Characteristics of Bacillus Anthracis Isolated From Pigs in Papua and New Guinea.

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INTRODUCTION.

The organism of porcine anthrax in the Territory has previously been examined by 1 *AMVSU* (1946), *and Carne* (1958). *The former isolated the bacillus from cases of anthrax in the Eastern Highlands District and discussed its close resemblance to classical strains of* Bacillus anthracis. *Carne examined subcultures of this organism and on the basis of several factors* (a) *lowered pathogenicity for laboratory animals and* (b) *the failure to form spores, concluded that the New Guinea organism may be an atypical strain of* Bacillus anthracis. *Subsequent work by the same worker established the pathogenicity of the organism for laboratory animals and pigs. Anderson* (1960) *described briefly anthrax of pigs in Highlands Districts of the Territory. Egerton* (1965), *has given more detail on the field aspects of the disease.*

In this paper it is proposed to present results of laboratory investigations into the characteristics of the New Guinea strain of Bacillus anthracis, *and of investigations into the possibility of immunizing against the disease.*

MATERIALS AND METHODS.

UNLESS otherwise stated the organism used in the investigations was derived from a field case of porcine anthrax at Ningil village, Lumi Subdistrict, Sepik District in April, 1962.

Bacteriology.

Media. The medium used for propagation has been tryptose agar (Difco) and stock cultures have been maintained on slopes of the same medium. Stains used for morphological descriptions have been Gram stain, giemsa and crystal violet.

Biochemical Reactions. Media used in the determination of biochemical characteristics were those described by Knight and Proom (1950), and Smith, Gordon and Clark (1946).

Preparation of Spore Suspensions. Heart blood from guinea pigs which died following inoculation with the organism were spread on agar plates and incubated aerobically for 48 hours.

Colonies were scraped off into distilled water suspended evenly by shaking with glass beads and heated at 80 degrees C. for five minutes in a water bath. Following this, spores were washed and centrifuged three times with distilled water. Spore counts were made by the method of Miles and Misra. Suspensions were stored at 4 degrees C.

LD50 *Estimations.* Groups of five mice and five guinea pigs were inoculated intra-peritoneally with measured doses of anthrax spores.

Survival of the Organism. Estimations of the survival time of spores and vegetative organisms in various environments were made.

Soil. 10 g. soil samples (5 per cent.-6 per cent. water content) from the enzootic area, were inoculated with 1,000,000 spores in 1 cc. water. Qualitative estimations of survival were made at monthly intervals and at twelve months, counts of surviving spores were made. Sterilized and non-sterilized duplicates of soil samples were used, and the samples maintained at room temperature (18-28 degrees C.).

Carcases. The carcases of five mice which died following anthrax inoculations were kept at 4 degrees C. and examined at intervals for the persistence of the organism. The thoracic and abdominal cavity of one mouse was opened and kept at 30 degrees C. for two days before placing in the refrigerator.

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Immunity Studies.

Goats. Two adult goats were vaccinated with 0.5 cc. McGarvie Smith anthrax vaccine. Fourteen days later the goats were challenged, one intravenously the other subcutaneously with 20,000 spores of a virulent suspension. Two controls were inoculated with the same dose by the same routes.

Pigs. (*a*) *Establishment of Immunity.* Four native Berkshire cross pigs were vaccinated subcutaneously with 0.5 cc. McGarvie Smith anthrax vaccine. Three weeks later these animals and four unvaccinated controls were challenged intravenously with virulent anthrax spores.

(*b*) *Duration of Immunity.* Twenty-one pigs, Berkshire cross, were vaccinated at about three months of age with 0.5 cc. McGarvie Smith vaccine subcutaneously behind the ear. At intervals of three months, groups of these pigs were challenged with intravenous doses of spores shown to be lethal for an unvaccinated control at the time of challenge. Other pigs from the station herd at Goroka vaccinated six months previously to the experimental group were also challenged at quarterly intervals.

Experimental per Os Infection of Pigs.

Fifteen Berkshire pigs 4-6 months old were divided into three groups of four and one of three animals. In each group the pharynx of two animals was lacerated under light nembutal anaesthesia. Doses of *Bacillus anthracis* ranging from 10^5 spores to 10^8 spores were introduced into the buccal cavity of all pigs under light anaesthesia.

RESULTS.

Bacteriology.

Morphology and Staining Reactions. In smears from lesions in pigs *Bacillus anthracis* is a large rod usually with square ends which occurs singly and is surrounded by a loose capsule. When stained by the Gram method, the bacillus has an irregular foamy appearance. The capsule is best demonstrated with giemsa stain. When this is used the loose envelope-like nature of the organism is apparent. After exposure to the atmosphere recently isolated strains readily form spores, usually within 24 hours. The spores show as stain resistant sub-terminal structures which do not alter the shape of the bacillus.

Cultural Features. B. anthracis grows readily on nutrient agar. At 24 hours the colonies have a ground glass appearance and typically the margins of the colonies are irregular consisting of entwined chains of the organism growing from the periphery of the colony. On blood agar plates haemolysis does not occur. In gelatin, incubated at 20 degrees C. an inverted fir-tree type of growth occurs.

When *B. anthracis* is grown on 10 per cent. serum agar plates in an atmosphere of about 10 per cent. CO_2, mucoid colonies form. The mucoid nature of the colonies is apparent when they are touched with a loop, and is derived from the exaggerated capsule formation in organisms grown under these conditions. The ability to form spores under otherwise suitable conditions is lost after five or six subcultures away from an infected animal. Sporulation can be reinduced by passage of the organism in massive doses through a susceptible animal.

Biochemical Reactions. Acetoin is produced from Voges-Prosgauer reagent in which phosphate is replaced by sodium chloride. Tests for the digestion of starch, casein, gelatin and egg yolk are all positive. Nitrates are reduced and the Gibson Abdel Malek test is negative. Glucose, but not arabinose or xylose is fermented when included in an inorganic base medium.

Pathogenicity. (*a*) *Mice. Table* 1 below gives details of the inoculation of groups of five mice with varying doses of spores.

Table 1.

Inoculation Dose Spores	Deaths Day 2	Day 3	Day 4	Total
20,000	5/5	....	....	5/5
10,000	4/5	....	....	4/5
1.000	2/5	1/5	1/5	4/5
100	1/5	2/5	....	3/5
10	....	1/5	....	1/5

Using Thompson's moving average method of estimation of LD50 doses, the following results were obtained :—

2 days—1,340 spores.

3 days—112 spores.

4 days—76.4 spores.

(*b*) *Guinea Pigs.* A similar estimation using guinea pigs, indicated that the LD50 for this species was of the order of 4,250 spores at five

days post inoculation with the culture used. Individual doses of as low as 200 spores have however been shown to be lethal for guinea pigs.

(*c*) *Goats.* 20,000 spores inoculated intravenously and subcutaneously into two goats resulted in their death at four and five days respectively. Full autopsies were not carried out. It was shown that a terminal bacteraemia was present in each case.

(*d*) *Man.* Five days after the limited post mortem examination carried out on the goats above, the author developed a lesion on the dorsal aspect of the right index finger. The lesion arose as a small red bleb containing reddish serous fluid. Over the succeeding three days the lesion increased in size and a peripheral erythema occurred. Smears at this stage demonstrated the presence of fragmenting, irregularly staining Gram positive bacilli. An overnight culture resulted in the growth of typical anthrax colonies. By this time a lymphangitis involving the medial aspect of the forearm and the axillary lymph node had developed. Treatment with tetracycline was rapidly effective.

(*e*) *Pigs.* Pigs have proved difficult to infect other than by the intravenous route. It has been shown that 10^8 spores of a culture of which 1,000 spores are lethal for guinea pigs will regularly kill pigs when introduced intravenously. Death in these cases occurs in four days. Of nine susceptible pigs inoculated with doses of this order all have died.

Subcutaneous inoculation of pigs with quite large doses has variable and inconsistent results. Of five pigs inoculated subcutaneously with about 10^9 organisms of a Goroka isolate, two animals died and three recovered.

Attempts at experimental per os infection have been unsuccessful. Spore doses ranging from 10^5 to 10^8 were given to fifteen pigs, eight of which had a deliberately lacerated pharynx. No clinical signs of illness developed. Examination of the pharynx of pigs which had been lacerated showed apparent healing 48 hours after treatment.

Immunity.

Goats. 0.5 cc. McGarvie Smith spore vaccine was shown to protect two goats against challenge with a dose of spores which killed unvaccinated controls. The latter died four and five days after challenge, respectively.

Pigs.

(*a*) *Establishment of Immunity.*

It was demonstrated that three weeks after subcutaneous inoculation with spore vaccine a solid immunity was established. Two young adult pigs inoculated with 0.5 ml. vaccine resisted challenge by intravenous inoculation of 6×10^6 virulent spores. Two unvaccinated controls of similar age and body weight died within 72 hours after treatment with the same challenge. Two weaners immunized with 0.25 ml. vaccine similarly resisted challenge while controls died. The challenge dose in this case was 1.5×10^6 spores.

The vaccinated animals showed no signs of illness in the period under observation (six weeks) after challenge. Signs of illness appeared in controls within 24 hours of challenge.

(*b*) *Duration of Immunity.*

Pigs vaccinated in January, 1963, were shown to have maintained immunity up to September, 1964. Pigs which resisted challenge were removed from the experimental group. A sow aged about three years which was vaccinated in July, 1962, did not resist a challenge dose administered in June, 1964. *Bacillus anthracis* was re-isolated from the oedematous lungs and pleural fluid of this animal which died seven days post challenge.

Survival of the Organism.

Soil. Spores inoculated into soil samples at the rate of 100,000 per gram survived for at least twelve months. There was evidence of a decline in spore numbers especially in more acid soils. There appeared to be no difference in the survival rate in autoclaved and non-autoclaved soil samples. Details are presented in *Table* 2 below.

Table 2.—**Spore counts/gram of soil samples inoculated 12 months previously with 100,000 spores per gram.**

Soil Sample	pH	Autoclaved	Not Autoclaved
7191	4.9	8,000	4,000
7221	5.2	2,400	4,000
7330	5.8	22,000	8,000
7291	6.0	5,000	6,000
K1	7.8	400,000	1,200,000

The first four samples were from enzootic areas in the Highlands. Sample K1 was from the Veterinary Station at Kila Kila near Port Moresby. In the latter there is evidence of multiplication of the spores.

Carcases. (*a*) *Unopened.* It was not possible to culture *B. anthracis* from unopened carcases of experimental mice kept for longer than three days at refrigerator temperature.

Attempts at culture from the heart blood, liver and spleen were consistently unsuccessful.

(*b*) *Opened Carcases. B. anthracis* was cultured at regular intervals for up to three months following death from carcases in which sporulation had been induced by exposure to the atmosphere.

DISCUSSION.

Bacillus anthracis isolated from pigs in Papua and New Guinea behaved similarly in the laboratory to strains isolated in other countries. When freshly isolated it was morphologically the same and it sporulated readily. Its biochemical reactions were identical with those described by other workers.

In mice and guinea pigs small doses of spores were lethal. It was shown to be pathogenic for a fairly wide range of other hosts including man. The difficulty in setting up experimental infections in pigs other than by using heavy intravenous inoculations has confirmed the experience of other authorities. Pigs are recognized as having considerable resistance to anthrax (Sterne, 1959, Ferguson and Bohl, 1959). In other countries anthrax in pigs is usually associated with the ingestion of large numbers of organisms or viable spores, in contaminated feedstuffs.

In Papua and New Guinea, feeding of artificial rations to enclosed pigs is not commonly practised in the enzootic area. Infective doses for swine in the field must come from either the soil when pigs are grazing or through the consumption of pigs which have died of the disease. The infective dose for pigs *per os* would appear to be so high that it is difficult to believe that contaminated soil would be able to set up the infection. For this reason the ingestion of carcases is considered to be the usual source of infection. It is possible that in particular areas organisms may multiply in the environment but this would be contrary to accepted views on the life history of *B. anthracis.* Minett and Dhanda (1941) showed that, while in moist sterilized soils multiplication and subsequent sporulation occurred, *B. anthracis* did not multiply when in competition with soil and/or water organisms. These authors concluded that *B. anthracis* was an obligatory parasite.

A further complication to the understanding of the epizootiology of anthrax in pigs is that the only type of the disease seen here has been the pharyngeal type in which there is usually a localization of the organism in the throat area. The carcase of such a pig would not provide such a great source of infection as a herbivore which died of anthrax. It is possible that the septicaemic form of anthrax does occur in the Territory and is not recognized because of the absence of the characteristic symptoms seen in the pharyngeal form.

Under field conditions it might be postulated that the resistance of the native pig, on a subsistence diet and heavily parasitized, may be considerably lower than in experimental animals. Certainly when a favourable set of circumstances exists epizootics of anthrax result in the loss of many animals.

It has been shown that commercially available spore vaccines are quite effective in protecting against anthrax in pigs in this Territory. Immunity persists for at least eighteen months. Within the enzootic areas it should be possible, by widespread vaccination, to reduce the losses caused by anthrax. Vaccination campaigns should be instituted during the dry season in order to establish immunity before the onset of the wet season with its greater frequency of the disease.

Sterne (1959) has suggested that total eradication is possible if sufficiently intensive vaccination is carried out even in areas where husbandry is primitive. In Papua and New Guinea the biggest difficulty would be to get the co-operation of native owners. Experience with limited vaccination campaigns in the past has been disappointing. Activity at the present stage of development should be directed towards demonstrating at the field level that vaccination is effective. The realization of this by native owners which may be concurrent with some development of husbandry methods will allow a more intensive application of compulsory vaccination campaigns in the future.

There has been a tendency in the Territory to undertake localized vaccination to prevent the spread of an outbreak. Success has been claimed for vaccination of this type but has not been proven. Nicol (1933) stated that in South Africa this method of immunization proved disappointing. In the Territory environment it is considered that every effort should be made to avoid vaccinating pigs possibly in incubation. The loss of pigs following vaccination will bring the vaccine into disrepute and increase the difficulty of future full scale vaccinations.

SUMMARY.

Bacillus anthracis isolated from cases of porcine anthrax in Papua and New Guinea had the morphological and biochemical characteristics of classical strains of the organism. Its pathogenicity was of the same order and its host range similar.

There are some unanswered questions regarding the transmission of the disease. Commercially available vaccines are effective in the establishment of immunity. Use of these vaccines in the Territory has been discussed briefly.

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