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Phenotypic and molecular characterizations of *Yersinia pestis* isolates from Kazakhstan and adjacent regions

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Recent interest in characterizing infectious agents associated with bioterrorism has resulted in the development of effective pathogen genotyping systems, but this information is rarely combined with phenotypic data. *Yersinia pestis*, the aetiological agent of plague, has been well defined genotypically on local and worldwide scales using multi-locus variable number tandem repeat analysis (MLVA), with emphasis on evolutionary patterns using old isolate collections from countries where *Y. pestis* has existed the longest. Worldwide MLVA studies are largely based on isolates that have been in long-term laboratory culture and storage, or on field material from parts of the world where *Y. pestis* has potentially circulated in nature for thousands of years. Diversity in these isolates suggests that they may no longer represent the wild-type organism phenotypically, including the possibility of altered pathogenicity. This study focused on the phenotypic and genotypic properties of 48 *Y. pestis* isolates collected from 10 plague foci in and bordering Kazakhstan. Phenotypic characterization was based on diagnostic tests typically performed in reference laboratories working with *Y. pestis*. MLVA was used to define the genotypic relationships between the central-Asian isolates and a group of North American isolates, and to examine Kazakh *Y. pestis* diversity according to predefined plague foci and on an intermediate geographical scale. Phenotypic properties revealed that a large portion of this collection lacks one or more plasmids necessary to complete the blocked flea/mammal transmission cycle, has lost Congo red binding capabilities (Pgm^-), or both. MLVA analysis classified isolates into previously identified biovars, and in some cases groups of isolates collected within the same plague focus formed a clade. Overall, MLVA did not distinguish unique phylogeographical groups of *Y. pestis* isolates as defined by plague foci and indicated higher genetic diversity among older biovars.

INTRODUCTION

Interest in micro-organisms with potential for use as bioweapons has steadily increased in the last 5 years (Anisimov *et al.*, 2004; Chain *et al.*, 2004; Gage & Kosoy, 2005; Girard *et al.*, 2004; Lowell *et al.*, 2005), providing insight into the epidemiology, ecology, evolution and molecular diversity of pathogenic bacteria. *Yersinia pestis*, the aetiological agent of plague, has caused millions of deaths worldwide and holds greater historical significance for human health than perhaps any other microbe (Link, 1955; Perry & Fetherston, 1997), but little is understood about its maintenance in nature, and whether phenotypic diversity is important. Recent collaborative efforts have provided a synthesis of old and new information describing the phenotypic and molecular diversity of worldwide *Y. pestis* isolate collections (Anisimov *et al.*, 2004) and additional molecular characterizations have summarized the

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Abbreviations: FSU, former Soviet Union; JK, jackknife; KSCQZD, Kazakh Scientific Centre for Quarantine and Zoonotic Diseases; MLVA, multi-locus variable number tandem repeat analysis; NA, North American; SNP, single-nucleotide polymorphism; VNTR, variable numbers of tandem repeat.

evolutionary and epidemiological relationships of *Y. pestis* (Girard *et al.*, 2004; Huang *et al.*, 2002; Lowell *et al.*, 2005; Motin *et al.*, 2002; Parkhill *et al.*, 2001). This knowledge, combined with ecological properties and life-history traits, may help answer questions about epizootic plague cycles, *Y. pestis* transmission in nature and the resulting structure of plague foci (Anisimov *et al.*, 2004; Gage & Kosoy, 2005).

Defining plague foci on the basis of phenotypic, biochemical and ecological properties of bacterial isolates has proven useful. Anisimov *et al.* (2004) summarized the characteristics of *Y. pestis* isolates from central Asia, one of the hypothesized regions from which *Y. pestis* emerged as a flea-borne pathogen of rodents and other mammals (Achtman *et al.*, 2004). This summary described plague focus designations and geographical extent for each of the 43 purportedly distinct plague foci found in the southern and southeastern regions of the former Soviet Union (FSU), based on epizootic activity, main rodent hosts and main flea vectors. Subgroups of *Y. pestis* were also characterized using biochemical properties, rodent host and geographical region of isolation.

While these phenotypic properties have historically represented *Y. pestis* subtypes and helped define the foci from which they originated, relatively new molecular techniques have provided bacteriologists with tools capable of revealing genetic diversity in *Y. pestis*. Molecular typing methods such as multi-locus variable number tandem repeat analysis (MLVA) have helped to define *Y. pestis* population structure on several spatial scales (Achtman *et al.*, 2004; Girard *et al.*, 2004; Lowell *et al.*, 2005). On local scales, where isolates were collected within a few kilometres of each other, MLVA genotyping has demonstrated matches between environmental isolates and associated human plague infections in the southwestern United States (Lowell *et al.*, 2005), and genotype relationships of *Y. pestis* isolates among 19 prairie dog colonies in Arizona (Girard *et al.*, 2004). At broader scales, MLVA has inferred relationships between biovars based on clustering algorithms, but has not typically been used to show hierarchical relationships between the biovars (Achtman *et al.*, 2004; Klevytska *et al.*, 2001). Recent single-nucleotide polymorphism (SNP) analyses, however, have indicated that biovar Antiqua is polyphyletic (Achtman *et al.*, 2004; Chain *et al.*, 2006), and that the atypical Antiqua isolate 'Angola' and the *Y. pestis* 'Pestoides' isolates are ancestral to all three biovars (Achtman *et al.*, 2004).

While phenotypic and molecular characterizations have provided valuable insight into worldwide *Y. pestis* collections and their relationships, studies examining collections of *Y. pestis* isolates from an intermediate scale across central Asia are lacking. Combining ecological, phenotypic and molecular information into single datasets could be especially useful for central-Asian isolates, as they potentially contain diversity not seen in parts of the world where *Y. pestis* is relatively new. The goals of this study were twofold. The first was to phenotypically characterize these isolates according to the tests commonly used by plague diagnostic and reference laboratories around the world (Chu, 2000). The second was to examine intermediate geographical scale MLVA on *Y. pestis* isolates that have potentially circulated in nature for thousands of years, generating MLVA relationships different from those in North American (NA) isolates.

METHODS

Isolate collection. The FSU was previously divided into five natural plague regions based on the rodent host species, the ecology of the area and the *Y. pestis* phenotypes generally found within these regions (Anisimov *et al.*, 2004). These natural plague regions were further subdivided by FSU scientists and designated 'autonomous plague foci', creating independent areas according to the predominant rodents and vectors, and also by administrative units (Anonymous, 1979). Sixteen autonomous plague foci reside within and adjacent to Kazakhstan. For this study, 48 isolates were selected from the *Y. pestis* collection at the M. Aikimbayev's Kazakh Scientific Centre for Quarantine and Zoonotic Diseases (KSCQZD), Almaty, Kazakhstan. These isolates were originally collected from 10 of the 16 autonomous plague foci within and adjacent to Kazakhstan (Table 1, Fig. 1). The isolates selected are representative of the mammal host, vector host and geographical diversity found in Kazakhstan. The isolate source, geographical origin and collection year are recorded in Table 1. Although some of the isolates were collected adjacent to Kazakh borders, all were kept in the Kazakh collection at KSCQZD and are here referred to as Kazakh isolates. Isolates were previously stored at KSCQZD at 4 °C on Hottinger's medium, until 1999, when they arrived at the Centers for Disease Control and Prevention (CDC) in Fort Collins, CO. There they were stored in Heart Infusion Broth (Beckton Dickinson) with 10 % (v/v) glycerol at −80 °C.

Phenotypic analyses. Kazakh isolate biovars were determined using nitrate reduction and glycerol fermentation tests (Devignat, 1951). Plasmid profiles were performed according to the method of Kado & Liu (1981), and the *caf-1*, *pla* and *repA1* genes were analysed by PCR according to Begier *et al.* (2006). Isolates lacking the 110 kb pMT1 plasmid were also confirmed for F1 negativity by Western blotting (Rahalison *et al.*, 2000). Amino acid auxotroph screening was performed by plating isolates on minimal medium containing or lacking the corresponding auxotroph amino acid, according to the method of Martinevsky (1973). Amino acids tested included leucine, tryptophan, cysteine, phenylalanine, threonine and arginine. Plates were incubated at 25 °C and 37 °C for 72 h and auxotrophs were defined by negative growth on the minimal medium lacking the corresponding amino acid, and positive growth on minimal medium containing the specifically required amino acid. As controls, five typical *Y. pestis* isolates were plated on the minimal medium and all isolates were plated on sheep blood agar. Bacteriophage lysis tests were also performed according to Cavanaugh & Quan (1953).

Haemin-binding capabilities were determined by plating each isolate on Congo red agar and incubating for 48 h at 25 °C (Burrows & Jackson, 1956; Surgalla & Beesley, 1969). The presence or absence of the *pgm* locus was verified using *irp2*-directed PCR. Each 50 µl PCR reaction contained 1 × PCR buffer with 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 U *Taq* polymerase (Promega), 0.5 ng *Y. pestis* DNA and 10 µM each of primers IRP2F (5′-TTTCCGGGGGCGTCTCAAC-3′) and IRP2R (5′-CTCGACCTTTTGCAGTTTGATGTG-3′). Primers were designed using Primer Select (DNASTAR v 7.0) and the *irp2* sequences of *Y. pestis* CO92 and KIM (GenBank accession nos NC_003143 and NC_004088, respectively). A negative control containing master mix only and a *Y. pestis* CO92 positive control

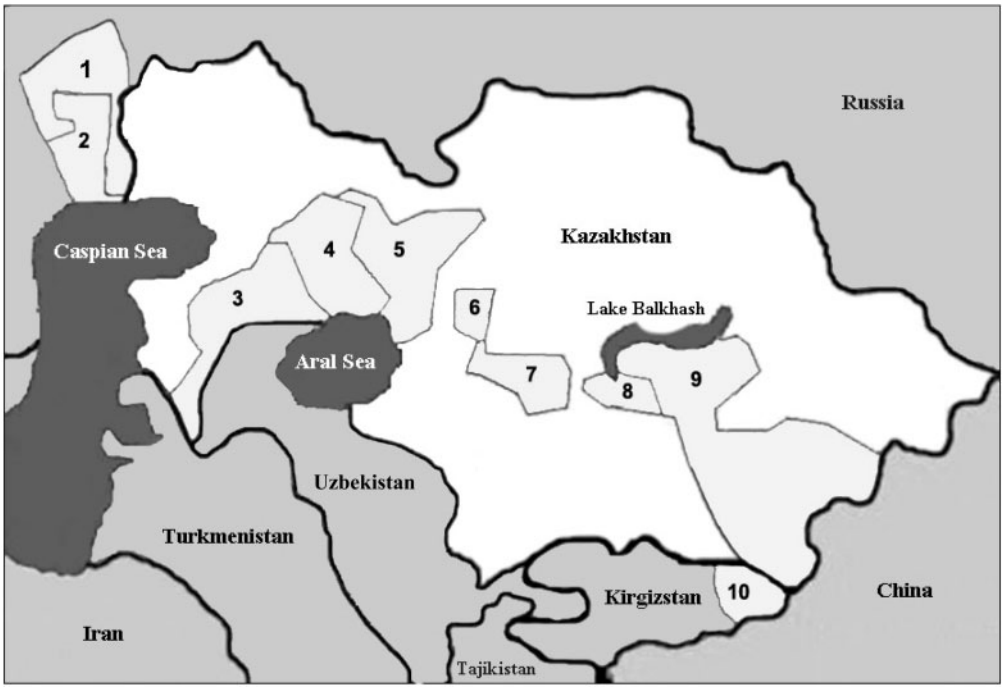

Fig. 1. Kazakh and adjacent autonomous plague foci from which the 48 Kazakh isolates were collected. 1, Volga-Ural steppe; 2, Volga-Ural sandy; 3, Ustyurt; 4, North Pre-Aral; 5, Pre-Aral Kara-Kum; 6, Betpak-Dala; 7, Muyun-Kum; 8, Tau-Kum; 9, Pre-Balkhash; 10, Sarydzhaz.

were included in the set of reactions. All reactions were amplified in a PTC-200 thermal cycler (MJ Research) with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. Products were 234 bp long and visualized by separating 5 µl reaction mixture on 2 % agarose gels containing 0.1 % ethidium bromide.

Sugar requirements and carbohydrate fermentation capabilities of all isolates were determined at KSCQZD according to the method of Aparin & Golubinskii (1989).

Phylogenetic analyses. MLVA was used to compare isolates from Kazakhstan and from southwestern USA (NA isolates) on three geographical scales. A geographically distant scale was represented by comparing Orientalis, Medievalis, and Antiqua isolates from Kazakhstan to Orientalis isolates from NA. An intermediate geographical scale was represented by comparing the Kazakh isolates from 10 autonomous plague foci, while the NA *Y. pestis* set represented a close geographical scale analysis with isolates from human cases in Arizona, New Mexico and Colorado (Four Corners region), compared to the animal or flea isolates obtained during the associated epidemiological investigations. These isolates were previously described by Lowell *et al.* (2005), but for this analysis an additional 25 variable number of tandem repeat (VNTR) markers were added to the dataset to complete the set described by Klevytska *et al.* (2001), plus marker M19 from the work of Girard *et al.* (2004), for a total of 42 markers.

DNA was extracted and VNTR marker genotypes were determined according to the method of Lowell *et al.* (2005). Each 20 µl PCR multiplex reaction for the additional 25 markers contained 1 × PCR buffer with 1.5 mM $MgCl_2$, 200 µM dNTPs and 0.5 U *Taq* polymerase (Promega), 1.0 µl DNA template (approx. 0.5 ng DNA) and one of the following multiplex phosphoramidite linkage dye-labelled primer sets: 0.1 µM primer M26 and 0.25 µM M36; 0.1 µM M15 and 0.25 µM M37; 0.1 µM M42 and 0.2 µM M49; 0.1 µM M51 and 0.2 µM M52; 0.1 µM M43 and 0.25 µM M54; 0.1 µM M66 and 0.2 µM M55; 0.2 µM M68 and 0.25 µM M65; 0.1 µM M69 and 0.2 µM M56; 0.2 µM M61 and 0.25 µM M70; 0.1 µM each of M73 and M74; 0.1 µM each of M72 and M76; 0.1 µM each of M75 and M79; and 0.1 µM M71. Primer sets were run in pairs, as more consistent amplifications were achieved with fewer primers per multiplex. Pairs were chosen based on the primer dye label and on the size of the PCR fragment. All 42 markers were also analysed for the Kazakh isolates according to Lowell *et al.* (2005) and the above primer combinations.

Of the 42 markers, three were variable and 34 were parsimony-informative. The phylogeny was inferred using equally weighted parsimony and 1000 tree-bisection-reconnection (TBR) searches with a maximum of 20 trees held per search. TBR branch swapping was then performed on all of the most parsimonious trees found with a maximum of 100 000 trees held, from which a strict consensus tree was calculated (Maddison, 1991). Jackknife (JK) support (Farris *et al.*, 1996) was inferred using 1000 replicates, each consisting of 10 TBR searches and a maximum of 20 trees held (Fig. 2). The atypical Antiqua Angola (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = genomeprj&cmd = Retrieve&dopt = Overview&list_uids = 16067) and *Y. pestis* strain 15-70 (Pestoides F) isolates (https://maple.lsd.ornl.gov/microbial/ypes_1570/) were chosen as outgroups (Farris *et al.*, 1996). Negative PCR results for Angola and Pestoides F were scored as missing data. All phylogenetic analyses were performed using PAUP 4.0b10 (Swofford, 2002).

Table 1. Phenotypic characteristics of the Kazakh isolates

Isolate ID	Origin*	Source‡	Date isolated (dd/mm/yyyy)	Passage no.#	Biovar§	Plasmid profile‖	Pgm¶	Biochemical properties**
North Pre-Caspian region†								
3785	Volga-Ural sandy	*Meriones* fleas	08/05/1971	13	M	110, 70, 9.5	White	T
3786	Volga-Ural sandy	*Meriones tamariscinus*/spleen	19/05/1998	6	M	110, 70, 9.5	White	T
3787	Volga-Ural sandy	*M. tamariscinus/Nosopsyllus laeviceps*	05/11/1998	6	M	110, 70, 9.5	Red	T
3788	Volga-Ural sandy	Human/blood	19/06/1997	7	M	110, 70, 45-50	Red	T
3823	Volga-Ural steppe	*Citellus pygmaeus/Nosopsyllus laeviceps*	25/04/1966	38	M	110, 70, 9.5	White	T, Try
3824	Volga-Ural steppe	*M. tamariscinus*/blood	21/04/1966	38	M	110, 70, 9.5	White	T
3825	Volga-Ural steppe	*M. tamariscinus/Haemaphysalis* sp. (tick)	25/04/1966	38	M	110, 70, 9.5	White	T
3826	Volga-Ural steppe	*M. tamariscinus*/fleas	21/06/1982	22	M	110, 70	White	T
3827	Volga-Ural steppe	*Citellus pygmaeus*	26/06/1997	7	M	110, 70, 9.5	White	T
Central-Asian Desert region†								
3789	Ustyurt	*Rhombomys opimus*/liver	10/05/1963	40	M	110, 70, 9.5	White	T, Leu
3790	Ustyurt	*M. meridianus*/blood	03/11/1964	40	O	110, 70, 9.5	White	T
3791	Ustyurt	*Spermophilopsis leptodactylus*/spleen	08/10/1965	39	M	110, 70, 9.5	White	T
3792	Ustyurt	*Mus musculus*/blood	21/11/1971	33	M	110, 70	White	T, Leu
3793	Ustyurt	*R. opimus*/lice	25/04/1957	41	M	110, 70	White	T, Leu
3794	Ustyurt	Camel/blood	06/08/1968	42	M	110, 70, 9.5	White	T, Leu
3795	Ustyurt	*R. opimus/Haemaphysalis* sp. (tick)	09/10/1973	35	M	110, 70, 9.5	White	T, Leu
3796	Ustyurt	*R. opimus/Echidnophaga oschanini*	24/10/1973	35	M	110, 70	White	Leu
3797	Pre-Balkhash	*R. opimus*/liver	31/05/1938	66	A	70	White	T
3798	Pre-Balkhash	*R. opimus*/blood	02/08/1949	55	M	110, 70, 9.5	White	T
3799	Pre-Balkhash	*R. opimus*/spleen	10/05/1950	54	M	110, 70, 9.5	White	T
3800	Pre-Balkhash	*R. opimus*/blood	08/05/1961	43	M	110, 70, 9.5	White	T
3801	Pre-Balkhash	*M. meridianus*/blood	07/09/1965	39	M	110, 70, 9.5	White	T
3802	Pre-Balkhash	*R. opimus*/fleas	01/06/1998	6	M	70, 9.5	White	T
3803	Pre-Balkhash	*R. opimus*/fleas	31/05/1993	11	M	9.5	Red	T, phage res.
3804	Pre-Balkhash	*R. opimus*/blood	15/07/1988	15	M	110, 70, 9.5	Red	T
3805	Pre-Balkhash	*R. opimus*/blood	17/06/1989	55	M	70, 9.5	White	T
3806	N. Pre-Aral	Human/blood	22/08/1955	59	M	110, 70	White	T
3807	N. Pre-Aral	Human/pharynx	03/07/1999	5	M	110, 70	White	T
3808	N. Pre-Aral	Human/bubo	14/08/1999	5	M	110, 70, 9.5	White	T
3809	N. Pre-Aral	Human/blood	07/08/1999	5	M	110, 70, 9.5	Red	T
3810	Muyun-Kum	*R. opimus* flea/*Coptopsylla lamellifer*	22/10/1962	44	M	110, 70	White	T
3811	Muyun-Kum	*R. opimus*	30/05/1969	37	M	110, 70, 9.5	White	T, Try
3812	Muyun-Kum	*R. opimus*/spleen	11/06/1972	40	M	110, 70, 9.5	Red	T, Leu
3813	Muyun-Kum	*R. opimus*	17/10/1998	7	M	110, 70, 9.5	Red	T
3814	Pre-Aral Kara-Kum	*R. opimus*/liver	08/05/1998	6	M	110, 70, 9.5	Red	T
3815	Pre-Aral Kara-Kum	Human/bubo	07/06/1999	5	M	110, 70, 9.5	Red	T
3816	Betpak-Dala	Unidentified fleas/burrow	21/10/1962	32	M	110, 70, 9.5	Red	T

Table 1. cont.

Isolate ID	Origin*	Source‡	Date isolated (dd/mm/yyyy)	Passage no.#	Biovar§	Plasmid profile‖	Pgm¶	Biochemical properties**
3817	Betpak-Dala	*Meriones libycus*	22/11/1983	21	M	110, 70, 9.5	Red	T
3818	Tau-Kum	Unidentified fleas/burrow	15/05/1966	38	M	110, 70, 9.5	White	T
3819	Tau-Kum	*R. opimus*/liver	09/10/1968	36	M	110, 70, 9.5	White	T, Try
3820	Tau-Kum	*R. opimus/Echidnophaga oschanini*	24/04/1971	33	M	110, 70, 9.5	Red	T
3821	Tau-Kum	*M. meridianus*/liver	18/04/1971	33	M	110, 70, 9.5	Red	T
3822	Tau-Kum	Unidentified fleas/burrow	29/05/1988	16	M	110, 70, 9.5	Red	T, Try
Tien Shan region†								
3828	Sarydzhaz	*Ixodes* ticks	24/08/1949	55	A	110, 70, 9.5	White	T, Leu
3829	Sarydzhaz	*Rhadinopsylla liventricosa*	12/05/1962	44	A	110, 70, 9.5	White	T
3830	Sarydzhaz	*Marmota baibacina*	28/06/1962	44	A	110, 70, 9.5	Red	T
3831	Sarydzhaz	*Ixodes* ticks	14/06/1966	40	O	70	White	T
3832	Sarydzhaz	*Marmota baibacina*	27/06/1971	33	M	110, 70	Red	T

*Origin is the autonomous plague focus contained within designated natural plague regions.

†Natural region containing autonomous plague foci.

‡Original biological source yielding *Y. pestis* isolate.

#Number of times isolate has been passed during storage.

§M, Medievalis; A, Antiqua; O, Orientalis.

‖Plasmid sizes: 110, pMT1; 70, pCD1; 9.5, pPCP1.

¶Pgm, pigmentation result on Congo red Agar: White, Congo red binding negative; Red, Congo red binding positive.

**T, typically ferments glucose, rhamnose, maltose and melibiose; Leu, leucine dependent; Try, tryptophan dependent; phage res., phage resistant.

RESULTS AND DISCUSSION

Phenotypic characteristics

Plasmid profiles revealed that many of the Kazakh isolates were missing one or more of the three unique *Y. pestis* plasmids. Two isolates lacked only pMT1, eight lacked only pPCP1, and two were missing both pPCP1 and pMT1 (Table 1). All isolate plasmid profiles corresponded to directed PCR for plasmid-borne genes, with the exception of isolate 3803. Although this isolate appeared to be missing pMT1 and pCD1, it was PCR positive for *caf-1* and *repA1*, and positive for F1 antigen by Western blotting, indicating chromosomal F1 integration (Protsenko *et al.*, 1991). It was possible that the pCD1 plasmid was atypical in size, a characteristic noted in some central-Asian isolates (Filippov *et al.*, 1990), and was not detected in the plasmid profile.

It has been established that the *Y. pestis* plasmids may spontaneously delete during laboratory storage (Perry & Fetherston, 1997), but recent studies have shown that isolates undergoing several passages under laboratory conditions were highly variable in their plasmid stability and that one subculture lost both the pPMT1 and pPCP1 plasmids after only four passages, while others lost none after 32 passages (Leal-Balbino *et al.*, 2004). Atypical isolates lacking plasmids have also been recovered in nature from around the world (Cavalcanti *et al.*, 2002), including FSU foci (Filippov *et al.*, 1990). Because plasmid profiles were not performed on our Kazakh isolates at the time of collection, it is not possible to determine if plasmid loss occurred naturally or during storage. These isolates may have circulated in nature for thousands of years, undergoing high passage during epizootic activity, and isolates lacking pPCP1 or pMT1 may have persisted in mammals if the route of transmission was oral or pulmonary (Hinnebusch *et al.*, 1998, 2000).

Thirty-two of the 48 Kazakh isolates (66 %) did not bind Congo red (Table 1) and were also negative for *irp2* PCR. Absence of the *pgm* locus including the yersiniabactin siderophore iron-acquisition system implies avirulence in these isolates (Hinnebusch *et al.*, 1996; Perry *et al.*, 2001). This result is most likely a laboratory artefact caused by spontaneous deletion because the isolates were passed many times during storage, or biased selection of pgm^- colonies during original bacterial isolation. This locus is known to spontaneously delete *en bloc* at a high rate (Perry & Fetherston, 1997) and mixed populations of pgm^+ and pgm^- isolates, with high frequency of pgm^- isolates, have been observed from the field (KSCQZD unpublished data; Cavalcanti *et al.*, 2002). If the majority of the bacterial colonies from the original post-epizootic isolations were pgm^-, selection of this phenotype for laboratory storage would be a likely scenario.

Nine leucine- and four tryptophan-dependent auxotrophic phenotypes were found in the isolate collection (Table 1). Seven of the nine leucine-dependent auxotrophs were from the Pre-Ustyurt autonomous focus and one was from the Muyun-Kum autonomous focus, both located in the central-Asian natural region, where leucine dependence is typically seen. The remaining leucine-dependent isolate (3828) was collected from the Sarydzhaz autonomous focus, located in the Tien Shan natural focus, an area that does not typically yield this phenotype. Tryptophan-dependent auxotrophs are not usually identified in Kazakh isolate subtypes or autonomous plague foci; however, considering the variety of amino acid auxotrophs noted in the subtypes (Martinevsky, 1973), finding these phenotypes in this collection was not a surprise. No additional amino acid auxotrophs were present.

Sugar fermentation characteristics for these 48 Kazakh isolates were typical for *Y. pestis* found in central-Asian plague foci. Isolate 3803 was phage resistant (Table 1).

Kazakh and NA MLVA comparison

NA (Orientalis) and Kazakh (Medievalis) isolates formed distinct clades. Kazakh isolates 3831 and 3790 fell in the NA clade because they are biovar Orientalis. The NA clade was supported by 87 % of JK replicates while the remaining Kazakh isolates formed a monophyletic group with 50 % JK support (Fig. 2). Markers M58, M59 and M52 were unique to biovar Orientalis.

Kazakh predefined foci versus Kazakh MLVA

The Kazakh phylogeny generally did not group isolates according to focus, with most isolates unresolved in a polytomy, although there were some exceptions (Fig. 2). Two isolates from Volga-Ural sandy (3786, 3787) clustered with 87 % JK support, two from Pre-Ustyurt (3789, 3794) with 57 % support, two from Pre-Balkhash (3800, 3801) with 68 % support, two from the Volga-Ural steppe (3823, 3825) with 67 % support, and several isolates from three different foci [3802, 3804, 3805, (3820, 3821), 3822, 3824, 3832] formed a clade with 76 % support. Within this clade, two isolates from the Tau-Kum focus (3820, 3821) were supported in 87 % of JK replicates. An additional group of isolates from the North Pre-Aral (3808) and Pre-Aral Kara-Kum (3815) were supported with 91 % JK (Fig. 2). These foci border one another, so it is possible that the isolates supported in this clade were collected in close geographical proximity.

Isolates that formed clades supported by at least 70 % JK differed by as many as four VNTR markers, and clades that were supported by between 50 and 70 % differed by as many as seven markers. Clades without support differed by at least eight markers.

This result is expected, given the local variation generated by relatively fast mutation rates in VNTR markers (Girard *et al.*, 2004) and by sampling on an intermediate geographical scale. Our sample set from Kazakhstan consisted of only a few isolates from each focus but the overall collection spanned most of the country. Isolates from Kazakh plague

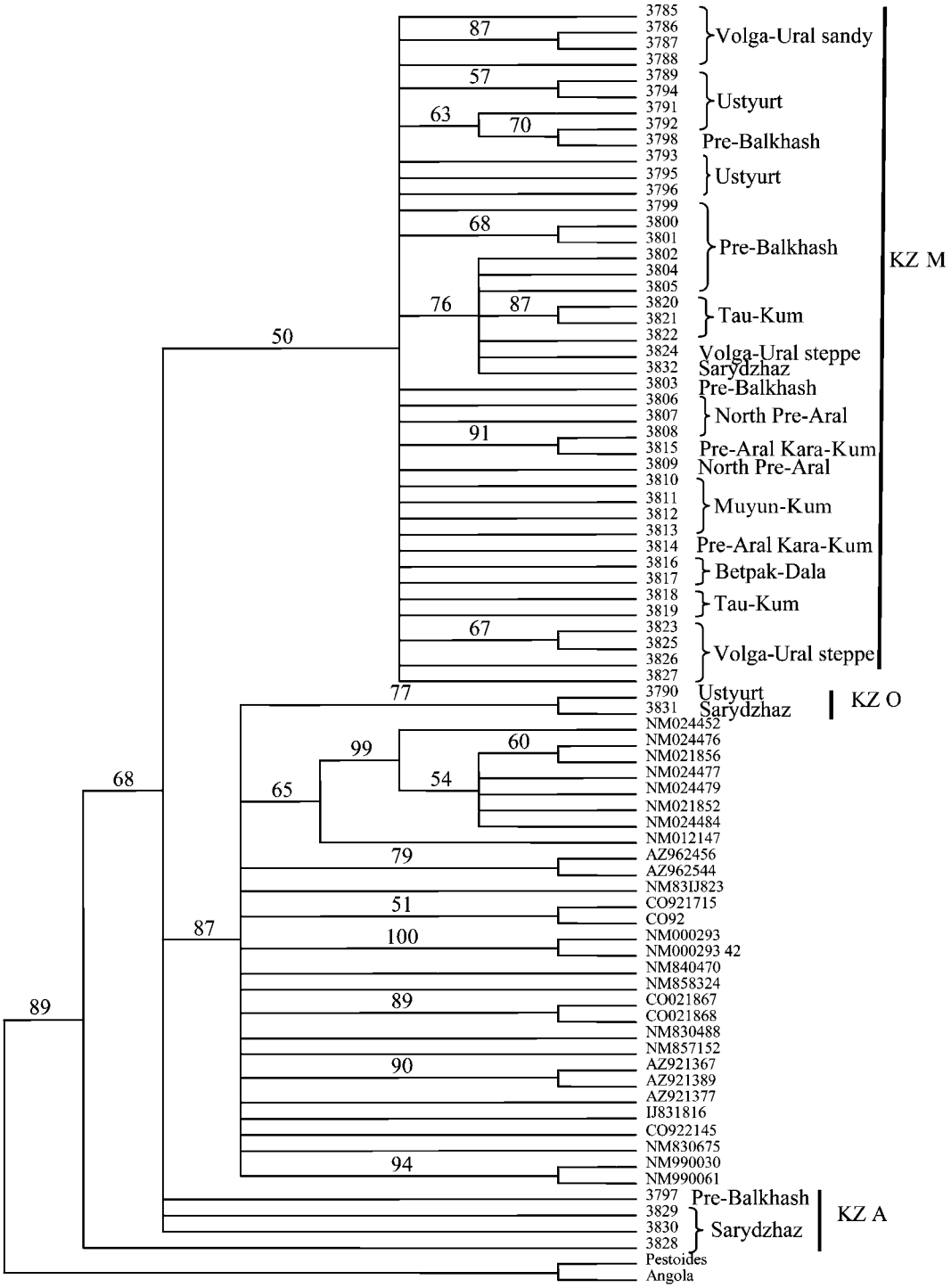

Fig. 2. Maximum-parsimony tree of Kazakh and NA isolates. Numbers above branches refer to jackknife support. Plague focus corresponding to each Kazakh isolate is also listed in Table 1. KZ M, Kazakh Medievalis isolates; KZ O, Kazakh Orientalis isolates; KZ A, Kazakh Antiqua isolates; NA O, NA Orientalis isolates. NA isolates are labelled with CDC accession numbers: NM, New Mexico; CO, Colorado; AZ, Arizona. Numbers following state abbreviation refer to the collection year and the CDC unique identifier.

foci that have potentially existed for thousands of years may have undergone adaptation to local hosts, vectors or micro-environments, creating a high level of genetic diversity throughout the larger geographical area. Even the relatively young NA isolates that clustered in geographical space (collected less than ~3 km apart) were strongly supported statistically (Girard *et al.*, 2004; Lowell *et al.*, 2005), providing evidence that individual *Y. pestis* clones causing plague epizootics tend to remain fairly isolated. While epizootics may eventually spread to adjacent rodent colonies after rapid animal die-offs like those observed within some rodent species, outbreaks typically remain isolated within a burrow system or rodent community for some time before the epizootic spreads to another location via dispersing juveniles or other mammals carrying infected fleas (Barnes, 1982; Girard *et al.*, 2004). This pattern of isolated epizootics with little dispersal would maintain local bacterial populations, and supports the hypothesis that *Y. pestis* populations are maintained in relatively small areas rather than on spatial scales defined by plague foci spanning several thousand square kilometres (Fig. 1). The low resolution of the Kazakh phylogeny using MLVA likely resulted from a combination of the geographical scale at which the Kazakh samples were collected, the relative age of Medievalis and Antiqua isolates, and the molecular marker mutation rates.

In contrast, MLVA distinguished between biovars and also provided information about their relative diversity. Biotypes Orientalis and Medievalis formed distinct clades (Fig. 2). Antiqua did not form a monophyletic clade, indicating that these isolates were more diverse in their MLVA profiles. Orientalis showed the least amount of marker variability with 87 % JK support for the NA clade, Medievalis showed only 50 % support, and Antiqua was not supported. This result showed signs of genetic diversity that reflect previous analyses that described Antiqua as ancestral to Orientalis and Medievalis (Achtman *et al.*, 1999; Devignat, 1951). Recent SNP analyses have demonstrated that one Antiqua isolate from Asia (Nepal 516) and several from Africa (Kenya) are polyphyletic and not ancestral to Asian Medievalis and worldwide Orientalis isolates, respectively (Achtman *et al.*, 2004; Chain *et al.*, 2006). However, Asian Antiqua isolates from Japan and the USSR are still shown ancestral to Asian Medievalis, and the atypical Antiqua isolate Angola is ancestral to all other biovars (Achtman *et al.*, 2004). When our MLVA tree was rooted with Angola and *Y. pestis* 15-70, the overall *Y. pestis* phylogeny was highly supported and one Kazakh Antiqua isolate (3828) formed a branch leading to the other *Y. pestis* biovars (Fig. 2).

These isolates provided insight into the phenotypic and molecular diversity that exists in Kazakh *Y. pestis*. If these isolates naturally lacked plasmids important for blocked flea transmission, this may suggest that direct mammal-to-mammal transmission or mechanical transmission by fleas may be important in epizootic cycles, but evidence for this is sparse. If plasmids were lost because of storage practices, perhaps researchers should consider other possible mutations that may occur *in vitro*. It was possible that laboratory storage also affected VNTR marker diversity in the faster-mutating markers (Girard *et al.*, 2004). Although MLVA did not group these isolates by focus, we are confident that additional collections of fresh isolates on local scales would yield similar results to those seen in the USA. While current molecular typing and whole-genome sequencing of *Y. pestis* have provided invaluable insight into the evolution and diversity of *Y. pestis*, much of this work has been performed on old isolates that have probably been stored in a similar manner to this set. Future studies should include phenotypic and genotypic analyses conducted on fresh field isolates and stored isolates that have undergone minimal passages to differentiate between naturally occurring diversity and laboratory artefacts.

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