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Rapid Antimicrobial Susceptibility Testing of *Yersinia Pestis* Strains from Kazakhstan Plague Foci

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Abstract

As far as new antibiotic-resistant *Yersinia pestis* strains appear in the climate of bioterrorism threats, plague susceptibility to antibacterials requires regular testing. *Yersinia pestis* should be put through rapid antimicrobial susceptibility tests. Since it grows slowly on agar media, the rapid *in vivo* test is especially required. The purpose of this research is to study *Yersinia pestis* strains from different Kazakhstan plague foci and to design an easy and fast method of antibiotic susceptibility estimation. All *Y.pestis* strains were susceptible to antibacterial agents traditionally recommended for plague treatment. All isolates were susceptible to ß-lactam antibacterials, including imipenem, fluoroquinolones, aminoglycosides and doxycycline. We have not detected any resistant *Y.pestis* strains during a 10-year investigation. We showed that preliminary rapid AST allows getting indices in 3-6 hours from the moment of *Y. pestis* isolation. Therefore, we recommend it for making a preliminary choice of antibacterial agents for plague treatment using HiComb MIC test for getting results that are more accurate. Strains resistant to antibacterials have not been detected during the 10-year research. The V.I. Ilyukhin's method and the HiComb MIC test are recommended for rapid assessment of microorganism sensitivity to chemotherapy and should be out by anti-plague institutions into practical contexts.

Keywords: *Y.pestis*, antibiotic susceptibility, plague foci, minimum inhibitory concentration, antibacterial agent

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INTRODUCTION

In Kazakhstan, 39% of territory (1.4 million squares km) is the plague foci area –plague epizootics with hundreds of virulent isolates from the rodents and fleas are registered there annually (Aikimbajev et al. 2003).

Plague is an enzootic infection caused by the bacterium *Yersinia pestis* and transmitted by fleas across many parts of Asia, Africa, North and South America. The natural reservoirs for plague are wild and urban rodents that can act as links between the sylvatic (natural) reservoir and the people (Perry and Fetherston 1997). *Rhombomys opimus* is considered as the main

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reservoir occupying large areas of Central Asia (Anisimov et al., 2004). Changes in land use that occur in many parts of the world increase the risk of people contacting with wild and urban rodents (Perry and Fetherston 1997).

According to serological and bacterial data available from the 4-year study by Zhao et al., marmot and long-tailed ground squirrel plague foci are located in the Altay Mountains and the Biezhentao Mountain. Of eight *Y. pestis* isolates that they studied, six were from new foci. This indicates its expansion. At this point, Zhao et al. suggested that Kazakhstan should strengthen the plague epidemiological surveillance in the wild, especially in an area bordering with China (Zhao et al. 2017).

Constant testing of *Yersinia pestis* sensitivity to antibacterial agents is important because antibiotic resistance genes can arise in bacterial plasmids (Galimand et al. 2006).

For example, in 1999, *Y. pestis* strains isolated from plague patients in Kazakhstan were resistant to chloramphenicol and had average sensitivity to gentamicin (Atshabar et al. 2000). It was also reported (Galimand et al. 1997, Guiyoule et al. 2001) that isolated from Madagascar patients *Y. pestis* strains had a plasmid of multidrug-resistance. In this regard, sensitivity of various isolates to antimicrobial agents must be checked regularly.

Moreover, *Y. pestis* is one of several agents that can be used as a biological weapon for bioterrorism purposes (Atlas 1998). Thus, defining the range of its sensitivity to different antimicrobial agents is of great importance.

There are some publications on *in vitro* sensitivity of *Y. pestis* strains to various antibacterial agents (Frean et al. 1996, Galimand et al. 1997, Rasoamanana et al. 1989, Wong et al., 2000). For example, Smith and et al. studied susceptibility of 78 Vietnamese strains to 14 antibacterials (Smith et al. 1995). The plague bacteria were sensitive to ceftriaxone, ciprofloxacin, ofloxacin and ampicillin. In 1996, Frean et al. (1996) studied *in vitro* sensitivity of 100 plague isolates from South Africa to new antibacterial agents. They marked out three oral antibacterial agents as extremely active against *Y. pestis* – two quinolones (levofloxacin and ofloxacin) and cefotaxime.

Doxycycline, ciprofloxacin and ofloxacin were effective on animals, but they were not used as first-choice drugs for plague treatment in people. At the same time, penicillin and cephalosporins were not effective during in vivo tests (Byrne et al. 1998).

It is manifestly obvious that results on sensitivity to antibacterial agents must be get as quick as if plague complications progress. Early and adequate (compliant with lab results) antibacterial treatment of patients with infectious diseases allows reducing the case fatality rates and the hospital stay period (Barenfanger et al. 1999). However, standard AST methods (serial dilution, disk diffusion testing) allow receiving results in 24-48 hours. This will be the biggest shortcoming of these methods if one deals with such bacteria (in our case, *Y. pestis)* that grow slowly on agar medium, but rapidly *in vivo* (Steinberger-Levy et al. 2016). Colorimetric methods didn't fit the study of especially dangerous pathogens at all (Tunney et al. 2004).

In recent years, a number of new methods have appeared that help to reduce the AST duration by reducing both the pre-isolation stage duration and the time required for determining antimicrobial resistance (Pulido et al. 2013, van Belkum and Dunne 2013). Some of these alternative methods (such as flow cytometry using live or dead bacteria (Jepras et al. 1997)) allow us to determine both the minimal inhibitory concentration (MIC) and the category of susceptibility. In 2016, Ida Steinberger-Levy et al. suggested applying an alternative approach to rapid AST that was based on the quantitative determination of changes in the expression levels of specific marker genes exposed to antibacterials at concentrations that inhibit growth (Steinberger-Levy et al. 2016).

Such testing takes only 7 hours in total. Above-mentioned methods are not time consuming and accuracy-demanding, but they do require expensive equipment and reagents. Most other methods (such as MALDI-TOF-MS (Burckhardt and Zimmermann 2011)) or magnetic bead rotation (Kinnunen et al. 2012)) allow determining more than just the category of susceptibility. MIC values are as important for patients with contraindications to antibacterials, pregnant women, children and strains with intermediate resistance as the categories of susceptibility.

We found a method for preliminary rapid testing of antimicrobial susceptibility to antibacterials that can be applied to especially dangerous infections. The V.I. Ilyukhin's method (Ilyukhin et al. 2007) does not require complex equipment and can be useful in epidemiological studies of plague. Quick and adequate preliminary recommendations on antibacterial agents to

select are essential when plague complications are in progress. At this point, testing results will allow improving the treatment.

The purpose of this research is to design a method for rapid estimation of antimicrobial susceptibility to chemotherapy, as well as to test the antimicrobial susceptibility of bacteria, isolated from the Kazakh plague foci. This goal was achieved my means of the V.I. Ilyukhin's method followed by a more accurate HiComb MIC test. In addition, we determined *Y. pestis* sensitivity to antibacterials using standard laboratory methods, such as serial plate agar dilution, disk diffusion testing and data comparison.

MATERIALS AND METHODS

Research was carried out in the Laboratory of Zoonotic Infections of M. Aikimbayev's Kazakh Scientific Center for Quarantine and Zoonotic Diseases (KSCQZD) in 2008-2017.

Bacterial Strains

In this research, we studied 200 strains of *Y. pestis* from the Living Culture Museum of KSCQZD. These strains were isolated over 19-year period in Republic of Kazakhstan (1998-2016). Strains were isolated from animal carcasses, humans and fleas. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as control microorganisms. Studies were conducted in a Biosafety Level 2 (BSL-2) laboratory.

AST Procedure

The test list of antibacterial agents included: amoxicillin, imipenem, cefalotin, cefoxitin, aztreonam, ofloxacin, pefloxacin, ciprofloxacin, streptomycin, gentamicin, amikacin, tobramycin, doxycycline, and chloramphenicol (produced by the Pasteur Research Institute of Epidemiology and Microbiology, Russia). They were used as prescribed by the producer. Strains of *Y.pestis* were identified by a routine laboratory technique according to the guidelines (Chu 2000, Nekrassova et al. 2001). Susceptibility of plague strains has been preliminary tested by means of a disk-diffusion method (Chu 2000, Nekrassova et al. 2001). At the same time, rapid testing was done on Hottinger agar medium with glucose and bromothymol blue additives. The growth rates of isolates have been updated with a period of one hour up to the point of color change. In growth inhibition areas, medium color remained, while in the growth areas, it turned yellow because of acidulation with splitting glucose. The results were analyzed in 18-24 hours. MIC values of 14 antimicrobial agents were determined by means of dilution agar dilution and HiComb MIC testing. Susceptibility was tested on Mueller–Hinton medium (HiMedia, India). We first plated the three or four colonies of each isolate on Hottinger Agar, and then incubated the plates at 28°C for 48 hours. Then, the colonies were suspended in Mueller–Hinton broth in order to obtain final inoculums size of 10^8 CFU/mL. Bacteria concentration was determined by turbidity standard. Plates with isolates inoculated into Mueller–Hinton agar (*Mueller Hinton Agar, HI MEDIA, REF M173-500G, NOV 2019, LOT 0000248260)* were incubated at 28°C for 48 hours. The follow-up was considered after incubation. The HiComb MIC testing (HiMedia, India) was carried out as outline in attached instructions. Non-treated culture was used as a reference point for growth, while the medium without bacterial cells and antibacterials was used as a reference point for sterility.

Statistical Analysis

Statistical data were processed using Excel-97 program to determine the average values, calculate standard errors, and determine the reliability of differences between mean values using the Student's t-test. Differences were assessed as significant at a probability of 95%, $p < 0.05$.

RESULTS

The studied plague strains were confirmed to be the strains of *Y. pestis* by standard criteria (Nekrassova et al. 2001). At mandatory stage of lab study, their sensitivity to antibacterials was tested following the procedure established in the international standards (Chu 2000).

Rapid AST revealed that medium with bacterial isolates on it that were cultivated at 28 °C turned yellow in areas where cultures were growing during 3-6 hours of incubation in response to acidulation. Visually, strain growth cannot be detected without using solutions. AST results obtained by this method significantly correlate with the results of HiComb MIC testing, agar dilution and disk diffusion testing. Thus, modified method of rapid AST of *Y. pestis* strains allows making preliminary recommendations on the antibacterial agents to select for plague treatment in 3-6 hours from the moment of culture isolation. All plague isolates were sensitive to all specified antibacterials (**Table 1**). These results correlate with data obtained earlier (Meka-Mechenko et al. 2009).

Table 1. Susceptibility testing of 200 *Y. pestis* isolates from natural plague foci of Kazakhstan

Antibiotic	MIC_{50}, µg/ml	MIC_{90}, µg/ml	Range, µg/ml	Breakpoints, µg/ml	S/IR (%)
Streptomycin	4	8	0.125–16	ND	100/0
Gentamicin	0.5	2	0.125–4	≤ 4 >8	100/0
Chloramphenicol	4	4	0.125–8	≤ 8 >16	100/0
Amoxicillin	0.5	0.5	0.125–0.5	≤ 8 >16	100/0
Imipenem	0.25	0.5	0.125–0.5	≤ 4 >8	100/0
Cefalothin	2	2	0.125–4	≤ 8 >32	100/0
Cefoxitin	2	4	0.125–4	≤ 8 >32	100/0
Aztreonam	<0.125	<0.125	<0.125–0.125	≤ 4 >32	100/0
Ofloxacin	<0.125	<0.125	<0.125–0.125	≤ 1 >4	100/0
Pefloxacin	<0.125	0.25	<0.125–0.25	≤ 1 >4	100/0
Ciprofloxacin	<0.125	<0.125	<0.125–0.125	≤ 1 >2	100/0
Amikacin	2	4	0.125–8	≤ 8 >16	100/0
Tobramycin	0.5	1	0.125–2	≤ 4 >8	100/0
Doxycycline	0.5	1	0.125–2	≤ 4 >8	100/0

ND, not determined S, susceptible; IR, intermediate or resistant

Of 14 chosen antibacterials, the most active against the plague were ciprofloxacin, pefloxacin, ofloxacin and aztreonam with MIC_{90} less than 0.125. This corresponds with the study of Frean et al. [11], who also stated that ofloxacin was highly active against the plague. There was no difference in sensitivity between isolates.

Results obtained by standard laboratory methods – serial plate agar dilution and disk diffusion testing – did not significantly differ from the HiComb MIC testing results.

DISCUSSIONS

Monitoring of *Y. pestis* sensitivity to antibacterial agents is of great importance if we refer it as knowledge of regional features of plague strains (Otang-Mbeng and Afolayan 2017). The outbreak of plague epidemics sparked by cross-country spillover or bioterrorism will require great financial support for plague anti-epidemic and prevention measures to take. In the Republic of Kazakhstan, Healthcare system has to be ready to take full-scale anti-epidemic actions.

Ciprofloxacin, doxycycline and, as an alternative, chloramphenicol are considered to be the most crucial antibacterials when it comes to plague post-exposure prophylaxis, while streptomycin, gentamicin and, as alternatives, levofloxacin, ciprofloxacin, doxycycline, moxifloxacin and chloramphenicol are recommended for treatment (Inglesby et al. 2000). It is known that most natural strains of *Y. pestis* are susceptible to these antibacterials. However, strains with plasmids that provide antibiotic resistance to both one and several types of agents have been removed in infected patients (Galimand et al. 2006). Although no evidence was found that antibiotic resistance had something to do with the virulence plasmids, a recent research on several isolated strains of *Y. pestis* has revealed many genes encoding resistance to antibacterials with transposon sequences (Galimand et al. 2006, Welch et al. 2007). This indicates that gene transfer to virulent plasmids is at risk.

As found previously, virulent plasmids of *Y. pestis* are unstable if cultivated at 35-37 °C (Ferber and Brubaker 1981, Portnoy and Falkow 1981), although it is the standard incubation temperature established by the Clinical and Laboratory Standards Institute (CLSI). At this point, incubation temperature of 28°C may do to determine the accurate susceptibility profile of recombinant strain, maintain plasmid stability, and obtain reliable data on antibiotic resistance. We considered this figure during the research.

Although there is a list of bactericidal and bacteriostatic antimicrobials against plague, we still have to define the sensibility of *Y.pestis* to antibacterial agents. There are a number of methods for the case, divided into 2 groups: dilution that allows finding MIC values and disc-diffusion that allows determining areas where bacterial colonies do not grow (Jorgensen and Turnidge 2015). Disc-diffusion and agar dilution are standard methods for sensitivity testing that are popular with anti-plague laboratories.

Whereas, HiComb MIC test is recommended by agent producers as a rapid method with a considerable number of advantages. As we found out, MIC values determined by serial dilution and by HiComb MIC testing are similar. Area diameter determined by disk-diffusion testing also correlated with MIC values and then significantly. HiComb MIC test is undoubtedly more favorable, as it allows getting reliable results quickly and without any difficulties that may come with other methods. Our tests, as well as studies conducted before by other authors (Balakhonov et al. 2013), confirm that this test can be used to estimate susceptibility of plague strains and put by anti-plague institutions into practical contexts.

The plague is a severe and rapidly progressing disease characterized by a high mortality rate (40–60% – bubonic plague, 30–50% – septicemic plague, and 100% –pneumonic plague) if not treated with proper antibacterials within 18–24 hours after the onset of symptoms (Inglesby et al. 2000). This is why we made an attempt to design a rapid and convenient method for preliminary AST. This method implies incubating plague bacteria plated on Hottinger agar with solutions added to the medium. It allows making preliminary recommendations in 3 hours after the plague bacteria isolation. We advise following up with a HiComb MIC test for justification.

MIC values were found for a number of specific strains of *Y. pestis* to use them as references in future tests. Standard MIC_{90}s values determined at 35°C reflect susceptibility to gentamicin, streptomycin, doxycycline, tetracycline, ciprofloxacin, levofloxacin, cotrimoxazole and chloramphenicol (Clinical and Laboratory Standards Institute 2010).

These values correspond with the reference design data and data from a recent study of 392 strains (Lonsway et al. 2011, Urich et al. 2012). Ours MIC values determined for gentamicin, streptomycin, doxycycline, ciprofloxacin and chloramphenicol at 28° C correlate with the values obtained in the above studies.

Taking all things together, this research shows that all *Y.pestis* strains were susceptible to antibacterial agents that are traditionally recommended (Chu 2000, Nekrassova et al. 2011) for treatmenting *Y.pestis* infections. All isolates were susceptible to ß-lactam antibacterials, including imipenem, fluoroquinolones, aminoglycosides and doxycycline. We detected no resistant *Y. pestis* strains over a 10-year investigation.

CONCLUSION

Antimicrobial susceptibility testing of 200 *Y.pestis* strains isolated from the natural plague foci of Kazakhstan for sensitivity to 14 convention antibacterials showed no resistance of plague strains to these antibacterials. Various AST methods (disk-diffusion, serial dilution, HiComb MIC testing) provided comparable MIC values. This article discloses the advantages that HiComb MIC testing has for anti-plague institutions. Rapid preliminary AST of *Y. pestis* strains using the V.I. Ilyukhin's method allows making recommendations on antibacterial agents to select against plague complications.

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