Plague (*Yersinia pestis*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *yersinia pestis.*

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1 PHLN summary laboratory definition

1.1 Condition:

Plague
Bacteria
Yersinia pestis infection

1.1.1 Definitive Criteria

Isolation of Yersinia pestis from bubo aspirates, blood/liver/spleen, cerebrospinal fluid (CSF), respiratory secretions, bone marrow or lymph nodes.

1.1.2 Suggestive Criteria

- Detection of *Y. pestis* by nucleic acid tests (NAT); OR
- Gram negative single or short-chained pleomorphic fat bacilli exhibiting bipolar (closed safety pin) polychromatic staining with Wayson or Wright-Giemsa stains from patients with clinical features of pneumonic/bubonic plague.

2 Introduction

Plague is a zoonotic disease caused by the bacterium *Yersinia pestis*, a Gram negative bacillus and facultative intracellular pathogen.

Plague holds a special place in history because of its occurrence in large epidemics, such as the Black Death that destroyed around a quarter of the population as it swept through Europe during the middle of the 14th century. The recurrence of epidemic plague in various European populations inspired an awe and horror matched only by influenza and smallpox.

The last plague pandemic began in mainland China in 1894 and lasted over a decade, spreading from Hong Kong to port cities around the world. It ravaged Bombay and San Francisco and other cities along the Pacific Coast of the United States. More than twenty-six million people were infected. Twelve million died[1]. Plague still occurs, and has been reported recently in human populations from India, Africa (and in particular Madagascar), South Asia, the United States of America, where it is epizootic in the western states[1] and southeastern Europe.[3, 5]. Between 1000 and 2000 cases of plague are reported every year to the World Health Organisation. The incidence of plague is probably under-reported due to deficiencies in detection and surveillance of the disease in some of these countries.

There have been three documented outbreaks of plague in Australia. The first of these occurred in Sydney in 1900 and resulted in 303 cases with 103 deaths[4]. This was followed by another outbreak in 1902 with a concurrent outbreak in Brisbane. Living conditions in Sydney were very poor at the time and the wharves and nearby housing were badly rat-infested.

Outbreaks of plague in humans always involve transmission of plague among the natural animal reservoir and are typically linked to squalid living conditions. In these situations, humans become incidental targets of the plague- carrying fleas (the most efficient insect vector of *Y. pestis* is the tropical rat flea, *Xenopsylla cheopis*) that feeds on rats which may be infected. During the Black Death, the only rat in Europe was the black rat (*Rattus rattus*), a species that is known to live in close association with human habitation[7]. Now urban and domestic rats (*R. rattus* and *R. norvegicus*) are the commonest mammalian vectors. A range of small mammals maintain sylvatic foci of plague such as prarie dogs, gerbils and marmots. A highly invasive and virulent disease, plague is one of three quarantinable diseases subject to international regulation. Every case must be reported to the WHO[1].

A single bite from an infected flea can disgorge as many as twenty-four thousand *Y. pestis* cells into the blood or lymphatic system[1]. Bubonic plague has an incubation period that ranges from one to eight days, victims begin to suffer fever and chills. Six to eight hours after the first symptoms occur, painful lumps called buboes begin to form under the surface of the skin, increasing in size and darkening as the body's tissues are attacked. Glands swell, causing so much pain, particularly in the neck, groin and armpits, that even comatose patients have been known to writhe in agony[1]. Bubonic plague is not transmitted from person to person.

The most severe form of the disease is pneumonic plague. In this form, the bacterium is easily passed from person to person by a cough or sneeze. This infection results in a fatal attack of pneumonia[1]. The incubation period for pneumonic plague is short – usually 2–3 days. The symptoms are sudden and often difficult to distinguish from other infectious diseases. They include rapid onset of fever, chills, malaise, myalgia, headache, and an acute pneumonia syndrome, accompanied by chest pain, dyspnea, and cough. Sputum production is usually watery and blood-tinged, but may be frankly bloody[5]. A delay in correct diagnosis can be fatal – treatment must be commenced within the first 24 hours. As *Y. pestis* is attacked by the body's immune system, it releases a potent toxin that leads to further collapse of the circulatory system.Patients experience severe pain prior to death. Victims of pneumonic plague will succumb within eighteen hours of the toxin's release, sometimes going into convulsions and delirium and usually lapsing into a coma towards the end[1]. Plague infection may also manifest as meningitis, or more rarely, pharyngitis[6].

Untreated bubonic or pneumonic plague can develop into septicemia, which frequently begins with gastrointestinal symptoms that then progress to systemic symptoms and disseminated intravascular coagulation, adult respiratory distress syndrome and circulatory collapse[5]. Septicaemic plague may occur as a complication of untreated bubonic or pneumonic plague[3].

Plague has been used as a biological weapon. The Japanese dropped plague-infested fleas from planes in Manchuria during World War Two. The former USSR successfully weaponised plague and developed multi-drug resistant strains whilst maintaining or enhancing virulence[1]. A biological attack could take the form of aerosol distribution of *Y. pestis*. Large numbers of pneumonia cases in a short period of time with a massive casualty rate might be the first indication of such an attack. The infectious dose by aerosol is approximately 100 – 500 organisms[6].

3 Laboratory diagnosis/tests

3.1 Culture

Yersinia pestis is a non-fastidious bacterium and is able to grow on a variety of suitable rich or selective microbiological media, including blood agar, MacConkey agar and CIN agar[2, 6].

3.1.1 Media

Yersinia pestis grows well on 5% sheep blood agar or chocolate agar. Two blood agar plates should be inoculated, one for incubation at 37 ºC and the other at 28 ºC. At the lower temperature *Y. pestis* will grow faster than most enteric bacteria (although other *Yersinia* spp will also grow well at this temperature) but is significantly slower at 37 ºC. Growth of *Y. pestis* also occurs on MacConkey agar. For contaminated specimens such as sputum selective media such as Yersinia CIN agar may be useful in addition to these media [5, 6].

3.1.2 Suitable specimens

A. Samples from acutely ill patients

- Blood for culture
- Sputum
- Tracheal or lung aspirates
- Aspirate from a bubo if present.
- If no fluid or pus is obtained, a small amount of sterile saline can be injected and then re-aspirated for culture (fine-needle aspirate)
- CSF if clinical features of meningitis
- Acute and convalescent sera

NB. Samples should be labelled as “High Risk” and forwarded to a PHLN laboratory for culture.

B. Samples from post-mortem

- Blood from a vein (if possible)
- Bubo aspirate
- Lymphoid tissue
- Lung tissue
- Bone marrow
- CSF if features of meningitis

NB. Extensive post-mortem examination is discouraged in cases of suspected plague because of the risk of aerosolising *Y. pestis* present in body fluids, drips, etc.[5, 6].

3.1.3 Test sensitivity

No quantitative data available.

After 48 hours incubation at 37 ºC, the colonies resemble those of a typical Gram-negative rod. It is therefore necessary to perform confirmatory tests to verify the identification. In broth culture after 24 hours at 37 ºC, *Y. pestis* produces structures that resemble stalactites. Crumbly clumps form at the side and the bottom of the tube whilst the remainder of the broth remains clear. These disappear after 48 hours incubation[5].

3.1.4 Test specificity

As there are no unique colony characteristics that aid in the identification of the organism, other confirmatory tests are necessary to confirm a diagnosis. A stained smear demonstrating bipolar staining of the bacilli should increase suspicion when suitable symptomology is evident[5].

3.1.5 Predictive values

A negative culture does not exclude plague, especially if there is some doubt over the age and storage in transit of the specimen. A positive culture in the absence of symptoms should be treated with utmost suspicion but should not lead to an automatic diagnosis of plague. Strains of *Y. pseudotuberculosis* can be very similar biochemically to Y. pestis, which can be problematic for automated bacteriological test systems.

3.1.6 Suitable acceptance criteria

On blood agar, tiny translucent colonies appear after 24 hours at 37ºC[2, 6]. After 48 hours incubation, colonies range in size from 1-2 mm in diameter, are grey-white to slight yellow in appearance, and are raised with an irregular “hammered copper” appearance[3]; colonies are not haemolytic. On MacConkey agar, the colonies are pinpoint and non-lactose fermenting. They tend to disappear after 2 - 3 days, presumably as a result of autolysis[6]. On CIN agar, colonies of *Y. pestis* will have deep red centres. Growth on CIN agar is slower than growth on blood agar, so colonies will typically be 1-2mm after 48 hour incubation at 25ºC.

3.1.7 Suitable internal controls

Blood agar is a fairly consistent product, but all batches must be validated for suitable performance using a documented internal quality control system. The consistent growth of fastidious and non-fastidious Gram-negative bacilli should be indicative of suitable performance.

3.1.8 Suitable test validation criteria

Biochemical identification can be obtained using API 20E, Microbact 24E or other similar commercial test systems, but these may not always be reliable[5]. The slow-growing nature of the organism necessitates the use of a heavy inoculum in order to detect biochemical activity within 24 hours of incubation. Because the results for most of the tests are negative, it must be confirmed that the isolate has actually grown (on a purity plate). Such tests should be regarded as presumptive identification only. Confirmation may be made using specific bacteriophage analysis and direct fluorescent antibody staining[5]. Specific rabbit antiserum raised against *Y pestis* (in-house), for tube agglutination, is also available.

3.1.9 Suitable external QC program

The RCPA Biosecurity QAP runs virtual modules that cover the identification of *Yersinia pestis* as well as specimen modules that cover near neighbours (*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*).

3.2 Identification of *Yersinia pestis*

There are two main steps likely to occur in the aetiological diagnosis of plague:

- Diagnostic laboratories – In the event of a patient returning from a plague endemic area or a deliberate biohazard release, clinical samples should be sent to a routine diagnostic microbiology laboratory for analysis. As soon as there is any suspicion of the organism being *Yersinia pestis*, all culture work should cease. The cultures should be forwarded immediately to a PHLN laboratory by arrangement with senior PHLN lab staff.
- Reference (PHLN) laboratories – Samples as described above or, where a presumptive diagnosis has already been made and clinical samples are being collected based on this diagnosis, these samples should be referred to a PHLN laboratory for culture and confirmation.

3.2.1 Presumptive identification

Staining – *Y. pestis* is a small (1 x 0.5µm) Gram-negative bipolar staining rod. A presumptive diagnosis of plague may be made if the direct stain made from a bubo, blood, or tracheal or lung aspirates from a patient with compatible clinical symptoms shows small bipolar staining rods. The Wayson stain or Wright-Giemsa can be used to demonstrate the closed safety-pin appearance of *Y. pestis*[5]. NB. Bipolar staining is not specific to *Y. pestis*. It is a feature of other facultative intracellular pathogens such as other *Yersinia* species*, Burkholderia mallei*, *Burkholderia pseudomallei* and *Francisella tularensis*.

- *Y. pestis* expresses an envelope glycoprotein known as the Fraction 1 (F1) antigen; while this antigen is immunologically reactive and has anti-phagocytic properties, absence of this

antigen does not generate avirulence in an isolate. However, given it is highly conserved among *Y. pestis* strains, it is a useful diagnostic target, especially on cultures.

- Identification test strips – a biochemical identification of *Y. pestis* from a suitable sample from a patient demonstrating compatible symptoms provides further presumptive evidence. NB. Biochemical test strips may not provide a clear cut identification.
- A direct fluorescent antibody stain is available in some laboratories.
- Laboratories using a Maldi-TOF instrument should be aware that the standard Bruker database may not provide reliable identification of *Y. pestis.* It is recommended that the biosecurity database that can be purchased as an add-on for the Bruker be used for any suspected isolates.
- PCR assays typically targeting plasmid genes such as *caf1, pla* and *lcrV*, as well as a chromosomal marker specific for *Y. pestis* are available.
- Some PHLN laboratories have access to the GeneXpert or Biofire Film Array system which has a bioterrorism panel that covers PCR identification of *Yersinia pestis.*

3.2.2 Definitive identification

Confirmation of the identity of presumptive *Y. pestis* requires specialised reagents with very limited distribution. These include specific bacteriophage for the lysis test[5].

3.2.3 Predictive values

A negative result in a biochemical test strip does not rule out *Y. pestis* as a diagnosis. Isolates must be confirmed by the methods outlined above.

3.2.4 Suitable test criteria

An isolate that gram stains as a gram negative coccobacillus, exhibits bipolar staining with an appropriate stain, biochemically conforms to*Y. pestis* and gives a positive specific bacteriophage lysis test.

3.2.5 Suitable internal controls

Each batch of reagents tested with positive and negative controls. Results of all QC testing recorded and the records maintained.

3.2.6 Suitable validation criteria

Correct reactions exhibited by a recognised control strain of *Y. pestis*.

3.2.7 External QC Program

RCPA Biosecurity QAP

3.3 Nucleic Acid Detection

Various in-house and commercial real time PCR assays have been developed for the detection of *Y. pestis* in animals using targets such as the plasminogen activator (*pla*) and murine toxin (*mlt*) genes[8]. No commercial validated kits are available for the detection of *Y. pestis* in humans[2].

3.4 Serological Diagnosis

Serology for *Y. pestis* is not widely available in Australian laboratories. However, an "in-house" test, developed from methods recommended by WHO *Yersinia* Reference Laboratory, Paris, is available. Serological diagnosis can be useful in culture-negative cases[6]. Acute and convalescent sera can be forwarded via jurisdictional reference lab to either the Health Protection Agency (HPA) in the UK or the CDC in the US.

4 References

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