5 isolates formed smooth colonies) and the ability to grow on the medium with sodium salicylate (1 isolate). Nevertheless, the complex of phenotypic traits allows all the isolates to be assigned to *M. tuberculosis.*

A system of three primers (Mt3, Mtb592, and Mtt640) was designed for analyzing deletions in RD7. The primer-binding sites for Mt3 and Mtb592 are located at the boundaries of the deletion sequence, and the target for Mtt640 occurs within it). If this system is applied to DNA with a deletion, the internal primer finds no binding site, and only external primers operate. As a result of the deletion, the binding sites for the

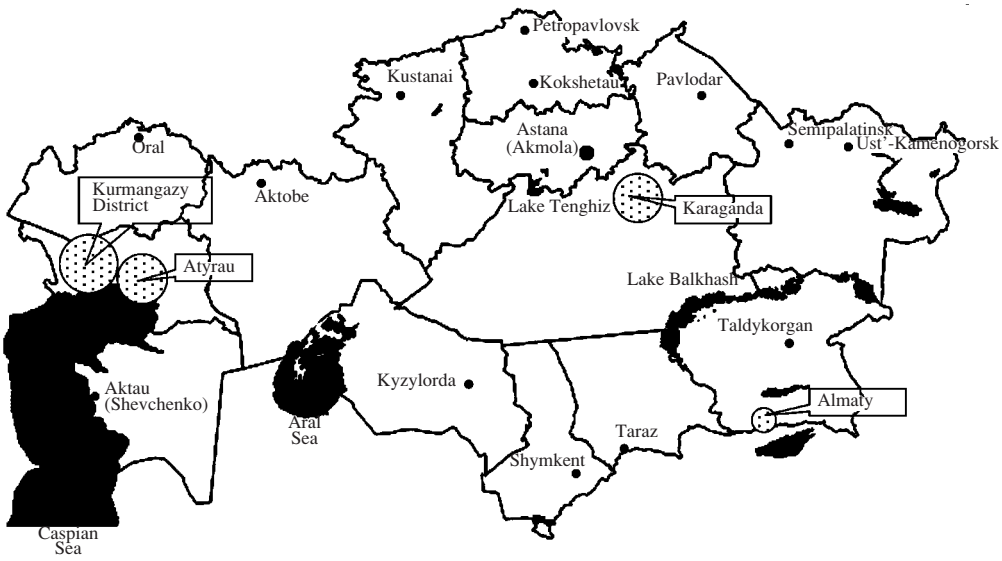

Fig. 1. Sites in Kazakhstan (circled) where strains were isolated. Circle diameter is linearly dependent on the number of strains isolated.

external primers are closer to one another and form an amplicon of an appropriate size, 574 bp. If the DNA sample to be analyzed contains no deletion, it contains binding sites for all the three primers, but the distance between the external primers is about 13 kb, which is too long for PCR under the assay conditions. Therefore, PCR generates only an amplicon between the internal primer and one of the external ones, and its length is 660 bp. The difference in amplicon size produced by the operation of different primer pairs allows the conclusion of the presence or absence of a deletion. The specificity of the primers was tested in experiments with DNA of reference strains of *M. tuberculosis* (with intact RD7), *M. bovis* (with deletion in RD7), and the following nontuberculosis mycobacteria: *M. kansasii*, *M. avium*, *M. vaccae*, *M. marinum*, *M. terrae, M. fortuitum*, and *M. gordonae*, which should produce no amplicons with this primer set. As was expected, PCR with *M. tuberculosis* and *M. bovis* yielded amplicons of the predicted sizes but no amplicons with nontuberculosis mycobacterium DNA (Fig. 2).

The primer set designed was applied to genotyping of the strains under study. All isolates showed amplicons with sizes corresponding to that obtained with the reference *M. tuberculosis* strain H37Rv (660 bp). The electrophoretic spectra are exemplified in Fig. 3.

Additionally, PCR-RFLP analysis of the gyrB gene fragment with *Rsa*I restriction endonuclease was performed for 21 isolates. It allows for *M. tuberculosis* and

Table 1. Culture and biochemical traits of *Mycobacterium* strains

Isolation site	Number of strains	Growth at 37°C	Colony morphology	Nitrate reductase activity	Catalase activity	Peroxidase activity	Growth with salicylate	Growth with TCH
M. tuberculosis H37Rv	1	Slow	R	+	+	+	–	+
Almaty	23	″	R (18/18)*	+ (22/22)*	+ (4/4)*	+ (4/4)*	– (22/22)*	+ (21/21)*
Atyrau	47	″	R (40/44)* S (4/44)*	+ (45/45)*	+ (16/16)*	+ (15/15)*	– (47/47)*	+ (21/21)*
Karaganda and Temirtau	46	″	R (46/46)*	+ (46/46)*	no data	no data	– (46/46)*	+ (46/46)*
Kurmangazy District	55	″	R (51/53)* S (2/53)*	+ (52/52)*	+ (23/23)*	+ (19/19)*	– (50/50)*	+ (23/23)*
Penitentiaries	6	″	R (2/2)*	+ (6/6)*	+ (6/6)*	+ (6/6)*	– (5/6)* + (1/6)*	+ (6/6)*

*Numerals in parentheses: number of strains with the corresponding trait/total number of strains tested.

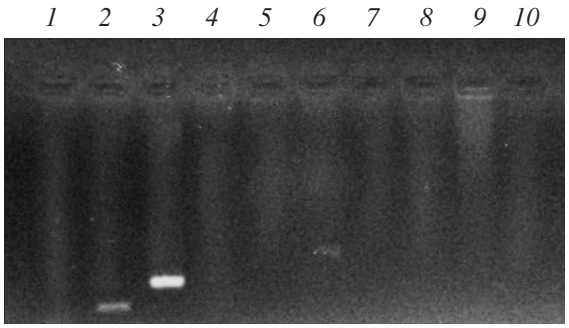

Fig. 2. Test of specificity of primers Mt3, Mtb592, and Mtt640. Here and in Fig. 3: primer concentration is 1 OD/ml each. Polymerase chain reaction was conducted as follows: a predenaturation step for 2 min at 95°C followed by 10 cycles: denaturation at 95°C for 30 s; annealing at 65°C for 30 s, and elongation at 72°C for 30 s; then by 30 cycles: 95°C for 30 s; 60°C for 30 s, and 72°C for 30 s. Lanes: *1*, negative control; *2*, *M. bovis*; *3*, *M. tuberculosis*; *4*, *M. kansasii*; *5*, *M. avium*; *6*, *M. vaccae*; *7*, *M. marinum*; *8*, *M. terrae*; *9*, *M. fortuitum*; *10*, *M. gordonae*.

M. africanum to be differentiated from other MTBc species [14, 20]. The genotyping of gyrB showed that all strains under study had restriction patterns that corresponded to *M. tuberculosis* (Table 2).

Smooth colony surface is a characteristic feature of *M. canettii* strains [26]. They are polymorphic for the *hsp65* gene, which allows them to be discriminated from other MTBc members. Restriction analysis of *hsp65* with *HhaI* endonuclease should yield the three following fragments: 260, 105, and 60 bp, whereas other MTBc members should yield the four following fragments: 185, 105, 75, and 60 bp [9]. Thus, *M. canettii* isolates can be identified by the size of the longest *HhaI* fragment. This test was carried out for 34 isolates, including the 5 with smooth colonies. All isolates tested demonstrated identical restriction fragment sets, the longest one corresponding to 185 bp (Fig. 4). Thus, the resulting restriction patterns differed from that expected for *M. canettii.*

The isolates were collected in western, southeastern, and central regions of Kazakhstan. Taking into account their geography, we may suggest that they were independent and this set was representative for the whole population of lung tuberculosis agents in whole Kazakhstan. All strains were identified as *M. tuberculosis* on the grounds of biochemical tests (presence of nitrate reductase, catalase, and peroxidase activities) and genotyping. Some isolates produced smooth colonies, which might have led to the suggestion that they belonged to *M. canettii*, but this suggestion was not confirmed by hsp65 genotyping. It is worth noting that the strains with smooth colonies were only isolated in the Atyrau region (the city of Atyrau, Kurmangazy District). Mycobacterial tuberculosis agents are often phenotypically variable; therefore, genetic criteria are currently considered preferable over phenotypic ones for isolate identification [5].

It is believed that deletions in RD7, RD8, and RD10 are evolutionarily conjugated; therefore, the whole MTBc can be divided into two groups according to the presence or absence of these regions. The first group contains these regions and includes only the following human pathogens: *M. tuberculosis*, *M. canettii*, and some *M. africanum* strains. The second group includes other MTBc strains that infect animals, although they sometimes cause the disease in humans. An exception in the second group is *M. africanum*, some strains of which have deleted RD7, RD8, and RD10 [5]. In this regard, RD7, RD8, and RD10 are convenient markers

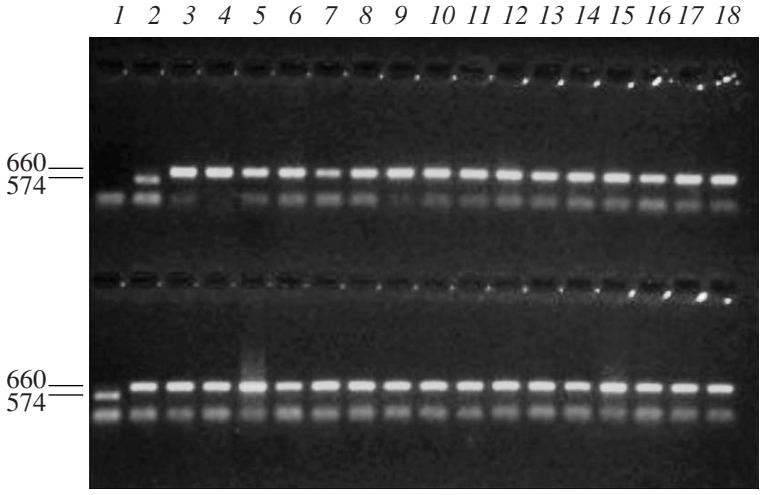

Fig. 3. Genotyping of deletions in DR7. Numerals on the left indicate locations and sizes of amplicons of *M. tuberculosis* (660 bp) and *M. bovis* (574 bp). Upper lanes: *1*, negative control; *2*, *M. bovis*; *3*, *M. tuberculosis*; *4–18*, isolates. Lower lanes: *1*, *M. bovis*; *2*, *M. tuberculosis*; *3–18*, isolates.

Table 2. Genotyping of strains with regard to gyrB

Strain designation	Isolation site	Lengths of *RsaI* restriction fragments of the gyrB replicon, bp	Strain designation	Isolation site	Lengths of *RsaI* restriction fragments of the gyrB replicon, bp
M. tuberculosis H37Rv	Control strain	360/560	14551	″	360/560
M. bovis	Control stain	360/480	14582	″	360/560
9	Kurmangazy District	360/560	16139	″	360/560
55	Ditto	360/560	302 K	Karaganda	360/560
74	″	360/560	A 137	Atyrau	360/560
106	″	360/560	A 253	Ditto	360/560
118	Atyrau	360/560	A 34	Kurmangazy District	360/560
120	Ditto	360/560	K 1109	Karaganda	360/560
128	″	360/560	K 1836	Ditto	360/560
184	Kurmangazy District	360/560	K 511014	″	360/560
14050	Almaty	360/560	K 1112	″	360/560
14491	Ditto	360/560			

that allow the differentiation of strains of human and animal origin except for the minor group of *M. africanum* strains, which can be differentiated by other tests. However, the described regularities were deduced from a study of strain collections that included few (sometimes single) strains representing various areas of the world, whereas little is still known about the prevalence of RD7, RD8, and RD10 deletions in natural regional MTBc populations. Spoligotyping of 455 isolates from Cameroon was performed by S. Niobe-

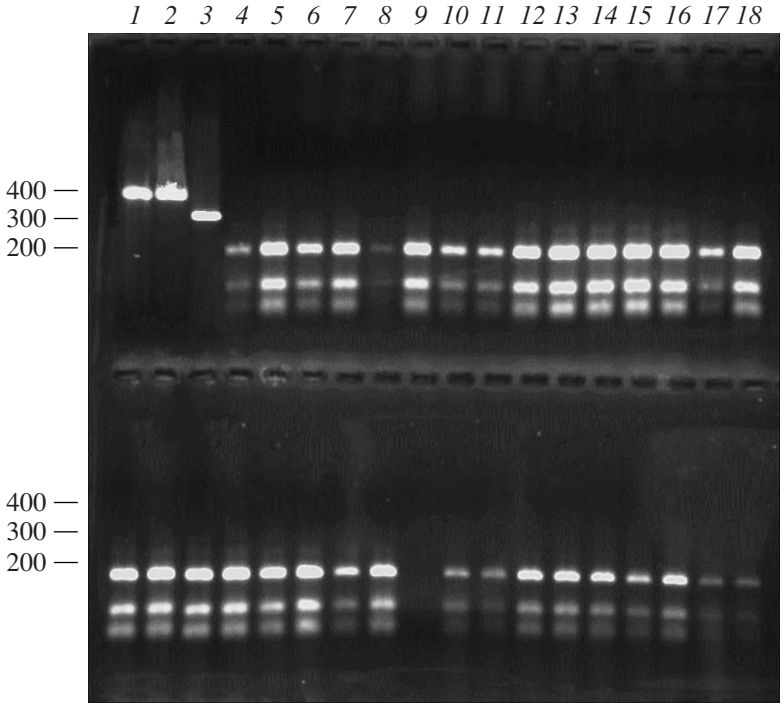

Fig. 4. Restriction analysis of hsp65 amplicons with *HhaI* restriction endonuclease. Lanes: *1*, undigested amplicon, 425 bp; *2*, undigested amplicon incubated in the restriction buffer without the enzyme; *3*, molecular weight marker, 300 bp; *4–18* in the upper row and *3–18* in the lower row, amplicons of isolates digested with *HhaI*; lane 4 in the upper row, *M. tuberculosis* H37Rv. Fragments 75 and 60 bp remained unresolved, yielding a fused band of elevated intensity. Numerals on the left indicate locations and sizes of corresponding fragments.

Eyangoh et al. [21], and RD typing including RD10 was also performed for 44 isolates [21]. 43 isolates (most of which were identified as *M. africanum*) had no RD10 deletions, and the only isolate in which this region was deleted was identified as *M. bovis.* A more complicated distribution of RD genotypes was observed in another study that was dedicated to the RD typing of four regional MTBc collections made in India (30 isolates), Libya (48 isolates), Angola (15 isolates), and Peru (38 isolates) [24]. In particular, RD10 was preserved in all India and Angola isolates and in 97% of Peru isolates, whereas RD8 was deleted from all isolates from India, while RD7 was deleted from 94% of Angola isolates and 84% of those from Peru.

All lung tuberculosis agent strains from Kazakhstan tested in our study had no deletions in RD7. Apparently, they all belonged to *M. tuberculosis.* We found no traces of other MTBc species in the population, including *M. bovis* (according to RD7 and gyrB typing). Our data also indicate the population stability of the RD7 region in MTBc strains isolated from humans. Further studies of regional MTBc populations would not only contribute to understanding evolutionary pathways of modern MTBc species, but also help the development of new diagnostic criteria and design of genotyping methods, which would take into account the species and regional variability of tuberculosis agents.

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