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Susceptibilities of *Yersinia pestis* to twelve antimicrobial agents in China

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Abstract

Following Clinical and Laboratory Standards Institute (CLSI) recommendations, the susceptibilities of twelve traditional or new recommended antimicrobial agents were evaluated by the agar microdilution method in 1012 strains of *Yersinia pestis* isolated from 1943 to 2017 in 12 natural plague foci in China. A clinical *Y.pestis* isolate (S19960127) isolated from a pneumonic plague outbreak in 1996 in Tibet Autonomous Region exhibited high-level resistance to streptomycin (the MIC was 4,096 mg/L). The strain S19960127 was still sensitive to other eleven antibiotics, i.e. ciprofloxacin, ofloxacin, kanamycin, chloramphenicol, ampicillin, ceftriaxone, cefuroxime, trimethoprim-sulfamethoxazole, tetracycline, spectinomycin and moxifloxacin. The remaining 1011 *Y.pestis* strains in this study demonstrated susceptibility to above mentioned twelve antimicrobial agents. Streptomycin is used as the first-line antibiotic against plague in many countries include China, thus antimicrobial sensitivity surveillance of *Y.pestis* isolates, including dynamically monitoring streptomycin resistant during various clinical plague treatment should be carried out routinely.

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

Background

Plague is an acute infectious disease caused by *Yersinia pestis* (*Y.pestis*), and it is mainly involved in wild rodents while parasitic fleas are considered as transmitting vectors [1]. There are various clinical types of plague, and the major three types are bubonic, pneumonic, and septicemic plague. Base on different biochemical properties, four biovars of *Y.pestis* have been recognized worldwide, i.e. *Y.pestis* orientalis, antiqua, mediaevalis, and pestoides (Microtus) [2, 3].

Up to now, the plague still is not eradicated worldwide. Traditional antimicrobials used for treatment and/or prophylaxis of plague patients include aminoglycosides (streptomycin and gentamicin), chloramphenicol, tetracyclines (doxycycline and tetracycline), and trimethoprim sulfamethoxazole [4] et.al. More newer antimicrobial agents, such as levaquin and moxifloxacin have been used in USA [5], and ciprofloxacin, ceftriaxone, ofloxacin were also used to cure pneumonic or bubonic plague in China [6, 7] and in other countries [8].

A few studies were performed to evaluate the susceptibility of traditional or newer antimicrobial agents in some countries [9]. However, a limited number of *Y.pestis* strains were involved in such antibiotics susceptibility evaluation in China. The plague is classed as A infectious disease in China, and at least 12 plague foci covering more than 1.4

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million square kilometers still exist [10], and there are different biovars *Y.pestis* strains which inhabit in different plague natural foci in China. In this study, we investigated susceptibilities to twelve antimicrobial agents in plurality of *Y.pestis* strains in China.

Methods

Strains in this study

A total of 1012 *Y.pestis* isolated from 1943 to 2017 in 12 natural plague foci in China were included in this study (Table1), in which 536 *Y.pestis* strains were once used in previous research work [11] were included in the collection of 1012 of *Y.pestis* in this study. The sources of these strains as follows: 570 rodent animals (Marmot, Rat, Mice, Chipmunks, etc.); 268 humans origination; 157 fleas; 14 artiodactyla (Tibetan sheep, goat); and 3 strain from other animals. These selected strains represent different biovars, genotypes in China. All the strains were collected in 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.

Table 1. *Y.pestis* used for antibiotic resistance evaluation in this study

Natural plague foci in China	Number of strains	Sources				Biovar.
		Humans	Hosts	Vectors	Others	
A: *Marmota caudate* focus on Pamirs plateau	3	0	3	0	0	Antiqua
B:*Marmota baibacina-Spermophilus undulates* focus in Tianshan mountains	64	9	37	18	0	Antiqua
C:*Marmota himalayana* focus on	545	166	329	49	1	Antiqua

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Qinghai-Gansu-Tibet grassland						
E:*Apodemus chevrieri-Eothenomys miletus* focus in highlands of northwestern Yunnan province	14	1	8	5	0	Antiqua
F:*Rattus flavipectus* focus in Yunnan-Guangdong-Fujian provinces	46	13	26	7	0	Orientalis
H: *Spermophilus dauricus* focus on Song-Liao plain	148	69	63	14	2	Medievalis; Antiqua
I:*Meriones unguiculatus* focus on Inner Mongolian plateau	126	5	74	47	0	Medievalis
J: *Spermophilus dauricus alaschanicus* focus on loess plateau in Gansu and Ningxia provinces	25	5	10	10	0	Medievalis
K:*Marmota himalayana* focus in Kunlun mountain	2	0	2	0	0	Medievalis
L: *Microtus brandti* focus on Xilin Gol grassland	5	0	7	1	0	Microtus
M:*Microtus fuscus* focus on Qinghai-Tibet plateau	11	0	5	6	0	Microtus
O: *Rhombomys opimus* focus in Junggar basin of Xinjiang	22	0	22	0	0	Medievalis
Total	1012	268	584	157	3	

The strains used in this study. The nomenclature of these plague foci are according the previous report [12].

Antibiotic Resistance Evaluation

Susceptibility testing for *Y.pestis* and corresponding CLSI quality control referenced methods [13]. Minimal inhibitory concentrations (MICs) were determined by the agar dilution method following National Committee for Clinical Laboratory Standards guidelines [14] and previous literatures [15, 16]. The MICs of antibiotics for *Y.pestis* strains were determined on 96-well plates of Cation-adjusted Mueller-Hinton agar by a multipoint inoculators with an inoculum of 10^4 CFU per spot. The cultures were incubated for 48 hours at 37°C[14]. Quality control strains (*Pseudomonas aeruginosa* ATCC27853and *Escherichia coli* ATCC 25922) were tested with each batch of *Y.pestis* isolates to validate the

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accuracy of the procedure. Corresponding procedure and interpretation follow the CLSI guidelines for rapidly growing gram-negative rods and previously literatures [14]. The population ranges of antibiotic susceptibilities in various originations of *Y.pestis* strains were evaluated through MIC50 and MIC90 values.

Twelve antimicrobial agents (Table 2) were obtained from the Chinese formal pharmacy. The stock solutions (5 mg/ml) were prepared in the appropriate solvents, based on the current CLSI recommendations [13]. Antibiotics were serially diluted 2-fold in camion-adjusted Mueller-Hinton Agar (CAMHA). The concentration range was 64 to 0.004 μg/ml for tetracycline, ciprofloxacin, chloramphenicol, ofloxacin, kanamycin, ceftriaxone, ampicillin, spectinomycin, cefuroxime, trimethoprim-sulfamethoxazole and moxifloxacin in the plates. For streptomycin, double dilution upper limit ranged up to 4096 mg/ml.

Antibiotic resistance genes of twelve antibiotics was scanned by PCR method in 1012 *Y.pestis* strains in this study. The oligonucleotide primers targeted for identify twelve antimicrobial agents resistance listed in Supplemental Table 1. In which, the oligonucleotide primers for the *strA* and *strB* genes (Plasmid–associated streptomycin resistance genes) were scanned by PCR to identify the conjugative plasmids pIP1202 and pIP1203 [17, 18]. PCR was performed using Taq DNA polymerase (Takara) with the following cycling protocol: Denaturing 5 min at 95°C; followed by 30 amplification cycles at 95°C for 50 s, Tm °C for 50 s, and 72°C for 1 min; the final extension at 72°C

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for 5 min. For the gene *gyr*A, *gyr*B, *parC* and the gene *rrs*, the sequence of PCR products were used to identify the mutation of corresponding antibiotics resistance mutations.

Results

Susceptibilities of *Y.pestis* to twelve antimicrobial agents

With the exception for a clinical *Y.pestis* isolate (S19960127) exhibiting resistance to streptomycin (the MIC was 4,096 mg/L, the upper limit of the dilution range)[11], all other *Y.pestis* strains in this study remained susceptible *in vitro* to 12 antibiotics agents (Table2). These antibiotics agent included those recommended for plague therapy (streptomycin, ciprofloxacin, chloramphenicol , kanamycin) and prophylaxis (sulfonamides and tetracycline)[4], as well as new antibiotics used (ceftriaxone, cefuroxime, spectinomycin, moxifloxacin) and others (ampicillin).

MICs of strains isolated from different sources, years and plague foci

Generally, there were no differences on MIC50 or MIC90 for the isolates regardless of the source of the isolates (human, rodents and fleas) and different natural plague foci (Table 2). In addition, only very limited changes on antibiotic susceptibilities for *Y.pestis* with different isolated years was detected (Table 3), and these changes were still various in the susceptible range for these antibiotics. Such observation also reflected plague natural ecological characteristics.

PCR screening results for antibiotic resistance-associated genes of twelve antibiotics

The PCR screening results for antibiotic resistance-associated genes of twelve antibiotics in this study were all negative. For the *gyr*A, *gyrB* and *parC* gene targeted for

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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 and the MICs of streptomycin were 4096 μg/ml[11], and other 1011 *Y.pestis* strains in this study remained susceptible to streptomycin *in vitro*. Using the PCR primers targeted to streptomycin resistance gene *strA* and *strB,* the results are negative for *Y.pestis* S19960127 and other 1011 *Y.pestis* strains. A novel mechanism of streptomycin resistance in *Y.pestis* was subsequently identified as the mutation in the *rpsL* gene[11].

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

Antibiotics	MIC(μg/ml)												
	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							
Ciprofloxacin	23	60	405	496	28								
Kanamycin								146	752	114			
Streptomycin									228	783			1
Ceftriaxone	308	593	111										
Ampicillin					224	594	193	1					
Chloramphenicol									467	540	5		
Spectinomycin										95	700	215	
Cefuroxime					47	348	469	148					
Tetracycline									22	460	530		
Trimethoprim-Sulfamethoxazole			396	591	21	2							
Moxifloxacin				13	743	256							

*CLSI MIC breakpoints for the broth microdilution method

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Table 3. MIC50 and MIC90 values of *Y.pestis* strains in various sources, natural foci and isolated

Antibiotics	**Source**				Natural plague foci#								
	Humans (268)		Hosts and fleas(744)		Focus C (545)		Focus I (126)		Focus H (148)		Focus B (64)		1943-1 (288
	MIC 50*	MIC 90	MIC 50	MIC 90	MIC50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50
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
Ciprofloxacin	0.06	0.06	0.03	0.06	0.06	0.06	0.03	0.06	0.03	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
Kanamycin	2	4	2	4	2	4	2	4	2	4	2	4	2
Streptomycin	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
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
Chloramphenicol	2	4	2	4	2	4	2	4	2	4	2	4	**2**
Spectinomycin	8	16	8	8	8	8	8	16	8	16	8	16	8
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**
Tetracycline	4	8	4	8	4	8	8	8	8	8	4	8	4
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

*MIC50 and MIC90, MICs for 50 and 90% of strains tested against strains in Cation-adjusted Mueller-Hinton ag

Note, here only listed the Natural plague foci that collecting number of *Y.pestis* above 40.

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Discussion

Y.pestis isolates were uniformly susceptible to the dominant antibiotics against Gram-negative bacteria in previous literatures [9, 19]. In China, with the exception that one clinical *Y.pestis* was found resistant to streptomycin[11], a large collection of *Y.pestis* strains in this study were generally found susceptible to antimicrobial agents, these antibiotics include traditionally recommended for the treatment of *Y.pestis* infections. Such results were similar to previous investigation in other counties or areas. For instance, no resistance to eight antimicrobials 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* to new antimicrobial agents was determined *in vitro*, Among oral antibiotics, two quinolones (ofloxacin and levofloxacin) showed extremely high antibacterial activity against *Y.pestis*, while cefotaxime was the most effective non-parenteral antibiotic [16]. In Madagascar, although resistance to multiple-drug or single resistance to streptomycin by plasmids had been documented in 1995 [17, 18], by scanning a total of 713 *Y.pestis* in Madagascar, no resistance of *Y.pestis* isolates was observed in humans, rats, or fleas in Madagascar after 1995 [20]. Generally, the plague host animals, especially for those in Sylvatic Plague Foci, were comparatively more far away from the human living environment, while the *Y.pestis* strains inhibited these plague hosts had relatively less exposure to antibiotics or bacteria with antibiotics resistance, thus the *Y.pestis* strains in natural were generally susceptible to antimicrobial agents.

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However, *Y.pestis* strain presenting multidrug-resistant traits to eight antimicrobial agents (streptomycin, chloramphenicol, tetracycline, sulfonamides, ampicillin, kanamycin, spectinomycin, and minocycline) was 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, a plasmid-mediated doxycycline resistance in *Y.pestis* strain was reported in Madagascar (isolated from a rat in 1998) [21]. Still another, resistance to streptomycin (25 mg/ml) was observed in one *Y.pestis* strain isolated from Vietnamese rats [22].

Evaluating the variation of MIC50 and MIC90 values of *Y.pestis* strains could provide reference information about antibiotic resistance changes in different backgrounds. In this study, generally, there were no differences on MIC50s or MIC90 among Chinese *Y.pestis* isolates regardless of the source of these isolates (human, rodents and fleas) or various natural plague foci (Table 3), as well as isolated years. Plague is generally occur among wild animals, and human plague occasionally originates from major reservoirs or domestic animals, such as *Marmota himalayana, Meriones unguiculatu, Spermophilus dauricus, Marmota baibacina, Spermophilus undulates, 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).

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In 1995, an isolate named 17/95 was isolated from a Plague patients in Madagascar exhibited multidrug-resistant traits to eight antimicrobial agents [17]. In addition, another isolate named 16/95 obtained in 1995 in Madagascar Plague patients [18] exhibited only streptomycin resistance. The MICs of streptomycin for Strain 17/95 (orientalis) were above 2,048 mg/L [17], while the MICs of streptomycin for 16/95 (orientalis) were 1,024 mg/L [18]. The resistance to streptomycin was conferred by 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 a streptomycin phosphotransferase activity [17].

Streptomycin is the preferred choice for the therapy of plague in China. A clinical *Y.pestis* isolate exhibited resistance to streptomycin [11]. The strain (biovar antiqua) was isolated from a pneumonic plague outbreak in 1996 in China, belonging to the *Marmota himalayana* Qinghai–Tibet Plateau plague focus. This was the first report that *Y.pestis* streptomycin resistance was present in China, and a novel mechanism of streptomycin resistance in *Y.pestis* was identified, i.e. Mutation at 128bp in the *rpsL* gene [11]. Subsequently, same streptomycin resistance mechanism was reported in Madagascar. Corresponding resistance *Y.pestis* strain was identified and circulated in one pneumonic plague outbreak in the Faratsiho district in Madagascar in 2013. Another plague case with *rpsL* gene mutation was found in in a region of Madagascar in 1987 [24].

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

In China, there were various plague foci where different reservoirs or vectors inhibited, representing the most widely distributed, most complicated, and most active natural plague foci in world. In recent years, the human plague cases have been down to less than 10 cases per year in China, and all these cases were limited in remote places with low population such as in Inner Mongolia, Gansu, Qinghai Province. In this study, the susceptibilities of *Y.pestis* isolates to twelve antimicrobial agents provided a corresponding antibiotic resistance baseline in China. In addition, the emergence of streptomycin resistance in *Y.pestis* in China represents a critical public health problem . In one side, the emergence of resistance to streptomycin in *Y.pestis* would render the plague case treatment failure, thus corresponding antibiotic monitoring should be

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real-timely carried out during those plague cases treatment. On the other side, the *Y.pestis* strain resistant to streptomycin could be involved in transmission in pneumonic plague outbreak. Such situation occurred in the pneumonic outbreak in Tibet, China in 1996[11]. Lately, same phenomenon and mechanism was reported in one pneumonic plague outbreak in 2013 in Madagascar [24]. and the investigation in Madagascar considered these streptomycin resistant strains may spontaneously arise 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 case should be performed routinely. And, the causes of *rpsL* mutation causing streptomycin resistance in *Y.pestis* need be further studied by experimental measures.

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CONFLICTS OF INTEREST

The authors have no competing interests.

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