VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 12, Number 3, 2012
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DOI: 10.1089/vbz.2011.0748

Phenotypical Characterization of Mongolian *Yersinia pestis* Strains

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Abstract

Although Mongolia is regarded as one of the possible places of plague radiation, only few data are available from Mongolian *Yersinia pestis* strains. In this study a total of 100 Mongolian *Y. pestis* strains isolated from wild mammals and their parasites between the years 1960 and 2007 were analyzed for their phenotype. All strains grew well on selective Cefsulodin-Irgasan-Novobiocin agar and were positive for the F1-antigen, the F1-gene (*caf1*), and the plasminogen activator gene (*pla*). Biochemical analyses using the API20E® system identified 93% of the strains correctly as *Y. pestis*. The BWY in-house system consisting of 38 biochemical reactions was used to differentiate among *Y. pestis* subspecies *pestis* biovars Antiqua and Medievalis and also between the subspecies *microtus* biovars Ulegeica and Caucasica. Antibiotic susceptibility testing according to Clinical and Laboratory Standards Institute-guidelines identified one strain as being multiresistant. This strain was isolated from a wildlife rodent with no anthropogenic influence and thus suggests naturally acquired resistance.

Key Words: *Yersinia*—Plague—Microbiology—Rodent-Borne—Vector-Borne.

Introduction

In human history, no other disease claimed more victims than plague. The causative agent of plague, *Yersinia pestis*, caused approximately 200 million deaths during the three major pandemics in the 6th, 14th, and 19th century (Duplaix 1988). Especially, the massive decimation of the European population by the Black Death in the 14th century had a dramatic impact on the cultural, social, and economic development in Europe (Ziegler 1969, Twigg 1984). At present, natural foci of *Y. pestis* can be found on all continents except Australia and Antarctica, covering 6%–7% of the dry land (Perry and Fetherston 1997). According to the WHO, 1000 to 5000 human cases and 100 to 200 resulting fatalities were detected annually in the last two decades. The estimated number of unreported cases is considered to be much higher (Stenseth et al. 2008). The generally increasing incidence of human plague caused the WHO to classify it as a reemerging disease and *Y. pestis* is classified as a category A biological agent (Anisimov et al. 2004, Gage and Kosoy 2005, Koirala 2006). Being a multi-host multi-vector zoonosis (Anisimov et al. 2004) involving more than 233 mammal species (Rall 1960, Khrustselevskij 1978) and 263 flea species (Goncharov 1983, Serzhan and Ageyev 2000) in addition to other hematophagous arthropods like ticks and lice (Pollitzer 1954, Girard 1955, Hoogstraal 1980), the plague transmission cycles show a high complexity (Stenseth et al. 2008). Phylogenetic analysis suggests Central Asia (Cui et al. 2008, Li et al. 2009, Morelli et al. 2010), and ecological studies indicate more specifically Transbajkalian and Mongolian steppes (Suncov and Suncova 2006, 2009) as the place of origin for *Y. pestis*. In this region the bacterium evolved from *Y. pseudotuberculosis* 15,000 to 20,000 years ago (Achtman 2004). So far, only few data are available for *Y. pestis* strains from Mongolia, one of the possible places from where the plague radiation may have occurred (Galdan et al. 2010). Most studies on *Y. pestis* are based on a highly limited amount of strains from the Americas and Africa reflecting a minimal genetic diversity. Studies on Central Asian strains which are genetically and phenotypically more diverse are crucial for the understanding of this highly pathogenic agent (Anisimov et al. 2004). The goal of this study was the phenotypic characterization of Mongolian *Y. pestis* strains using available methods thus validating these methods for Central Asian strains.

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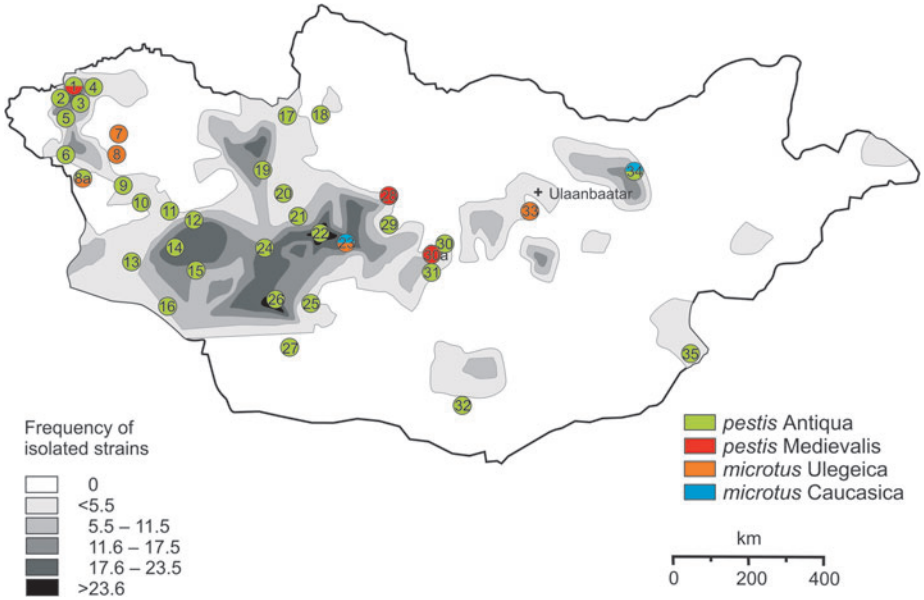

FIG. 1. Physical map of Mongolia showing isolation sites and distribution of different *Yersinia pestis* biovars. The occurrence of natural plague foci investigated in this study matches with the occurrence of human plague cases that is shown in frequency per year. Numbers in circles represent spatial clusters as given in Supplementary Table S1. Color images available online at www.liebertonline.com/vbz

Materials and Methods

Strains

This study comprised of 100 *Y. pestis* strains collected in Mongolia by the National Center of Infectious Diseases with Natural Foci (NCIDNF) within the years 1960 to 2007 (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/vbz). The spatial coverage included 13 Aimags (provinces) and 38 Soums (sub-provinces). The 57 collection sites were merged in 37 spatial clusters (Fig. 1). The strains were isolated not only from wild rodents, mainly *Marmota sibirica* known to be the primary host for *Y. pestis* and their hosting parasites such as the flea *Oropsylla silantiewi*, but also from animals and vectors barely described to transmit *Y. pestis* so far such as ticks and lice (Table 1, Supplementary Table S1).

DNA-preparation

Thermolysates were prepared from *Y. pestis* strains grown at 28°C on Columbia blood agar for 24 h. Briefly, a 10 μL loop full bacteria was resuspended in AE-buffer (Qiagen, Hilden, Germany) and heated at 95°C for 30 min. Cell debris was removed by centrifugation at 10,000 *g* for 10 min. For subsequent polymerase chain reaction (PCR) 2 μL of the supernatant was used.

Strain confirmation

All strains were investigated for the presence of the *Y. pestis*-specific virulence plasmids pPCP1 and pMT1 using real-time PCR assays targeting the *pla*- and *caf1*-genes as previously described (Tomaso et al. 2008, Riehm et al. 2011).

Lateral flow rapid test

An immunochromatographic test targeting the F1-capsule antigen of *Y. pestis*, the Crystal F1 Rapid test (Span Diagnostics,

Table 1. Isolation Frequency and Host Distribution of *Yersinia pestis* Isolates Investigated in This Study

	Order	*Species*	*Number of* Yersinia pestis *isolates*
Hosts	Rodents	*Lasiopodomys brandti*	9
		Marmota sibirica	70
		Meriones meridianus	1
		Spermophillus undulatus	8
	Lagomorphs	*Ochotona daurica*	1
		Ochotona pallasi	5
	Carnivores	*Mustela eversmannii*	1
Vectors	Fleas	*Amphalius runatus runatus*	1
		Amphipsylla primaris mitis	3
		Citellophilus sungaris sungaris	6
		Ctenophyllus hirticrus	1
		Frontopsylla frontalis baikal	1
		Frontopsylla hetera	1
		Neopsylla mana	2
		Oropsylla silantiewi	62
		Paradoxopsyllus integer	1
		Paramonopsyllus scalonae	4
		Rhadinopsylla li li	1
	Ticks	*Ixodes crenulatus*	2
	Lice	*Linognathoides palaearctus*	3

See also Supplementary Table S1. The majority of strains (70) were isolated from *Marmota sibirica*, or their hosting flea species *Oropsylla silantiewi* (62).

Table 2. List of *Yersinia pestis* Strains Misidentified by the API20E®-Test

Strain	BWY (ref: Li et al. 2009[a])	NCIDNF (in house)	API20E® determination	Relative probability	Absolute probability
MNG 0003	*pestis* Medievalis	*Y. pestis*	*Pasteurella pneumophila/haemolytica*	57.6	0.86
MNG 1681	*pestis* Antiqua	*Y. pestis*	*Klebsiella pneumoniae ozaenae*	50.1	0.70
MNG 1683	*pestis* Antiqua	*Y. pestis*	*Pantoea* sp.	49.0	0.51
MNG 3096	*microtus* Ulegeica	*Y. pestis*	*Escherichia vulneris*	41.3	0.80
MNG 0111	*pestis* Antiqua	*Y. pestis*	*Pasteurella pneumophila/haemolytica*	56.9	0.90
MNG 2181	*pestis* Medievalis	*Y. pestis*	*Pasteurella pneumophila/haemolytica*	56.9	0.90
MNG 3020	no identification	*Y. pestis pestis*	n.d.		

Strains were correctly identified by the in-house system (BWY-system, Merlin, Germany) and the NCIDNF.
[a]Result was obtained using data on the fermentation of rhamnose, melibiose, arabinose, glycerol, nitrate reduction, and urease activity.
NCIDNF, National Center of Infectious Diseases with Natural Foci.

Surat, India) (Chanteau et al. 2003, Tomaso et al. 2007) was carried out for identification. The test was performed according to the manufacturer's instructions. The strains were grown at 37°C on Columbia blood agar for 24h because F1 production is induced best at 37°C. Briefly, a 1 μL loop of bacterial cell mass was manually suspended in 200 μL of 0.9% NaCl-solution. Then the test stripe was dipped and read out after 15 to 20 min.

Growth on selective media

All strains were plated on Cefsulodin-Irgasan-Novobiocin (CIN; BD, Heidelberg, Germany) agar at 28°C for 24 h and on Columbia blood agar (BD) at 28°C for 24 h to compare the growth of *Y. pestis* on selective and nonselective media.

Biochemical investigations

All strains were tested by the API® 20E bacteria identification System (bioMérieux, Nürtingen, Germany) according to the manufacturer's instruction. The results were analyzed by API® Lab 3.3.3 (bioMérieux).

In addition to the API 20E test, strains were also analyzed using a biochemical *in-house* assay (BWY-system, Merlin, Germany) consisting of 38 biochemical reactions as described earlier (Neubauer et al. 2000). Results were documented manually. For subspecies and biovar identification, previously published data were used on the bacterial fermentation of rhamnose, melibiose, arabinose, glycerol, nitrate reduction, and urease activity (Li et al. 2009).

Antibiotic susceptibility testing

A selection of 50 strains was tested for its minimal inhibition concentration using the following E-test (bioMérieux, Solna, Sweden) agar diffusion stripes: Chloramphenicol, ciprofloxacin, streptomycin, tetracycline, trimetroprim/sulfomethoxacole, gentamicin, and doxycycline on Mueller Hinton II agar at 37°C for 24 h as recommended by the Clinical and Laboratory Standards Institute (CLSI 2006) guidelines. Results were determined using the references of CLSI.

Results

All strains grew well on nonselective Columbia blood agar and selective CIN medium and could be confirmed as *Y. pestis*, by specific *pla*- and *caf*-1-gene PCR. All strains were also positive for F1-capsule antigen production.

Of the 100 strains tested 93 (Table 2) were successfully identified as *Y. pestis* by the API20E® system but could not be further differentiated to the biovar level.

In contrast the BWY system was able to differentiate between the *Y. pestis* subspecies *pestis* biovars Antiqua and Medievalis and also between the *Y. pestis* subspecies *microtus* Ulegeica and Caucasica (Table 3).

Table 3. Number of *Yersinia pestis* Strains Identified as Subsp. *Microtus* Biovars Ulegeica and Caucasica and Subsp. *pestis* Biovars Antiqua and Medievalis and Their Distribution into the 37 Spatial Clusters

Subspecies/Biovar	Number of isolated strains	Fermentation of				Nitrate reduction	Urease activity	Spatial cluster (Fig. 1)
		Rhamnose	Melibiose	Arabinose	Glycerol			
microtus Ulegeica	7	+	+	+	+	—	—	7, 8, 8a, 23, 33
microtus Caucasica[a]	2	+	+	+	+	+	—	23, 34
pestis Antiqua	86	—	—	+	+	+	—	1–6, 8a—22, 24–27, 29, 30–32, 34, 35
pestis Medievalis	3	—	—	+	+	—	—	1, 28, 30a

[a]Biochemical identification doubtful; molecular investigations by SNP and MLVA (Li 2009, Morelli 2010) suggest *Y. pestis* subspecies *microtus* bv Xilingolensis (MNG 3129) and *Y. pestis* subspecies *pestis* bv Antiqua (MNG 3025). Subspecies *microtus* biovar Altaica could not be identified. The identification was performed as previously published by Li (2009), using the data for the fermentation of rhamnose, melibiose, arabinose, glycerol, nitrate reduction, and urease activity only.

Table 4. Antibiotic Susceptibility Profiles of the Mongolian *Yersinia pestis* Strain MNG 3122 and the Previously Published Madagascar-Strains 16/96, and 17/95 (Galimand Et al. 2006)

	Gentamicin	*Streptomycin*	*Tetracyclin*	*Doxycyclin*	*Ciprofloxacin*	*Trimethoprim-Sulfamethoxacol*	*Chloramphenicol*
CLSI guideline[a] for *Y. pestis*	S	S	S	S	S	S	S
Madagascar 16/95	n.d.	R	S	n.d.	n.d.	S	S
Madagascar 17/95	n.d.	R	R	n.d.	S	S (Trimethoprim)	R
Mongolian *Y. pestis* strain MNG 3122	R	I	R	R	S	R	R

[a]Clinical and Laboratory Standards Institute Performance (2006).
R, resistant; S, sensitive; I, intermediate; n.d., not determined.

Most of the analyzed plague-strains (86%) were identified as *pestis* Antiqua, followed by *microtus* Ulegeica (7%), *pestis* Medievalis (3%), and *microtus* Caucasica (2%). Two percent could not be identified via the available reactions. These results match the subspecies determination previously carried out by the NCIDNF to a high degree, although the subspecies *microtus* Altaica could not be confirmed (Table 4). For example, we identified three strains as *microtus* Ulegeica and one strain as *microtus* Caucasica. According to the NCIDNF all four were classified as *microtus* Altaica. (Supplementary Table S1)

Regarding antibiotic susceptibility testing, none of the analyzed strains showed a resistance to the seven applied antibiotics with the exception of one strain. This strain (MNG 3122) was resistant to the following analyzed antibiotics (chloramphenicol, tetracycline, trimetroprim/sulfomethoxacole, gentamicin, and doxycycline). An intermediate reaction was detected to streptomycin. For this strain ciprofloxacin remains the only sensitive antibiotic that could be used in therapy (Table 4).

Discussion

The Center for Disease Control and Prevention in the United States considers *Y. pestis* as one of the most important agents that could be used in a bioterroristic scenario. Consequently *Y. pestis* is placed in the highest risk group (category A) because of its easy dissemination, high mortality rate and psychological impact thus posing a threat to the national and international security. Therefore every endeavor has been made in order to assure preparedness for a plague-incident. Adequate therapy with antibiotics plays a crucial role. First-line antibiotics for plague treatment are aminoglycosides and/or doxycyclin. Ciprofloxacin and tetracyclines are recommended in a mass casualty setting (Inglesby et al. 2000).

According to Morelli et al. (2010), Li et al. (2009), and Cui et al. (2008), Central Asia and hence Mongolia is considered as the most probable location from where the radiation of *Y. pestis* occurred. Data acquired in this area provide essential information concerning the phylogeny diversity of *Y. pestis*. However, so far only as few as 4 Mongolian strains were analyzed in detail (Cui et al. 2008, Li et al. 2009).

In this study a comprehensive collection of 100 plague-strains isolated from wildlife animals, their dens and parasites but not from humans were investigated in order to get an insight into primal conditions of the natural plague foci in Mongolia. The study covers the whole spectrum of biovars, including the nonhuman-pathogenic ones, like the different biovars of the subspecies *microtus*. The resulting data enable prospective comparison of rural and urban conditions thus clarifying anthropogenic effects on the epidemiologic situation in Mongolia.

Most of the commercially available test systems (API20E, Vitec) for biochemical identification of *Y. pestis* had been evaluated with an extremely limited number of *Y. pestis* strains from Africa and the Americas (Wilmoth et al. 1996, Russell et al. 1997). Hence some authors speculated that these tests have only a low sensitivity (Anisimov et al. 2004) because they do not cover more distantly related strains. However, our results demonstrate that the API20E system correctly identified 93% as *Y. pestis*, including the distantly related subspecies *microtus*. Therefore the API20E test can be used as a rapid screening test for *Y. pestis* at the species level for both human and animal material.

The BWY-system was able to differentiate *Y. pestis* even at the subspecies/biovar level. Only few discrepancies occurred in the subspecies determination previously carried out by the NCIDNF. Whereas classical biotyping used by the NCIDNF is very resource intensive, demanding both, qualified personnel and funds, an automated read-out software is available for the BWY-system that can easily be established in routine laboratory diagnosis though it has to be adapted to the new biochemical profiles in Central Asia. In addition it can be complemented by manual analysis, if necessary. Therefore the BWY-system is a practical tool for the identification of *Y. pestis* at the biovar level in any laboratory.

The only aberration to the data acquired by the NCIDNF was the lack of identification of subspecies *microtus* biovar Altaica by the BWY-system. This biovar differs by only one reaction, the nonfermentation of arabinose, making it very susceptible to minimal defects in the test-layout, and misinterpretation. The identification of strains 3025 and 3129 as *Y. pestis microtus* biovar Caucasica by the BWY-system is also doubtful because biovar Caucasica is regarded as being devoid of the pPst plasmid and therefore should be negative for the *pla* gene (Anisimov et al. 2004). Furthermore by applying a molecular typing approach using multiple locus variable number tanden repeat analysis (MLVA) and single nucleotide polymorphism (SNP) analysis (Li et al. 2009, Morelli et al. 2010), strains 3025 and 3129 were identified as *Y. pestis pestis* biovar Antiqua and *Y. pestis microtus* biovar Xilingolensis, respectively (data not shown). We therefore conclude that the BWY-system can not correctly differentiate among the various biovars of the subspecies *microtus*. The discrepancy between the biochemical and molecular identification of strain

3025 (*Y. pestis microtus* bv Caucasica vs. *Y. pestis pestis* bv Antiqua) can not be explained at the present time and needs further investigation.

According to recent literature (Arsen'eva et al. 2010), the reliability of biochemical and morphological methods for the identification of *Y. pestis* has been doubted. Especially atypical *Y. pestis* strains that occur in natural plague-foci in Central Asia might not be correctly identified using existing biochemical assays. However, in this study in which we included various atypical strains, we did not encounter any significant problems using biochemical methods for correct identification of *Y. pestis* at both the species and the biovar level.

In this study we have identified one strain (MNG 3122) that is resistant to almost all antibiotics commonly used in prophylaxis and therapy of plague (Table 2, Supplementary Fig. S1). The resistance profile of this strain reduces the usable antibiotics to one single drug, Ciprofloxacin. The identification of a multi-resistant *Y. pestis* strain in Mongolia is the first of its kind. Up to date only two other multi-resistant plague-strains, isolated from patients on Madagascar in 1995 are known (Galimand et al. 2006). The antibiotic susceptibility profiles of these two strains, however, differ from the strain of this study (Table 2). The multiresistance of the two strains from Madagascar could be attributed to the presence of an additional plasmid, derived from other Gram negative bacteria (Guiyoule 2001, Hinnebusch et al. 2002, Galimand et al. 2006). Whether the multiple resistance of stain MNG 3122 is also based on the presence of an additional plasmid has to be further investigated.

This study has elucidated the phenotypical heterogeneity of *Y. pestis* strains in Mongolia. It could also be demonstrated that despite this heterogeneity, classical biochemical identification systems, that is, the API20E test are useful for the identification of *Y. pestis*. Furthermore, biochemical assays such as the BWY in-house test can be used for typing *Y. pestis* strains from this very old plague focus even below the species level. Previously established real-time PCR assays are reliable for the identification of *Y. pestis* including biovars of the subspecies microtus. The isolation of a multi-resistant strain in addition to the known multi-resistant strains from Madagascar is alarming and underlines the importance of cultivation of the bacterium and susceptibility testing.

Acknowledgment

The authors gratefully acknowledge the excellent assistance of Baatar Tserenkhuu, Robert Schneider, Philipp Vette, and Gabriele Echle. For the crucial support with material and knowledge the authors thank the NCIDNF and the great people of Mongolia. This project was supported by a grant from the DAAD (German Academic Exchange Program).

Disclosure Statement

No competing financial interests exist.

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