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THE LABORATORY DIAGNOSIS OF PLAGUE

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Abstract. Several diagnostic methods for *Yersinia pestis* infections are available including culture, serological, molecular and chromatographic assays. These techniques may be applied to human clinical specimens as well as rodents and other animals sampled in the course of plague surveillance. All the methods have their merits depending on the time available and the information required. Time is of the essence in the diagnosis of plague. Rapid diagnostic techniques capable of detecting *Y. pestis* directly in clinical samples, infected animal tissue and fleas will facilitate speedy diagnosis. The culture methods are reliable but relatively slow and insensitive. The serological ELISA tests are sensitive but in the case of the antibody assay, rely on a detectectable humoral immune response. Antigen can be detected at an earlier stage in the infection. DNA probes lack sensitivity, as 10^5-10^6 organisms are needed for reliable detection. The PCR is sensitive and can detect as few as 10 *Y. pestis* organisms, but cannot distinguish between live and dead bacteria. A recently-developed chromatographic assay is very specific and sensitive and takes only 10 minutes to obtain a result, but thorough field testing is awaited.

Key words: plague, *Yersinia pestis*, diagnosis, culture, serology, molecular biology, polymerase chain reaction.

INTRODUCTION

Plague is an acute systemic infection caued by *Yersinia pestis*. The infection, known since antiquity, has caused three authenticated pandemics. The third pandemic, after a slow advance from its origin on the Central Asian Plateau, reached the Chinese coast in the late 19th century. Throughout history a connection between rat mortality and the appearance of human plague has been noted (WU LIEN-THE *et al.*, 1936). According to excerpts from his diary quoted by LAGRANGE (1926), Alexandre Yersin, after whom the organism was named, detected plague in Hong Kong rats in 1894 and demonstrated the causative organism. Plague spread rapidly around the world from China and new wild rodent plague reservoirs were established in many parts of the world, including South America, the western United States and southern Africa. Plague diagnosis, surveillance and control have subsequently been researched in South Africa for more than 80 years. Laboratory diagnosis of plague is required for both human cases and for plague surveillance. In this brief review we emphasize the methods used in our own laboratory, and mention potentially useful new techniques.

METHODS

Culture

Smears of clinical material can be prepared under almost any circumstances and are very important in the identification of the organism. The plague bacilli can be demonstrated, often in large numbers, in these smears. *Y. pestis* is a Gram-negative cocco-bacillus that ranges from 0.5-0.8 μm in diameter and 1-2 μm in length and demonstrates typical bipolar staining with Grams or Waysons stains. Blood, bubo aspirates and sputum may be cultured for the presence of *Y. pestis*. It is important that clinical specimens are taken before antimicrobial therapy has been started. These samples are plated onto blood agar and incubated at 28° C for up to seven days. *Y. pestis* is a fairly slow-growing organism but will grow quite satisfactorily on a variety of ordinary media. The optimal growth temperature is 28° C, but growth can be obtained at temperatures ranging between 2° C and 45° C. In culture, the organism shows pleomorphism depending on the medium and temperatures used. The colonies of *Y. pestis* are opaque, smooth and round, although in some instances, irregular edges have been noted. When grown in liquid medium *Y. pestis* typically forms a deposit at the bottom of the tube and the supernatant remains relatively clear. *Y. pestis* is catalase-positive but oxidase-negative and is non-haemolytic. The organism is identified in biochemical tests, *e.g.* these tests demonstrate acid but not gas from glucose, mannitol and salicin; there is no acid reaction in sucrose, rhamnose and melibiose and the Voges-Proskauer test is negative. For confirmation *Y. pestis* is lysed by specific bacteriophage at 20° C (BAHMANYAR & CAVANAUGH, 1976). Various virulence factors can be demonstrated by using special media but this is not required for routine identification. Overgrowth of *Y. pestis* by other bacteria may be circumvented by animal inoculation. Ordinary white mice or guinea pigs can be used but WILLIAMS *et al.* (1982a) demonstrated the advantage of using laboratory-reared African multimammate mice (*Mastomys coucha* (Smith, 1834), 36 chromosome species) in the diagnosis of plague. Several atypical phenotypes of *Y. pestis* could be isolated from *M. coucha* after experimental inoculation but some could not be recovered from the other rodent species tested. If the material being cultured is likely to be contaminated with other organisms, it is diluted 1:10 with normal saline and inoculated intraperitoneally into two female *M.coucha*, following intraperitoneal injection of 100 g of Fe_2SO_4. At 48 hours post-inoculation, one of the rats is killed by CO_2 inhalation. Isolation of *Y. pestis* by streaking impressions of freshly cut portions of spleen and liver onto blood agar plates is attempted. The second female rat is killed 72 hours post-inoculation, and isolation of *Y. pestis* is likewise attempted. Triturated reticuloendothelial organs, especially bone marrow, of dead rodents collected in the course of surveillance activities, are usually best inoculated into laboratory animals as a first step in culturing *Y. pestis*. A useful method of transporting dead rodents to the laboratory for attempted plague bacillus isolation, particularly if delays are anticipated, is to first dust them with insecticide and then pack them in salt in screw-cap jars as described by BALTAZARD *et al.* (1956). Great care must be taken to avoid laboratory-acquired infections when culturing *Y. pestis* and all such work should ideally be done in a class 2 biohazard hood. Likewise, fleas from susceptible hosts pose a plague transmission risk for field and laboratory staff and adequate precautions must be taken when working with them.

Serological tests

The first specific plague haemagglutination test was developed by CHEN & MEYER (1954). Passive haemagglutination (PHA) relies on tanned sheep red cells, sensitised with F1 antigen. WILLIAMS *et al.* (1982b) initiated comparative studies using the PHA test and the F1 enzyme-linked immunosorbent assay (ELISA) for the confirmation of clinically suspected human plague; the latter was shown to be much more specific and sensitive. Likewise, SHEPHERD *et al.* (1984) demonstrated that by using an F1 ELISA, the number of non-specific reactors in dog sera were cut by almost two-thirds compared to PHA. PHA does however have the benefit of not requiring genus-specific anti-immunoglobulin, making it simple and cheap, especially for field surveillance where the same test can be applied to many different species of animals. ELISA has the major advantage that specific IgG and IgM titres can be determined, from which conclusions can be drawn regarding the period of time elapsed since infection. In our laboratory serological tests are performed on acute and convalescent (if available) blood specimens for antibody and antigen detection by ELISA, the latter being especially useful when antibiotics have been given before cultures are attempted. The ELISA plates are coated with monoclonal antibody specific for *Y. pestis* F1. The test conformation varies, depending whether antigen capture or antibody detection is to be performed, but it is a straightforward sandwich ELISA technique using peroxidase-labelled anti-immunoglobulin second-step antibodies for visualisation or quantitation by spectrophotometer (WILLIAMS *et al.*, 1986, 1988). Other serological techniques, such as complement fixation, immunofluorescence, agar gel precipitation, latex agglutination, and radioimmunoassay, have been applied to plague diagnosis (ISACSON, 1984). A new approach to F1 antigen detection is a fibre optic biosensor which is claimed to rapidly and safely detect fluorescence of bound immune complexes (CAO *et al.*, 1995).

Molecular methods

Rapid detection of *Y. pestis* by DNA hybridisation is possible if suitable gene sequences such as those encoding species-specific virulence factors can be targeted (GEMSKI *et al.*, 1987; THOMAS *et al.*, 1990). Oligonucleotide probe hybridisation can demonstrate presence of *Y. pestis* in fleas but 10^5-10^6 organisms are needed needed to give reliable results (MCDONOUGH *et al.*, 1988). Molecular typing of *Y. pestis* isolates is used elucidate the epidemiology of present and past plague. Ribotyping, pulse field gel electrophoresis, repetitive sequence analysis, and plasmid restriction profile analysis have been or are currently being applied to *Y. pestis* (GUIYOULE *et al.*, 1994; PRENTICE & CARNIEL, 1995; personal communication, E. Carniel, Institut Pasteur, Paris).

Polymerase chain reaction

Amongst molecular techniques, the polymerase chain reaction (PCR) is an attractive method for detection of *Y. pestis* because it is rapid, highly specific and sensitive, and does not need pure cultures or radioactive reagents. A number of applications of the technique to plague diagnosis or surveillance have been described (CAMPBELL *et al.*, 1993; HINNEBUSCH & SCHWAN, 1993; NORKINA *et al.*, 1994). In our laboratory we are applying

nested PCR to environmental samples in order to study the ecology of *Y. pestis* during and between epidemics (unpublished). One possible disadvantage of PCR is that the viability of the target bacteria cannot be established. As few as 10 colony forming units of the organism can be detected, but as with other applications of the technique, PCR is susceptible to contamination and false positive results. Special precautions are needed to minimise these problems, which may put it out of reach of most routine diagnostic laboratories.

Chromatographic assay

This type of assay can detect *Y. pestis* F1 antigen, and anti-plague IgM and IgG within a short period. A current version is a one step hand-held assay (personal communication, Dr J. Burans, Naval Medical Research Institute, Bethesda, MD). Serum or homogenized sputum is added to the end of a strip on which the stabilised test reagents are present; the sample diffuses along the strip and after 10 minutes the test can be read. For antibody detection, colloidal gold-labelled anti-human IgM or IgG combines with IgM or IgG anti-F1 antibody in the specimen, forming a complex. The complex will combine with the F1 antigen present in the assay strip and produce a visible line. The plague F1 antigen capture chromatographic assay uses a colloidal gold marker attached to detector antibody (anti-F1 monoclonal antibody). This is present in the assay strip and will combine with the capture antibody (rabbit anti-*Y. pestis),* and a complex forms resulting in a visible line. This rapid test holds great promise for use in areas where laboratory facilities are limited, and the results of field trials are awaited with interest.

DISCUSSION

All the identification methods discussed have advantages and disadvantages and some are better suited to certain applications than others. Plague epidemiology, public health resources, and the standard of laboratory facilities vary so much across the plague endemic areas of the world that it is difficult to generalise about the utility of any particular test, apart from emphasizing the importance of basic staining and culture techniques, in conjunction with clinical assesment, in human cases. It is necessary to confirm suspected cultures or specimens by full bacteriological examination. Direct immunofluorescence of bubo aspirate, although a rapid test, has led to a missed plague diagnosis when reported negative, with a fatal outcome (CROOK & TEMPEST, 1992). The problems surrounding the diagnosis of plague during the recent epidemic in India (DAR *et al.*, 1994; KUMAR, 1995) show that clinical laboratory diagnosis is not always optimal, even in countries with a long history of plague. On the surveillance side, there are many techniques available and the local situation and resources will determine which should be used (BAHMANYAR & CAVANAUGH, 1976). The basic surveillance methods are aimed at identifying and enumerating potential mammalian host and flea vector species, and demonstrating plague infections in order to anticipate epizootics before they spill over into human populations. Experience has shown that methods using the capture and bacteriological examination of rodents rarely yield positive results except during actual epizootics. SHEPHERD & LEMAN

(1985) failed to culture *Y. pestis* from any of 4516 rodents of 27 species collected during surveillance activities in South Africa. Serological methods can be used to detect the presence of plague, but when applied to susceptible rodents in plague foci usually yield few positives, mirroring bacteriological results. These species do not survive to carry *Y. pestis* nor develop antibodies, making them unsuitable for monitoring purposes. On the other hand, RUST *et al.* (1971) showed that dogs are relatively resistant to plague and develop high antibody titres, ideal attributes of sentinel animals. Seropositivity rates in dogs can be used to accurately identify epicentres and the direction of spread of outbreaks (ISACSON, 1984). The discovery that sibling species of *Mastomys*, an important bridging host in southern African plague ecology, varied in susceptibility to plague (ISACSON *et al.*, 1983) had major implications for plague surveillance, both in southern Africa and elsewhere. Modern molecular techniques will play an ever more important role in plague surveillance, but cheap, simple and robust versions are needed to make them accessible to many laboratories in plague endemic areas. Whatever techniques are used for plague identification, the safety of laboratory workers must be foremost, and specially designed and dedicated facilities, together with adequate staff training, are the ideal.

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