INVESTIGATION OF *YERSINIA PESTIS* IN *XENOPSYLLA ASTIA*

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Abstract. In this study, at the Department of Parasitology in the Pasteur Institute of Iran, *Xenopsylla* were allowed to feed on mice infected with *Yersinia pestis.* After 24-48 hours, they were killed by ether and kept in alcohol (70%) for 20 minutes. They were then examined for pathological signs and bacilli in different tissues and organs, longitudinally and cross-sectionally. The samples were studied using conjugated antibody and fluorescence microscopy. The results of this study revealed that the bacilli are abundant in the proventriculus after 6 hours, but it was found in other organs, rarely.

INTRODUCTION

Plague is an infectious disease of wild rodents, transmitted from one rodent to another by the bite of a flea. It has caused several epidemics in many parts of the world (Arkiev, 1982). Humans are accidental hosts, when bitten by an infected rat flea. After an incubation period of 5-7 days, the symptoms begin with high fever, chill, headache and painful lymphadenopathy, commonly with enlarged, tender nodes in the grain or axils. Vomiting, diarrhea and hypotension appear. Altered mental status, and renal and cardiac failure, and finally signs of pneumonia and meningitis can appear (Jawetz *et al*, 1987).

There are three plague forms of medical importance: A) *Bubonic plague* is the classic form of the disease. The mortality rate is up to 90% in untreated patients; B) *Pneumonic plague* is a rare but deadly form of the disease that is spread via respiratory droplets through close contact with an infected individual; C) *Septicemic plague* is generally defined as occuring in a patient with positive blood cultures but no palpable lymphadenopathy. The mortality rate is 30 to 50% (Hull *et al*, 1987).

At present, 90% of plague cases are reported from Southeastern Asia, especially Vietnam, Myanmar, Nepal, Africa and South America (Suntsov *et al*, 1997).

Since AD 541 there have been three plagues pandemics.

First pandemic: the Justinian plague began in Pelusium, Egypt, after arriving from Ethiopia (AD 541-544). It quickly spread thorugh the Middle East Mediterranean Basin with a limited foray into Mediterranean Europe, after that, the 2nd through 11th epidemics occurred in 8 to 12 cycles. This pandemic eventually affected the entire known world, North Africa, Europe, Central and Southern Asia, and Arabia.

Second pandemic: during the 8th to 14th centuries, Europe seemed to have coped with most epidemic diseases and it experienced a 300% population increase between the 10th to 14th centuries. The first epidemic (AD 1347 to 1351) that later became known as the Black Death, killed an estimated 17 million to 28 million Europeans.

Third pandemic: the third pandemic started in 1893 in the Chinese province of Yunnan. Troop traffic from the war in that area caused rapid spread of disease to the Southern coast of China. It reached Hong Kong and Canton in 1894, and Bombay in 1898; by 1899/ 1900 to Africa, Australia, Europe, North and South America. Plague killed 12.5 million *ie* in India, between 1898 and 1980.

The agent of plague is the *Yersinia pestis* bacillus. It is rod shaped, non-motile, gram-negative bacillus that exhibits striking bipolar staining with special stains. Three biotypes of *Y. pestis* are recognized on the basis of conversion of nitrate to nitrite and fermentation of glycerol. The biotype antiqua is positive for both characteristics. Orientalis forms nitrite but does not ferment glycerol, while medievalis ferments glycerol, but does not form nitrite from nitrate.

Ribotyping has identified 16 patterns that can be organized into three classical biotypes. The antiqua ribotype O occurred in the first pandemic. The second pandemic was caused by medievalis ribotype O. The third pandemic was caused by orientalis ribotype B.

There are two active foci of plague in Iran (Kurdistan, Ardebil) southwest of Iran. The reservoirs are four species of the genus *Gerbillidae* in Iran. They

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are *Meriones persicus, M. libycus, M. tristrami, M. vinogradovi.*

Over 1,500 species of fleas have been identified, while only a small number (over 31) of these are proven vectors. The vectors in the plague foci of Iran are rat' s flea spp. These are *Xenopsylla astia, X. buxtoni, X. stenoponia, X. conformis* and *Pulex irritans.* When the flea feeds on a rodent infected with *Y. pestis*, the ingested organisms multiply in the gut of the flea and block its proventriculus, so that no food can pass through, therefore the flea becomes hungry and feeds on blood frequently, transmiting the *Y. pestis* to rats, other animals or humans.

MATERIALS AND METHODS

Fleas are in the order Siphonaptera. They do not have wings but their coxa are very strong for jumping. The gastrointestinal organs are very important in the transmission of plague from rodents to humans. It is include stomodeum, mesenteron, and proctodeum (Fig 1). The preventericul is the most important part for the growth of *Y. pestis*.

In this study 20,000 *Xenopsylla astia* were colonized in insectariums (Department of Parasitology, Pasteur Institute of Iran). They were fed with blood powder in glass jars (20×30×50 cm) at 25°-28°C with 85% humidity.

Flea infection

*Yersinia pestis***,** strain 64-1 pkp, isolated from rat fleas in Kurdistan, were grown in peptone broth and peptone agar media at 28°-32°C for 48 hours. 0.5 ml of *Y. pestis* suspension containing 6×10^6 living bacilli, were inoculated into guinea pigs intraperitoneally. After 3 days, plague bacteria were seen in the blood stream. The infected guinea pigs were kept in glass jar (20×30×50 cm) with 200 hungry *Xenopsylla* for blood feeding. After 2-3 hours, the fleas were collected with an aspirator. When a flea feeds on a rodent infected with *Y. pestis*, the ingested organisms multiply in the gut of the flea and, helped by coagulase, block its proventriculus six hours after the infectious meal. Then, they were killed with ether and kept in alcohol 70% for 20 minutes for removing common surface bacteria, and washed with physiological saline.

Bacterial migration in the flea

Preparation of flea tissues. Since tissues and organs are usually too thick for transillumination, techniques have been developed for obtaining thin, translucent sections. In most cases, tissues must be sliced into thin sections (by microtomes) before they can be examined.

Fixing. The simple fixatives most commonly used are a solution of formalin in saline and 2% solutions of buffered glutarate dehydrate. The infected fleas were kept in 10% formalin for 3 days. Then they were kept into Bouin's solution for 20 minutes and washed with distilled water. To remove the water from the fleas' tissues, they were kept into Autotechnicom Tissue Processor for 23 hours.

Embedding. In order to obtain thin sections with the microtome, tissues must be infiltrated with embedding substances that import a rigid consistency to the tissue. The embedding material for light microscopy is paraffin. The process of embedding has 2 main steps, dehydration and clearing. The water of the flea tissues must be extracted by bathing in a graded series of mixtures of ethanol with water, from 70% to 100% ethanol. Then, the ethanol is replaced by a solvent miscible with embedding medium (such as xylene). As the tissues become infiltrated with solvent, they become transparent (clearing step). The fleas were placed in melting paraffin at 58°-60°C to be blocked. Cross sections and longitudinal tissues of several organs of the flea body (in paraffin) were sliced by the microtome to a thickness of 1-10 μm. The sections were floated on warm water and transferred to glass slides.

Staining. The basic dyes are toluidine blue and methylene blue on hematoxylin; hematoylin stains the cell nucleus and acidic structures blue. Each section was stained with hemotoxilin blue and was examined by common light microscope and one other was kept in a refrigerator (-20°C).

Fluorescent staining. The slides were placed in a desiccator jar to remove moisture, for 10 minutes. Then the slides were stained with conjugated serum antibody of *Yersinia pestis* for 30 minutes at room temperature. Then they were washed with PBS for 5 minutes and moisture removed. The slides were studied with a drop of glycerin (10%) and fixed with fluorescent microscope glass.

RESULTS

In this study, we colonized 20,000 *Xenopsylla astia*, being 4,000 fleas studied in 5 groups. Unfed *X. astia* were fed on guinea pig with *Y. pestis.* All fleas were subsequently placed in glass jars for six hours; two, four, six and eight days. Sections of several parts of the flea bodies were prepared as described previously. First, they were stained with hematoxylin and studied by light microscope. The internal system

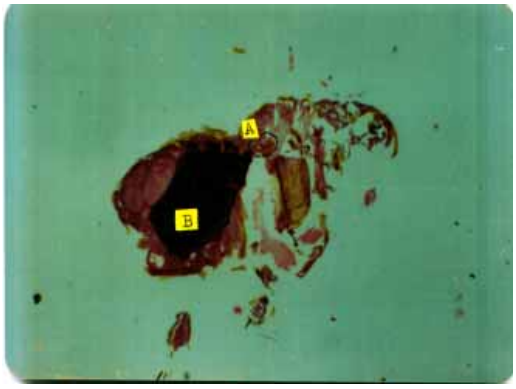

Fig 1- Longitiudinal section of hematoxylin-stained flea- A:proventriculus B:ventriculus.

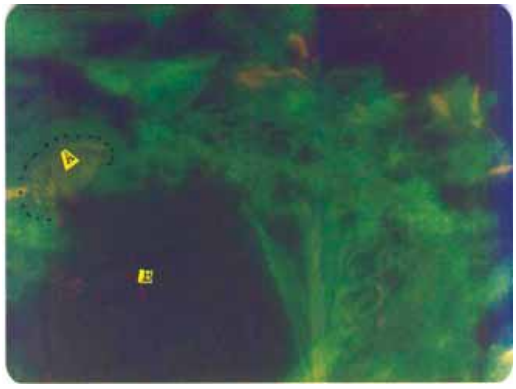

Fig 2- Longitudinal section of fluorescent antibody-stained flea A:proventriculus B: ventriculus.

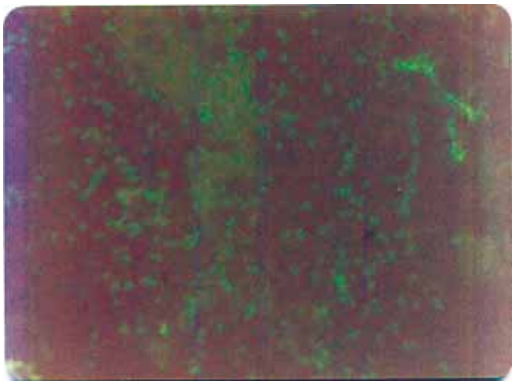

Fig 3- *Yersinia pestis* bacilli.

of the flea's body is shown in Fig 1. The other sections of the same parts of the tissues were stained with fluorescent antibody specific for *Y. pestis* (Fig 2). The result of this study showed that the method used here was appropriate to detect *Y. pestis* bacilli after six hours in the midgut of fleas. A large number of *Y. pestis* were observed in the proventriculus (Fig 3).

The preparations of infected flea tissues were examined in the days after the blood meals by fluorescent-specific antibody to *Y. pestis*. Positive results were detected from five to six days after the blood mealing only in the proventriculus part. The majority of fleas examined were heavily infected and ventricular masses of *Y. pestis* were detected. Other parts of the stomach showed a few *Y. pestis* organisms because the stomach was blocked by large *Y. pestis* masses in the proventriculus.

This research shows the migration of *Yersinia pestis* in fleas. The bacilli were aspirated with the blood of infected animals into the midgut and grow in the proventriculus. The results of bacteriological fluorescent antibody examination are shown. In general, a large number of bacteria were in the midgut, especially in the proventriculus.

DISCUSSION

The laboratory diagnosis of plague is based on bacteriological and or serological evidence. Samples for analysis can include blood, bubo aspirates, sputum, cerebrospinal fluid of patients, and scrapings from skin lesions. A positive fluorescent antibody test can be used as presumptive evidence of a *Y. pestis* infection. Fluoresent antibody staining techniques for the rapid identification of *Y. pestis* in animal tissues have been developed. Alternative methods for diagnosing plague have been developed, including enzyme-linked immunosorbent assay (ELISA) and PCR analysis and DNA hybridization studies. Several PCR assays have been developed for plague diagnosis and identification of *Y. pestis* in fleas (Hinnebusch and Schwan, 1993). Recently molecular techniques have been proposed as a means of more rapidly identifying plague bacteria in fleas.

We report here a fluoresent antibody method using sections of fleas tissues. This assay provides much greater sensitivity. Large numbers of the plague organisms appeared to be lysed during the first few hours after the infectious blood meal. Although the numbers of *Y. pestis* present in each flea increased and a few organisms were stained brilliantly enough for diagnostic purposes after the third or fourth day.

Cavanaugh and Randall (1959) reported that *Y. pestis* organisms in fleas yield only very dim fluorescence with antibodies specific to *Y. pestis* fraction 1b antigen. The reasons for this phenomenon undoubtedly are based upon antigenic changes that occur in the plague organisms while in the flea. In this assay, we used the somatic antibody of whole cell *Y. pestis* bacilli. It was virtually distinguishable from the staining achieved with antibody to *Y. pestis* fraction 1b. Our fluorecent antibody staining of flea tissues was very sensitive and able to detect a few *Y. pestis* bacilli in a flea. This specific polyclonal antibody could be used routinely to detect *Y. pestis* in fleas after blood meal from infected animals.

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