



ORIGINAL ARTICLE

# Susceptibilities of *Yersinia pestis* to Twelve Antimicrobial Agents in China

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## Abstract

**Objective:** Streptomycin is the preferred choice for therapy of plague in China and other countries. However, *Yersinia pestis* exhibiting plasmid-mediated antimicrobial agent-resistant traits had been reported in Madagascar. In this study, we evaluated the susceptibility of traditional or newer antimicrobial agents used for treatment and/or prophylaxis of plague.

**Methods:** Following Clinical and Laboratory Standards Institute (CLSI) recommendations, the susceptibility of 12 antimicrobial agents was evaluated by the agar microdilution method in 1,012 strains of *Y. pestis* isolated from 1943 to 2017 in 12 natural plague foci in China.

**Results:** One clinical *Y. pestis* isolate (S19960127) was found to be highly resistant to streptomycin, while the strain was still sensitive to other 11 antibiotics, that is, ciprofloxacin, ofloxacin, kanamycin, chloramphenicol, ampicillin, ceftriaxone, cefuroxime, trimethoprim-sulfamethoxazole, tetracycline, spectinomycin and moxifloxacin. The remaining 1,011 *Y. pestis* strains in this study demonstrated susceptibility to the above-mentioned 12 antimicrobial agents.

**Conclusion:** Antimicrobial sensitivity surveillance of *Y. pestis* isolates, including dynamic monitoring of streptomycin resistance during various clinical plague treatments, should be carried out routinely.

**Keywords:** *Yersinia pestis*; Antimicrobial Susceptibility; Streptomycin; China

## Edited by:

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Received: May 18 2022

Revised: June 11 2022

Accepted: June 20 2022

Published Online: July 26 2022

## BACKGROUND

Plague, an acute infectious disease caused by *Yersinia pestis* (*Y. pestis*), is mainly found in wild rodents, and parasitic fleas are considered transmitting vectors [1]. Various clinical types of plague exist, primarily bubonic, pneumonic and septicemic plague. On the basis of their biochemical properties, four biovars of *Y. pestis* are recognized worldwide: *Y. pestis* orientalis, antiqua, mediaevalis and pestoides (microtus) [2,3].

To date, the plague has not been eradicated worldwide. Traditional antimicrobial agents used for treatment and/or prophylaxis in patients with plague include

aminoglycosides (streptomycin and gentamicin), chloramphenicol, tetracyclines (doxycycline and tetracycline) and trimethoprim sulfamethoxazole [4]. Newer antimicrobial agents, such as levaquin and moxifloxacin, have been used in the USA [5], and ciprofloxacin, ceftriaxone and ofloxacin have also been used to cure pneumonic or bubonic plague in China [6,7] and in other countries [8].

Several studies have evaluated the susceptibility of traditional or newer antimicrobial agents in some countries [9]. However, a limited number of *Y. pestis* strains have been assessed for antibiotic susceptibility in China. Plague and Cholera

are classified as two Class A notifiable infectious diseases in Chinese Information System for infectious Disease Control and Prevention, and at least 12 plague foci covering more than 1.4 million square kilometers still exist [10]. Different biovars of *Y. pestis* strains inhabit the natural plague foci across China. In this study, we investigated the susceptibility of *Y. pestis* strains in China to 12 antimicrobial agents.

## METHODS

### Strains in this study

A total of 1012 *Y. pestis* strains isolated from 1943 to 2017 in 12 natural plague foci in China were included in this study (Table 1), among which 536 strains had been used in previous research [11]. The sources of these strains were as follows: 570 from rodent animals (marmots, rats, mice, chipmunks, etc.); 268 from humans; 157 from fleas; 14 from artiodactyla (Tibetan sheep and goats); and 3 from other animals. These selected strains represented different biovars and genotypes in China. All strains were collected in the National *Y. pestis* Preservation Center in QIEDC. All experimental activities with high bio-safety risk, such as the culture of *Y. pestis* and antibiotic susceptibility testing, were performed in the biosafety level-3 laboratory of QIEDC.

### Antibiotic resistance evaluation

Susceptibility testing for *Y. pestis* and corresponding CLSI quality control reference methods were performed [13]. Minimal inhibitory concentrations (MICs) were determined with the agar dilution method, according to National Committee for Clinical Laboratory Standards guidelines [14] and previous studies [15,16]. The MICs of antibiotics

for *Y. pestis* strains were determined on 96-well plates containing cation-adjusted Mueller-Hinton agar (CAMHA) with multipoint inoculators with an inoculum of  $10^4$  CFU per spot. The cultures were incubated for 48 hours at 37° [14]. Quality control strains (*Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC 25922) were tested with each batch of *Y. pestis* isolates to validate the accuracy of the procedure. Corresponding procedures and interpretation followed the CLSI guidelines for rapidly growing gram-negative bacilli and previous studies [14]. The population ranges of antibiotic susceptibility in various originations of *Y. pestis* strains were evaluated on the basis of MIC50 and MIC90 values.

A total of 12 antimicrobial agents (Table 2) were obtained from the Chinese formal pharmacy. The stock solutions (5 mg/ml) were prepared in the appropriate solvents, on the basis of current CLSI recommendations [13]. Antibiotics were serially diluted 2-fold in CAMHA. The concentration range was 64–0.004 µg/ml for tetracycline, ciprofloxacin, chloramphenicol, ofloxacin, kanamycin, ceftriaxone, ampicillin, spectinomycin, cefuroxime, trimethoprim-sulfamethoxazole and moxifloxacin in the plates. For streptomycin, the double dilution upper limit was as high as 4096 mg/ml.

Antibiotic resistance genes of 12 antibiotics were assessed by PCR in the 1012 *Y. pestis* strains. The oligonucleotide primers targeted for identifying resistance to the 12 antimicrobial agents are listed in Supplemental Table 1. The oligonucleotide primers for the *strA* and *strB* genes (plasmid-associated streptomycin resistance genes) were used in PCR to identify the conjugative plasmids pIP1202 and pIP1203 [17,18]. PCR was performed with Taq DNA polymerase (Takara) with the following cycling protocol: Denaturation

**TABLE 1** | *Y. pestis* strains used for antibiotic resistance evaluation in this study.

Natural plague foci in China	Number of strains	Sources				Biovar
		Humans	Hosts	Vectors	Others	
A: <i>Marmota caudate</i> focus on Pamirs plateau	3	0	3	0	0	Antiqua
B: <i>Marmota baibacina-Spermophilus undulates</i> focus in Tianshan mountains	64	9	37	18	0	Antiqua
C: <i>Marmota himalayana</i> focus on Qinghai-Gansu-Tibet grassland	545	166	329	49	1	Antiqua
E: <i>Apodemus chevrieri-Eothenomys miletus</i> focus in highlands of northwestern Yunnan province	14	1	8	5	0	Antiqua
F: <i>Rattus flavipectus</i> focus in Yunnan-Guangdong-Fujian provinces	46	13	26	7	0	Orientalis
H: <i>Spermophilus dauricus</i> focus on Song-Liao plain	148	69	63	14	2	Mediaevalis; Antiqua
I: <i>Meriones unguiculatus</i> focus on Inner Mongolian plateau	126	5	74	47	0	Mediaevalis
J: <i>Spermophilus dauricus alaschanicus</i> focus on loess plateau in Gansu and Ningxia provinces	25	5	10	10	0	Mediaevalis
K: <i>Marmota himalayana</i> focus in Kunlun mountain	2	0	2	0	0	Mediaevalis
L: <i>Microtus brandti</i> focus on Xilin Gol grassland	5	0	7	1	0	Microtus
M: <i>Microtus fuscus</i> focus on Qinghai-Tibet plateau	11	0	5	6	0	Microtus
O: <i>Rhombomys opimus</i> focus in Junggar basin of Xinjiang	22	0	22	0	0	Mediaevalis
Total	1012	268	584	157	3	

The strains used in this study. The nomenclature of these plague foci is as previously reported [12].

**TABLE 2** | Antimicrobial MIC distributions for *Y. pestis* isolates in this study.

Antibiotics	MIC ( $\mu\text{g/ml}$ )													MIC50	Susceptible*	Resistant*
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	4096			
Ofloxacin			50	470	411	81								0.12		
Ciprofloxacin	23	60	405	496	28									0.03	$\leq 0.25$	
Kanamycin								146	752	114				2		
Streptomycin									228	783		1	4	4	$\leq 4$	$\geq 16$
Ceftriaxone	308	593	111											0.015		
Ampicillin					224	594	193	1						0.25		
Chloramphenicol									467	540	5			2	$\leq 8$	$\geq 32$
Spectinomycin										95	700	215		8		
Cefuroxime				47	348	469	148							0.25		
Tetracycline									22	460	530			4	$\leq 4$	$\geq 16$
Trimethoprim-sulfamethoxazole			396	591	21	2								0.06	$\leq 2$	$\geq 4$
Moxifloxacin				13	743	256								0.06	$\leq 2$	$\geq 4$

\*CLSI MIC breakpoints for the broth microdilution method.

for 5 min at 95°C; 30 amplification cycles at 95°C for 50 s, Tm °C for 50 s and 72°C for 1 min; and a final extension at 72°C for 5 min. For the genes *gyrA*, *gyrB*, *parC* and *rrs*, the sequences of PCR products were used to identify the corresponding antibiotic resistance mutations.

## RESULTS

### Susceptibility of *Y. pestis* to 12 antimicrobial agents

With the exception of a clinical *Y. pestis* isolate (S19960127), which exhibited resistance to streptomycin (MIC of 4,096 mg/L, the upper limit of the dilution range) [11], all other *Y. pestis* strains in this study were susceptible *in vitro* to 12 antibiotic agents (Table 2). These antibiotic agents included those recommended for plague therapy (streptomycin, ciprofloxacin, chloramphenicol and kanamycin) and prophylaxis (sulfonamides and tetracycline) [4], as well as new antibiotics (ceftriaxone, cefuroxime, spectinomycin and moxifloxacin) and others (ampicillin).

### MICs of strains isolated from different sources and plague foci in different years

Generally, we observed no differences in MIC50 or MIC90 for the isolates regardless of their source (humans, rodents and fleas) and natural plague foci (Table 2). In addition, only very limited changes in antibiotic susceptibility for *Y. pestis* isolated in different years was detected (Table 3). But the susceptibility to these antibiotics varied still within sensitive ranges. This observation also reflected plague natural ecological characteristics.

### PCR screening for genes associated with resistance to 12 antibiotics

The PCR screening results for genes associated with resistance to 12 antibiotics were all negative. For the *gyrA*, *gyrB*

and *parC* gene targeted for ciprofloxacin, ofloxacin, moxifloxacin, as well as the gene *rrs* targeted for kanamycin, no corresponding mutations associated with ciprofloxacin, ofloxacin, moxifloxacin, or kanamycin resistance were found in the sequences of PCR products.

*Y. pestis* S19960127 was found to be resistant to streptomycin, which has an MIC of 4096  $\mu\text{g/ml}$  [11]. The other 1011 *Y. pestis* strains in this study remained susceptible to streptomycin *in vitro*. The results of PCR with primers for the streptomycin resistance genes *strA* and *strB* were negative for *Y. pestis* S19960127 and the other 1011 *Y. pestis* strains. A novel mechanism of streptomycin resistance in *Y. pestis* was subsequently identified as mutation of the *rpsL* gene [11].

## DISCUSSION

*Y. pestis* isolates were uniformly susceptible to the dominant antibiotics against Gram-negative bacteria described in previous studies [9,19]. In China, with the exception of one clinical *Y. pestis* strain found to be resistant to streptomycin [11], a large collection of *Y. pestis* strains in this study were generally found to be susceptible to antimicrobial agents, including antibiotics traditionally recommended for the treatment of *Y. pestis* infections. These results were similar to those of previous investigation in other counties or areas. For instance, no resistance to eight antimicrobial compounds was identified in 392 *Y. pestis* isolates from 17 countries [9] in North America, South America, Asia and Africa. In 1996, the susceptibility of 100 South African *Y. pestis* strains to new antimicrobial agents was determined *in vitro*; among oral antibiotics, two quinolones (ofloxacin and levofloxacin) showed extremely high antibacterial activity against *Y. pestis*, whereas cefotaxime was the most effective non-parenteral antibiotic [16]. In Madagascar, although multiple-drug or

**TABLE 3** | MIC50 and MIC90 values of *Y. pestis* strains from various sources, natural foci and isolated years.

Antibiotics	Source		Natural plague foci#						Isolated years										
	MIC90	MIC50	Hosts and fleas (744)	Focus C (545)	Focus I (126)	Focus H (148)	Focus B (64)	1943–1970 (288)	1971–1990 (236)	1990–2017 (488)	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90		
Ofloxacin	0.03	0.25	0.12	0.25	0.03	0.25	0.03	0.25	0.03	0.25	0.03	0.25	0.03	0.25	0.03	0.12	0.03	0.12	
Ciprofloxacin	0.06	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Trimethoprim-sulfamethoxazole	0.06	0.06	0.06	0.06	0.03	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.12
Kanamycin	2	4	2	4	2	4	2	4	2	4	2	4	2	4	2	4	2	4	4
Streptomycin	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Ceftriaxone	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.03
Ampicillin	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.5
Chloramphenicol	2	4	2	4	2	4	2	4	2	4	2	4	2	4	2	4	4	4	4
Spectinomycin	8	16	8	8	8	8	8	16	8	16	8	16	8	16	8	8	8	8	16
Cefuroxime	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	1
Tetracycline	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	4	8	8
Moxifloxacin	0.12	0.25	0.12	0.25	0.12	0.25	0.12	0.25	0.12	0.25	0.12	0.25	0.12	0.25	0.12	0.25	0.12	0.25	0.25

\*MIC50 and MIC90, MICs for 50 and 90% of strains tested against strains in CAMHA. # Only natural plague foci with more than 40 collected *Y. pestis* strains are listed. #: The bold values show the varied values of MIC50 or MIC90 in different year segments.

single-drug resistance to streptomycin conferred by plasmids had been documented in 1995 [17,18], testing of a total of 713 *Y. pestis* strains in Madagascar revealed no resistance of *Y. pestis* isolates in humans, rats or fleas in Madagascar after 1995 [20]. Generally, the plague host animals, particularly for those in sylvatic plague foci, were relatively remote from human living environments, and the *Y. pestis* strains inhabiting these hosts animals or fleas had relatively less exposure to antibiotics or bacteria with antibiotic resistance. Thus, the *Y. pestis* strains found in nature were generally susceptible to antimicrobial agents.

However, a *Y. pestis* strain presenting multidrug-resistance to eight antimicrobial agents (streptomycin, chloramphenicol, tetracycline, sulfonamides, ampicillin, kanamycin, spectinomycin and minocycline) has been found in Madagascar [17]. A multidrug-resistant *Y. pestis* strain was isolated from a marmot in Mongolia in 2000, but the genetic characteristics and transferability of the strain were not reported [21]. In 2018, plasmid-mediated doxycycline resistance in a *Y. pestis* strain was reported in Madagascar (isolated from a rat in 1998) [21]. Still another case of resistance to streptomycin (25 mg/ml) has been observed in one *Y. pestis* strain isolated from Vietnamese rats [22].

Evaluation of the variations in MIC50 and MIC90 values of *Y. pestis* strains would provide valuable reference information regarding antibiotic resistance changes in different backgrounds. In this study, we generally observed no differences in MIC50 or MIC90 values among Chinese *Y. pestis* isolates, regardless of their source (humans, rodents and fleas), natural plague focus (Table 3) or year isolated. Plague generally occurs among wild animals, and human plague occasionally originates from major reservoirs or domestic animals, such as *Marmota himalayana*, *Meriones unguiculatus*, *Spermophilus dauricus*, *Marmota baibacina*, *Spermophilus undulatus*, *Ovis aries* [23], cats and dogs, as well as some rodents (*Mus musculus*, *Allactaga sibirica*, *Microtus oeconomus*, *Cricetulus migratorius* and *Ochotona daurica*) or wild animals (lynxes, badgers and foxes).

In 1995, an isolate named 17/95, isolated from a patient with plague in Madagascar, exhibited multidrug-resistant traits to eight antimicrobial agents [17]. In addition, another isolate named 16/95, obtained in 1995 in a patient with plague in Madagascar [18], exhibited only streptomycin resistance. The MIC of streptomycin for strain 17/95 (*orientalis*) was above 2,048 mg/L [17], whereas that for strain 16/95 (*orientalis*) was 1,024 mg/L [18]. The resistance to streptomycin was conferred by a conjugative plasmid (pIP1202 in *Y. pestis* strain 17/95; pIP1203 in *Y. pestis* 16/95 strain), and the high-level resistance was due to the presence of streptomycin phosphotransferase activity [17].

Streptomycin is the preferred therapy for plague in China. A clinical *Y. pestis* isolate has exhibited resistance to streptomycin [11]. The strain (biovar *antiqua*) was isolated from a pneumonic plague outbreak in 1996 in China, in the *Marmota himalayana* Qinghai–Tibet Plateau plague focus. This was the first report of *Y. pestis* streptomycin resistance in China, and a novel mechanism of streptomycin resistance

in *Y. pestis* was identified: mutation at 128 bp in the *rpsL* gene [11]. Subsequently, the same streptomycin resistance mechanism was reported in Madagascar. A *Y. pestis* strain with corresponding resistance was identified and found to be circulating in a pneumonic plague outbreak in the Faratsiho district in Madagascar in 2013. Another plague case with *rpsL* gene mutation was found in a region of Madagascar in 1987 [24].

Antimicrobial therapy is the simplest component of the complex therapy required for patients with plague. Besides the streptomycin is considered as one of the most effective antibiotics for the treatment of plague [4]. In China, combination antibiotic therapy had been used in the treatment of human plague patients; for example, using streptomycin in combination with ciprofloxacin, norfloxacin or ceftriaxone sodium can shorten the course of disease and decrease the dosage of streptomycin [6,7]. In 2004, pneumonic plague occurred in Qinghai, and treatment with ceftriaxone sodium and ofloxacin combined with streptomycin and significantly shortened the course of disease [6]. In 2009, a plague outbreak occurred in Xinghai County, Hainan Prefecture, Qinghai province [7]. Treatment with streptomycin and ciprofloxacin in combination cured all patients within 18 days, a course markedly shorter than the general duration of the disease.

In China, various plague foci containing different reservoirs or vectors exist, representing the most widely distributed, most complicated and most active natural plague foci in the world. In recent years, fewer than ten human plague cases per year have been reported in China, all of which have been limited to remote areas with low populations, such as in Inner Mongolia, Gansu, Qinghai Province. In this study, the susceptibility of *Y. pestis* isolates to 12 antimicrobial agents provided an antibiotic resistance baseline in China. In addition, the emergence of streptomycin resistance in *Y. pestis* in China is a critical public health problem. This resistance would result in treatment failure; thus, antibiotic monitoring should be performed in real time during treatment of those plague cases. Furthermore, *Y. pestis* strains resistant to streptomycin may be involved in transmission of pneumonic plague outbreaks, as occurred in Tibet, China in 1996 [11]. A similar phenomenon and mechanism were reported in a pneumonic plague outbreak in 2013 in Madagascar, and these streptomycin resistant strains were believed to have spontaneously arisen in *Y. pestis* in the absence of antibiotic selective pressure [24]. Thus, antimicrobial sensitivity surveillance of *Y. pestis* isolates in animal plague epidemics or in human plague cases should be performed routinely. In addition, the causes of streptomycin resistance due to *rpsL* mutation in *Y. pestis* must be further studied experimentally.

#### ACKNOWLEDGEMENTS

We thank Mu Guo of the Yunnan Institute for Endemic Disease Control and Prevention for amending the manuscript. We are grateful to colleagues in CDCs or corresponding organizations for plague control and prevention in areas including Xinjiang, Yunnan, Jilin, Gansu, Ningxia, Shanxi, Hebei, Guizhou, Tibet and the Inner Mongolia Autonomous Region (province).



This work was supported by the national Key Research and Development Plan (No.2021YFC1200204), the National Health Commission Project for Key Laboratory of Plague Prevention and Control (2019PT310004), the Science and Technology Plan Project in Qinghai Province (2019-ZJ-7074) and the Key Scientific and Technology Project of Inner Mongolia Autonomous Region (2021ZD0006).

## CONFLICTS OF INTEREST

The authors have no competing interests.

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