

Brief Report

MULTIDRUG RESISTANCE IN
YERSINIA PESTIS MEDIATED BY
A TRANSFERABLE PLASMID

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Y*ERSINIA pestis* is the causative agent of plague, a zoonotic disease transmitted to humans through flea bites and typically characterized by the appearance of a tender and swollen lymph node, the bubo. Human-to-human transmission can occur, through either the bite of fleas (bubonic plague) or respiratory droplets, causing an overwhelming infection called pneumonic plague.

The last plague pandemic began in Hong Kong in 1894 and spread throughout the world, establishing many endemic foci. Antibiotics and enforcement of public health measures significantly decreased the morbidity and mortality associated with the disease but did not allow its eradication. In fact, plague is now considered a reemerging disease¹ for at least three reasons. First, there has been an increase in the number of cases reported to the World Health Organization.² Second, plague reappeared in 1994 in an epidemic form in countries, including Malawi, Mozambique, and India, where it had been silent for 15 to 30 years. Third, the number of foci is gradually expanding in certain countries. In the United States for instance, the number of states reporting cases of human plague increased from 3 in the 1950s to 13 in the 1990s.³

Streptomycin, chloramphenicol, and tetracycline are used to treat plague, and tetracycline and sulfonamides are recommended for prophylaxis.⁴ Classically, *Y. pestis* isolates are uniformly susceptible to the antibiotics active against gram-negative bacteria.⁵⁻⁷

We report high-level resistance to multiple antibiotics, including all the drugs recommended for plague prophylaxis and therapy, in a clinical isolate of *Y. pestis*. The resistance genes were carried by a plasmid that could conjugate to other *Y. pestis* isolates. This report should serve as a warning of the risk of the spread of resistance in *Y. pestis*, a species previously considered universally susceptible to antibiotics.

METHODS

Patient and Strains

The properties of the strains used are listed in Table 1. *Y. pestis* 17/95 biotype orientalis was isolated in 1995 in the Ambalavao district of Madagascar from a 16-year-old boy.⁵ The patient presented with fever, chills, and myalgia suggestive of malaria and was treated with quinine. Three days later, the appearance of a right inguinal bubo with high-grade fever (temperature, 41°C), delirium, and prostration led to the diagnosis of plague. The bubo was punctured, and the patient was treated with twice-daily intramuscular injections of streptomycin (2 g per day for 4 days) and oral trimethoprim-sulfamethoxazole (2 g per day for 10 days). The patient recovered but had severe asthenia for more than a month.

Media and Resistance Studies

Brain-heart infusion broth and agar (Difco) were used. The minimal inhibitory concentrations of antibiotics were determined on Mueller-Hinton agar (Sanofi Diagnostics Pasteur). The cultures were incubated for 48 hours at 28°C for *Y. pestis* and for 18 hours at 37°C for *Escherichia coli*. Chloramphenicol acetyltransferase and aminoglycoside-modifying enzymes were assayed in supernatants (centrifuged at 100,000×g) after ultrasonic disintegration.^{12,13} Matting on filters was performed as described previously.¹⁴

Nucleic-Acid Techniques

Isolation of plasmid DNA, cleavage of restriction fragments, and purification of DNA fragments from agarose type VII (Sigma Chemical) were performed as described elsewhere.⁹ Purified DNA fragments to be used as probes were labeled with [α -³²P]deoxycytidine triphosphate by nick translation. Hybridization was carried out under highly stringent conditions.⁹ The polymerase chain reaction (PCR) was performed on a DNA thermal cycler (model 2400, Perkin-Elmer Cetus). Double-stranded DNA sequencing was performed by the dideoxynucleotide chain-termination method¹⁵ with a modified T7 DNA polymerase and [α -³⁵S]deoxyadenosine triphosphate.

RESULTS

Antibiotic Resistance of *Y. pestis* 17/95

Disk-agar diffusion tests showed that *Y. pestis* 17/95 was resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin, sulfonamides, tetracycline, and minocycline. Resistance to ampicillin was due to the production of a beta-lactamase, and resistance to chloramphenicol was due to the production of a chloramphenicol acetyltransferase. Resistance to kanamycin was due to synthesis of a type I 3'-aminoglycoside phosphotransferase. The strain was also resistant to high levels of streptomycin-spectinomycin as a result of the production of 3''-9-aminoglycoside adenylyltransferase. *Y. pestis* 17/95 was resistant to sulfonamides⁶ but remained susceptible to trimeth-

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TABLE 1. PROPERTIES OF THE BACTERIAL STRAINS STUDIED.*

STRAIN		CHARACTERISTICS OF THE PLASMIDS			SOURCE
DESIGNATION	DRUG RESISTANCE	DESIGNATION	TRANSFERABILITY†	DRUG RESISTANCE	
<i>Y. pestis</i>					
17/95		pIP1202 pFra, pPla, pYV	Yes	Ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Wild strain ⁵
17/95-I 6/69 6/69c 6/69cN	Nalidixic acid	pFra, pPla, pYV pFra, pPla, pYV pFra, pPla pFra, pPla			Plasmid loss in 17/95 Wild strain Plasmid loss in 6/69 Spontaneous nalidixic acid-resistant mutant of 6/69
6/69cNR	Nalidixic acid	pIP1202 pFra, pPla	Yes	Ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Conjugation of 17/95 with 6/69cN
<i>E. coli</i>					
K802N	Nalidixic acid				Wood ⁸
BM4354	Nalidixic acid	pIP1202	Yes	Ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Conjugation of 17/95 with K802N
BM4355	Nalidixic acid	pIP1202-1	Yes	Ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides	Derivative of BM4354
BM4356	Nalidixic acid	pIP1202-2	Yes	Ampicillin, kanamycin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Derivative of BM4354
BM4357	Nalidixic acid	pIP1202-3	Yes	Ampicillin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Derivative of BM4354
BM4358	Nalidixic acid	pIP1202-4	Yes	Chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides	Derivative of BM4354
RR1	Streptomycin				Sambrook et al. ⁹
BM4359	Streptomycin	pIP1202	Yes	Ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Conjugation of 17/95 with RR1
DB10‡	Nalidixic acid, streptomycin				Datta et al. ¹⁰
BM4340	Nalidixic acid, streptomycin	pIP1202	Yes	Ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides, tetracycline-minocycline	Conjugation of BM4359 with DB10

*The pFra, pPla, and pYV plasmids are the three commonly found in *Y. pestis*.¹¹

†A transferable plasmid was one that was transferred by conjugation.

‡This strain is susceptible to fusidic acid, macrolides, lincosamides, and streptogramins A.

TABLE 2. MINIMAL INHIBITORY CONCENTRATIONS OF VARIOUS ANTIBIOTICS AGAINST THE BACTERIAL STRAINS STUDIED.

STRAIN	MINIMAL INHIBITORY CONCENTRATION								
	AMPICILLIN	CHLORAMPHENICOL	KANAMYCIN	STREPTOMYCIN	SPECTINOMYCIN	TETRACYCLINE	MINOCYCLINE	SULFONAMIDE	TRIMETHOPRIM
milligrams per liter									
<i>Y. pestis</i>									
17/95	2048	128	2048	>2048	2048	1024	512	1024	1
17/95-I	1	4	1	4	4	8	4	8	0.5
6/69cN	1	4	1	4	4	8	4	8	1
6/69cNR	2048	256	2048	>2048	2048	1024	512	1024	1
<i>E. coli</i>									
K802N	8	8	1	4	4	8	4	8	1
BM4354	1024	1024	1024	256	256	256	32	2048	1

oprim (Table 2), and no synergism was detected between the two drugs by the checkerboard method.¹⁶

A small percentage of *Y. pestis* 17/95 spontaneously lost the resistance determinants en bloc (1 of 100 colonies tested after 10 days of incubation in the absence of antibiotics), and this clone, 17/95-I, was studied further.

All the resistance genes were transferred by conjugation from *Y. pestis* 17/95 to avirulent *Y. pestis* 6/69cN at a frequency of 1.5×10^{-2} per donor colony-forming unit. Selection for transfer of one of these resistance characters revealed the transfer of all six. The minimal inhibitory concentrations of antibiotics for the parent strain, the clone that had lost the resistance determinants, strain 6/69cN, and a strain obtained by the conjugation of 17/95 with 6/69cN are shown in Table 2.

Plasmid DNA from *Y. pestis* 6/69, 17/95, and 17/95-I was extracted and digested with *EcoRV* (Fig. 1A, lanes 1, 2, and 3). Comparison of the restriction profiles indicated that strain 17/95 contained fragments corresponding to an additional plasmid, designated pIP1202, of approximately 150,000 bp, as estimated by pulsed-field gel electrophoresis (data not shown).

Characterization of Plasmid pIP1202

Plasmid pIP1202 was transferred by conjugation from *Y. pestis* 17/95 to *E. coli* K802N and RR1 at frequencies of approximately 1×10^{-2} . The minimal inhibitory concentrations of antibiotics for *E. coli* K802N and strain BM4354, obtained by conjugation of *Y. pestis* 17/95 with *E. coli* K802N, are shown in Table 2. The retransfer of pIP1202 from *E. coli* BM4359 to *Y. pestis* 6/69cN and *E. coli* K802N occurred at frequencies of 1.1×10^{-4} and 5.7×10^{-5} , respectively.

Approximately 5 percent of *E. coli* BM4354 did not contain plasmid pIP1202. Approximately 1 percent had lost part of the resistant determinants, generating plasmids pIP1202-1, -2, -3, and -4 (in Table 1), which were used to assess the incompatibility of the plasmids in experiments performed by reciprocal conjugation. Plasmid pIP1202-2 exhibited strong incompatibility with pIP55-1, which belongs to the Inc6-C group.¹⁷ Hybridization with a probe specific for Inc6-C replicons¹⁸ was detected only with plasmid DNA from the parental strain 17/95 and the *E. coli* strain that had acquired pIP1202 by conjugation from 17/95, confirming that this plasmid belongs to incompatibility group Inc6-C (data not shown).

Analysis of Plasmid DNA

Partial sequencing of an 864-bp PCR product obtained with oligodeoxynucleotides specific for *bla*_{TEM-1} indicated the presence of a cytosine at position 317 (numbering according to Sutcliffe¹⁹), confirming that resistance to ampicillin was due to the

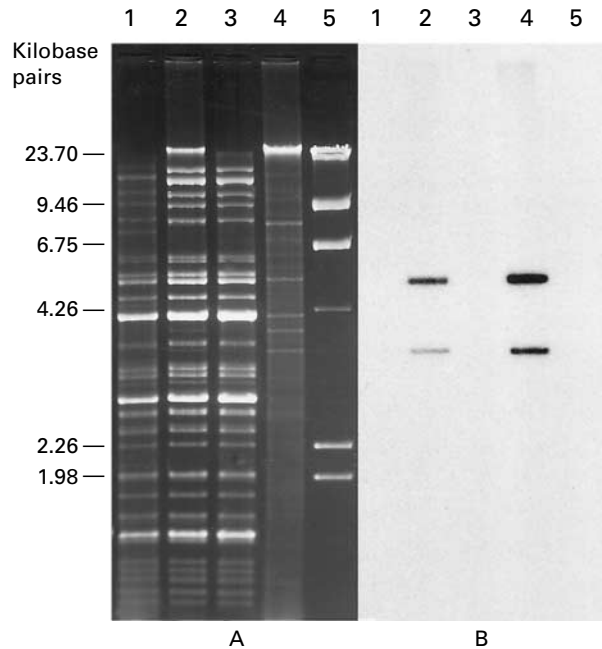


Figure 1. Analysis of Plasmid DNA by Agarose-Gel Electrophoresis (Panel A) and Hybridization (Panel B).

Plasmid DNA from *Y. pestis* 6/69 (lane 1), 17/95 (lane 2), and 17/95-I (lane 3) and *E. coli* BM4359 (lane 4) was digested with *EcoRV*, fractionated by agarose-gel electrophoresis (Panel A), transferred to a nitrocellulose sheet, and hybridized to the ³²P-labeled *tet(D)* PCR product (Panel B). Lane 5 shows fragments obtained by the digestion of bacteriophage lambda DNA with *HindIII* and used as standards for molecular size.

presence of the gene for TEM-1 penicillinase. The probe corresponding to *catI* hybridized to pIP1202 DNA, and the plasmid conferred to *E. coli* DB10 resistance to fusidic acid,²⁰ indicating that resistance to chloramphenicol was due to the production of a type I chloramphenicol acetyltransferase. Probes internal to the *aph(3')-I* and *aad(3'')*(9) genes hybridized to pIP1202 DNA, confirming that resistance to kanamycin was due to the synthesis of a type I 3'-aminoglycoside phosphotransferase and that resistance to streptomycin-spectinomycin was due to the production of a 3''-9-aminoglycoside adenylyltransferase (data not shown). The *tet(D)* gene was detected by hybridization (Fig. 1B), PCR, and sequencing of 520 internal base pairs. PCR amplification and sequencing of a 488-bp fragment internal to the *sulII* determinant confirmed that resistance to sulfonamide was due to the production of a drug-resistant dihydropteroate synthase. The *sulII* gene is nearly always located in conserved segments of integrons in Tn21-like elements that are carried by large conjugative plasmids.²¹ We used specific primers to amplify fragments internal to the integrase gene and

the truncated ORFIV from Tn21-like elements from pIP1202 DNA by PCR, and the two genes were found to flank *aad(3'')*(9) alone.

DISCUSSION

Y. pestis is considered universally susceptible to antibiotics recommended and widely used for prophylaxis and treatment of plague.⁴ In recent studies the isolates tested were susceptible in vitro to all antibiotics active against gram-negative bacteria,^{6,7} with the exception of tetracycline in rare cases.²²

Multidrug-resistant *Y. pestis* 17/95 was isolated in 1995 in the Ambalavao district of Madagascar from a patient who presented with symptoms of bubonic plague.⁵ Despite extensive surveillance of strains of *Y. pestis* isolated between 1926 and 1995 in Madagascar, no multidrug-resistant strain was detected.^{5,22} Strain 17/95 was resistant not only to all the antibiotics recommended for therapy (chloramphenicol, streptomycin, and tetracycline) and prophylaxis (sulfonamides and tetracycline) of plague⁴ but also to drugs that may represent alternatives to classic therapy, such as ampicillin, kanamycin, spectinomycin, and minocycline. The isolate remained susceptible to cephalosporins, other aminoglycosides, quinolones, and trimethoprim, and treatment with trimethoprim, despite its lack of synergism with sulfonamides, most likely led to the patient's recovery.

The resistance determinants were carried by the conjugative plasmid pIP1202. Several observations strongly argue for an origin of this plasmid in a member of the Enterobacteriaceae family. The resistance genes carried by pIP1202 were closely related in structure to plasmid-borne determinants commonly found in enterobacteria. The Inc6-C origin of replication of pIP1202 was typical of plasmids of this group of bacteria with a broad range of hosts, and the plasmid was easily transferred in vitro from *E. coli* to *Y. pestis*.

The site of the putative genetic transfer remains unknown. Enterobacteria are usually confined to the intestinal lumen of the host, whereas *Y. pestis* circulates in lymphatic vessels, the spleen, the liver, blood, and sometimes the lungs. However, intestinal enterobacteria and *Y. pestis* may come into contact when gut bacteria invade the bloodstream. Alternatively, if pIP1202 originated in an invasive pathogen, the contact between the two microorganisms may have occurred in the blood or in deep tissues. A third possibility is that intimate contact between *Y. pestis* and the donor was achieved outside the mammalian host, perhaps in the gut of a flea that ingested blood infected with both microorganisms.

The fact that the multidrug-resistant plasmid was highly transferable in vitro to other strains of *Y. pestis*, where it was stable, is of great concern. It is likely that this type of replicon can also be transferred among strains of *Y. pestis* in their natural environment and,

therefore, that resistance may spread locally in this species. Even more alarming, the observation that *Y. pestis* is able to acquire, under natural conditions, a resistance plasmid, regardless of its true origin, indicates that such a clinically ominous event may occur again.

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