

BODY OF REPORT

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Objective: The purpose of the present study is to investigate the presence of Pseudomonas pseudomallei (Whitmore's bacillus) in Thailand, its distribution, and its role as causative agent of melioidosis in animals and in man. Melioidosis, a polymorphic disease or group of diseases more or less restricted to Southeast Asia, has usually been regarded as a rare, but highly fatal disease in man. This dictum has arisen largely from the fact that the organism has been isolated from man only infrequently and then, usually at post mortem examination, in association with severe disease. In neighboring Malaysia and Viet Nam, the organism

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is widely distributed in nature and has been isolated quite frequently from the water and soil samples examined by laboratory workers in those areas. Despite the frequency with which the organism may be isolated from the external environment, clinical melioidosis is still rather rarely reported and seems to occur primarily in debilitated or traumatized individuals and those in prolonged and intimate contact with the natural sources of infection. The observations that a number of military men stationed in Southeast Asia have become infected, in some cases to manifest the disease on their return home, suggests that it could become of importance to military operations. The incidence and distribution of the disease among exposed rural populations are largely unknown because of the sparsity of physicians, medical treatment centers and, especially, laboratories without which the diagnosis of melioidosis cannot be accomplished because of the diversity of clinical manifestations which may be presented. A recent serological survey has suggested that subclinical or unrecognized melioidosis may be rather prevalent in Thailand despite the fact that, so far as we have been able to determine, only three cases have been reported in the Thai medical literature. This low incidence of reported melioidosis in Thailand, at the present state of knowledge, may be a reflection of a genuinely low rate of occurrence. On the other hand, it could be a vast underestimate because of lack of orientation of physicians to the problem and a general failure to recognize cases and to identify the organism. It is the objective of the present study to attempt to determine the true state of affairs. To our knowledge, no previous studies of this nature have been attempted in Thailand.

Description: Strains of Pseudomonas pseudomallei from various sources have been collected and examined by biological and biochemical tests to develop procedures which will aid in their recognition. Their pathogenicity for laboratory animals, hamsters and, in some cases, chick embryos, has been tested. Antisera against representative strains have been prepared for evaluation of serological homogeneity, for use as diagnostic antisera in the recognition and identification of new isolates, and for standard reagents in serological tests. Soil and water samples from various areas in Thailand are being tested for the presence of Ps. pseudomallei by hamster inoculation and by bacteriological techniques developed for the purpose. A serological test, an indirect hemagglutination procedure employing chicken erythrocytes sensitized with melioidosis antigens, has been developed and will be used to evaluate the distribution of antibodies reactive with melioidosis antigens among population groups and representative animal species. Attempts are being made to isolate Ps. pseudomallei from clinical specimens.

Progress: Fifty-five stock strains and colonial variants, from several sources (USAMRU, Malaysia; Pasteur Institute, Paris; WRAIR), were examined and found to exhibit considerable variation in their biochemical reactions, particularly in the fermentations. However, all were uniform in their reactions in the following tests which are now used, together with agglutination in pooled melioidosis (rabbit) antiserum, for identification of new isolates:

Kligler's Iron Agar: No change or alkaline reaction in normal incubation time; sometimes acid reaction at slant surface; H₂S, negative.

Motility: Positive in hanging drop although semi-solid mannitol motility agar gives discrepant results.

Cytochrome oxidase: Positive (Pathotec rapid paper test).

Urease: Negative (Medium of Rustigian and Stuart, Baltimore Biological Laboratory).

Citrate: Utilized (One rough variant did not) (Simmons citrate agar).

Indol Prodn: Negative.

Mannitol: Negative.

Nitrates: Reduced.

Gelatin: Proteolysis.

Voges-Proskauer: Negative.

Dextrose: Acid Prodn in broth.

Direct Hemagglutination: Negative (Slide test with chicken RBC).

Variable reactions were obtained with litmus milk and the following carbohydrates: Lactose, sucrose, salicin, mannose, arabinose, dulcitol, inositol and maltose. Variable reactions, as well as variable colonial morphology were given on MacConkey's agar. The latter observations likewise did not always correlate with the lactose fermentation test. Thirty-two strains were tested for sensitivity to antibiotics by the disc method on trypticase soy agar. All were sensitive to: tetracycline (30 mg); kanamycin (30 mg); neomycin (30 μ g); chloramphenicol (30 μ g), and ampicillin (15 μ g). All were resistant to polymyxin (300 units) and colimycin (10 μ g). Two strains were found to be sensitive to dihydro-streptomycin (10 μ g), 9 strains slightly sensitive and 21 strains resistant.

Thirty-six of the 37 strains tested were found to kill weanling hamsters, generally within 2 to 3 days, following intraperitoneal inoculation of 10^3 - 10^4 cells. The gross pathology was generally consistent with a septicemic infection usually with hyperemia or hemorrhagic abscesses at the injection site, local abscesses in the liver and spleen, hyperemic and hemorrhagic intestines and occasional kidney involvement. The lungs were generally not obviously affected. In every instance, direct streaking of a drop of heart blood from moribund animals led to profuse growth of colonies of Ps. pseudomallei (only) on crystal violet glycerol agar or trypticase soy agar. There, they could be promptly recognized by characteristic colonial morphology, odor and rapid agglutination with specific antiserum in slide agglutination tests. Virulence titrations of a few selected strains and colonial variants thereof revealed that hamsters succumbed following inocula of less than

5 viable cells. No significant differences in hamster virulence between smooth and "medusa type" colonies were noted.

The seven strains which were tested were found to be lethal for 13 day chick embryos inoculated allantoically, within 3 to 4 days, with inocula of the order of 100 organisms and more rapidly with larger inocula, although essentially no deaths were obtained within 24 hours even with inocula of the order of 10^7 cells.

Attempts have been made to recover the organism from clinical specimens, primarily enteric, which, up to this time, have been sent to the Department of Bacteriology and Immunology for other purposes. From a total of 274 pseudomonas-like cultures (based on reactions in Kligler's iron agar) tested by slide agglutination in known Ps. pseudomallei antisera, 5 positive reactions were obtained. Four of the five strains were tested and found to be avirulent for hamsters. Biochemical reactions also precluded their identification as Ps. pseudomallei. The fifth strain, which has not been tested in hamsters, has tentatively been identified as Pseudomonas eisenbergii. It is estimated that pseudomonas-like strains are picked from approximately 16.8% of the specimens submitted. (In 5 consecutive series of 100 specimens, pseudomonas-like cultures were isolated from 15%, 16%, 33%, 6%, and 14% respectively). Therefore these observations represent approximately 1700 specimens. The failure to obtain positive results should not be weighted too heavily at this point since the vast majority of specimens have been stools and rectal swabs which are known to be rarely positive even in frank cases of melioidosis. Only 19 blood cultures yielded pseudomonas-like strains which were subjected to agglutination tests during the period under study. The results do speak in favor of the relative specificity of this method of rapid recognition of Ps. pseudomallei and of the relative specificity of the antisera used, but point out, at the same time, that agglutination in the antisera cannot be regarded as an absolute criterion of identity of a strain as Ps. pseudomallei. While all the strains of Ps. pseudomallei we have tested do agglutinate in this serum, occasional organisms which are not Ps. pseudomallei may also do so although, it must be said, the reactions are generally weak and slow compared to the specific agglutination of melioidosis strains.

A total of 99 water samples, 78 of which were from klongs and other sources in the Bangkok-Thonburi-Paknam-Nontaburi area (sent to us for other purposes by the Thai Department of Health), 10 from klongs, ponds and streams in the vicinity of Udorn and Nong Kai, and 11 from the vicinity of Ubol, were tested for the presence of Ps. pseudomallei by intraperitoneal inoculation of 5 hamsters each with 2.0 ml amounts. Heart blood from sick or dead hamsters was streaked on nutrient agar containing crystal violet and glycerol and, in parallel, on trypticase soy agar. Suspicious colonies were tested with melioidosis serum by slide agglutination and subjected to biochemical tests. Similar procedures were employed with 20 soil samples, 10 from the Udorn area and 10 from Ubol, taken at the same sites as the water samples. Soil was suspended at a concentration of 10% in sterile normal saline, shaken thoroughly, allowed to settle and 2 ml of the supernatants were inoculated into each of 5 hamsters. Isolation of Ps. pseudomallei, 2 colonial

types, was accomplished from one source, a soil sample taken near the bridge over the Moon River in Ubol city. Four strains of organisms, which were isolated from sick or dead hamsters, (2 from water samples in the Udorn area, 1 from a soil sample in Udorn, and 1 from a water sample in Nong Kai) agglutinated to some extent in slide tests with melioidosis serum but are not Ps. pseudomallei. Tentatively, they have been identified as Achromobacter species. One water sample from the Bangkok area yielded an organism, by this procedure, which has been identified as Shigella sonnei form I. It should be pointed out that the samples tested so far are not necessarily from the best sources for isolation of melioidosis organisms. It is hoped that during the ensuing months and especially during the rainy season, that more appropriate samples can be collected.

An indirect hemagglutination technique employing chicken erythrocytes sensitized with melioidosis antigens, based upon one proposed by Dr. S.Z. Ileri (FAO Report No. 1707), has been developed and adapted to the microtiter apparatus. An antigen pool for this purpose has been prepared by mixing filtered extracts of sonically disrupted formalinized cell suspensions of 7 representative strains and colonial types in dilutions found to yield the highest titers with a pooled melioidosis antiserum prepared by immunization of rabbits with formalinized cell suspensions. The serum pool has a hemagglutination titer of 1:10, 240 against chicken erythrocytes sensitized with the antigen pool. Normal rabbit sera (pre-adsorbed with normal chicken erythrocytes) have no activity (at 1:10). This antigen will be used in serological tests on human and animal sera to evaluate the extent of distribution of melioidosis antibody and, potentially, in diagnostic tests for detection of chronic melioidosis in patients. In 20 sera, from healthy boy scouts in the Bangkok area, one reactor (titer 1:80) was found. A single serum sample from an American female with a prolonged low grade fever of unknown origin was negative in this test.

Summary: In the early stages of a survey to determine its presence and distribution in Thailand, Ps. pseudomallei was isolated from hamsters inoculated with a soil sample taken near the Moon River in Ubol. Other soil and water samples taken in the vicinity of Ubol, Udorn, Nong Kai and the Bangkok area have been negative for Ps. pseudomallei although, on occasion, other organisms which agglutinate to some extent in melioidosis serum have been isolated from sick and dead hamsters which had been inoculated with the soil and water samples. Attempts to isolate Ps. pseudomallei from clinical specimens have, up to this point, been consistently negative. However, the nature of the specimens thus far examined, predominantly enteric in origin, does not favor a positive result. A serological micro-test for antibodies against melioidosis antigens, an indirect hemagglutination procedure using sensitized chicken erythrocytes, is being applied to determine the distribution of serum antibodies in man and animals in Thailand. Thus far, one positive reactor has been found among 21 human sera tested.

Conclusions: It is too early in the course of this investigation, which was initiated in the last quarter of 1964, to draw any firm conclusions regarding the distribution or importance of Ps. pseudomallei as a causative agent of disease in

Thailand. The organism has been isolated, perhaps for the first time in the external environment in Thailand, from a soil sample taken near the Moon River in Ubol. Caution must be exercised in the identification of Ps. pseudomallei by serological means alone because some heterologous organisms, from soil and water samples as well as from clinical specimens, agglutinate in melioidosis antisera. The relation of these agents to serological reactivity of human and animal sera with melioidosis antigens also needs careful evaluation.