AN ANAEROBIC ORGANISM ASSOCIATED WITH "BONE-TAINT" IN BEEF

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(With Plate I)

Introduction

Through the courtesy of the Director of the Norfolk Agricultural Research Station, Sprowston, Norfolk, we were able to examine in the summer of 1938 an outbreak of "bone-taint" in home-produced beef. The cattle were killed in a local slaughter-house, and the sides hung to cool and set for about 24 hours above the killing floor, after which they were transported to a refrigerator. Some 2–3 days later, on cutting up the hind quarters, a most offensive odour was detected emanating from the deep parts of the tissues near the bone. There was no obvious change on the surface of the quarters before cutting, nor for some considerable distance below the surface but the tissue adjacent to the bone was greenish-brown in colour. Selecting the worst case, the femur and anterior portion of the pelvis together with as much undisturbed adjoining tissue as possible (without opening the hip joint)[1] were removed to the laboratory for examination.

Preliminary examination of the sample

On dissecting away the tissue round the head of the femur a brown slime was seen under the periosteum for nearly the whole length of the bone, but thickest round the neck. The tissue for half a centimetre or so near the bone was also discoloured. Smears from the slime showed large sporing rods in great numbers usually about $6 \times 2\mu$, but some up to 10μ, long. This organism was also abundant in the synovial fluid, in the articular cartilage of the head, and in the marrow in the shaft of the bone. Samples from various areas inoculated into Robertson's medium and Veillon broth containing tissue were cultured anaerobically at 37° C. The cultures contained the large rod mixed with cocci and possibly other rods. Since smears and sections of the tissue showed only the large rod in great numbers it was assumed that other organisms found in the original cultures were contaminants and steps were taken to obtain the large rod in pure culture.

[1] The word "joint" is used throughout in the anatomical and not the butcher's sense.

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Histological examination

Samples for histological examination were removed with aseptic precautions, from the following sites:

(1) Periosteum with attached muscle.

(2) Tissue surrounding the iliac artery, about 8 cm. from the shaft of the bone.

(3) Muscle and connective tissue approximately 5 cm. from the head of the femur.

(4) Bone with portion of muscle attached.

Sections of all samples consistently contained the organism in large numbers. Within the iliac artery the bacilli could be seen in the blood clots, and in these clots the organisms were usually in short chains and did not show the unstained bands characteristic of those found elsewhere. In muscle the bacilli tended to be concentrated along the bands of connective tissue and between the muscle bundles. More rarely they were found lying between the individual muscle fibres. Small arteries within the muscle bundles were usually packed with the organisms. In the periosteum the bacilli were widely distributed throughout the whole section, in the compact bone tissue they occurred in small clumps throughout the Haversian canals, while near the centre of the shaft they were found intermixed with marrow cells.

Sections of a superficial lymph gland (probably inguinal) from the same material, made after storage at $-20°$ C. for several months, did not show the organisms.

Isolation of the organism

As mentioned previously, the bacillus was first cultivated directly from the infected tissue in Robertson's medium, in company with some non-sporing organisms. After the formation of spores the cultures were heated at 80° C. and subsequent cultures produced the large rods only. Efforts to obtain a pure culture were at first unsuccessful as the organism could not be induced to grow on solid media in deep tubes or poured plates incubated anaerobically in a McIntosh and Fildes jar. Single sporing rods isolated by means of a micromanipulator failed to grow. Success was achieved only when a liver infusion peptone medium containing 1 % agar described by Turner and Davesne (1927) was used. In this medium stab or shake cultures gave colonies regularly within 24 hours at 37° C., growth occurring to within 1 cm. of the surface in deep tubes incubated aerobically. The colonies so formed were 2–3 mm. in diameter and woolly in appearance, the growth being rather diffuse round the periphery of the colony. Similar but somewhat smaller colonies were formed in 2 % agar. The medium was disrupted in several places, particularly when a heavy inoculum was introduced. Surface colonies have not been obtained.

Description of the organism

The organism is a spore-producing rod with rounded ends varying in length from 4–10 μ and in breadth 1·3–2·0 μ, but with average dimensions of 6–7 × 1·5–1·7 μ when smears from liver infusion cultures stained with fuchsin are examined. The spores are oval, subterminal, and markedly distend the rod. In young cultures in liquid or solid media the elements stain uniformly and frequently adhere in long chains. In older cultures in liquid media the organisms most commonly occur singly and on staining show a characteristic colourless or faintly stained band in the centre of the rod (see Pl. I, fig. 1). Smears made from 24 hr. cultures show the bacilli to be predominantly but not all Gram positive, but in older cultures the organisms are Gram negative. Motility has been observed only in cultures 8–10 hr. old. Flagella have not been demonstrated.

Cultural characters

The organism is a strict anaerobe which grows poorly, or not at all, in most media. Growth is slow in Robertson's medium, faster in brain medium and most luxuriant in liver infusion peptone medium. The last is the only medium found that will give growth without the addition of tissue. Added tissue, such as minced ox muscle or liver, is digested only to a very slight extent. In the liver infusion gas production is vigorous, and the organisms are deposited at the bottom within 48 hr., the supernatant fluid becoming clear. Spores are formed in most media but they are rarely numerous in less than 4–5 days.

The optimum temperature is close to 37° C. Growth occurs at 18° C., but not in 30 days at 15° C.

In a liver infusion medium, buffered with $M/10$ phosphate, good growth occurred from *p*H 5·8–7·6. There was feeble and limited growth at *p*H 5·5. The optimum *p*H zone is approximately 6·5–7·0.

Nutrient gelatin is liquefied in 24 hr. at 37° C. but only when tissue is added to give growth: coagulated ox serum was not liquefied. Similarly in milk there was no growth without the addition of tissue; slight acid production occurred in 5–7 days at 37° C. with no marked change during further incubation for 20 days.

In Spray's (1936) medium with added tissue and Robertson's medium, nitrite could not be detected by the Griess-Ilosva method at any time between 1 and 12 days. Nitrate was not destroyed. Media containing lead acetate were blackened slightly, but brain medium was not darkened. The Erhlich test for indol was positive in a sugar-free medium incubated for 10 days at 37° C.

Sugar fermentations were studied in the semi-solid medium of Spray(1936) but it was necessary to add tissue to induce growth. The production of acid was taken as the criterion of fermentation, since good growth in control tubes without added sugar showed no appreciable change of *p*H. Dextrose and

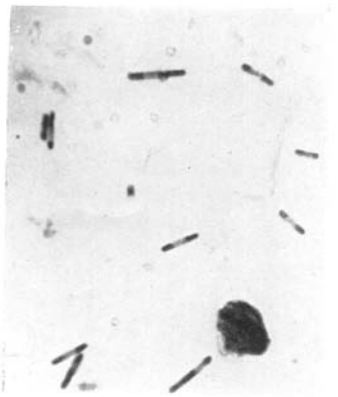


Fig 1 Smear from synovial fluid (×980) Showing characteristic banded appearance of rods

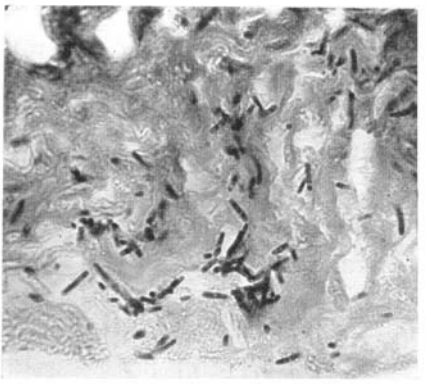
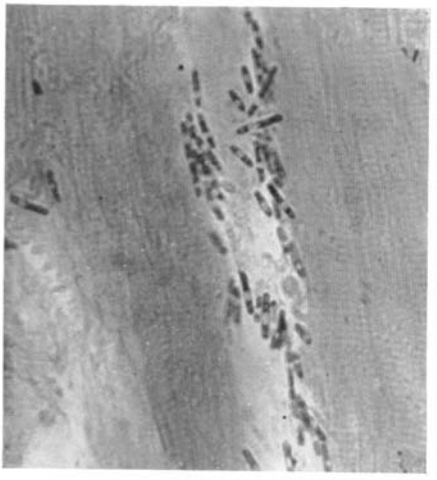


Fig 2 Section of periosteum (×420)



ig 3 Muscle fibres 5 cm from shaft of femur (×980). Mostly banded rods but a few show spores

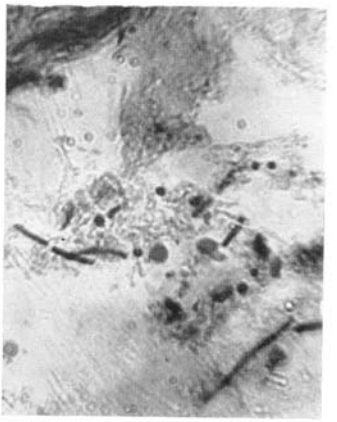


Fig 4 Non sporing rods in blood clot (×950)

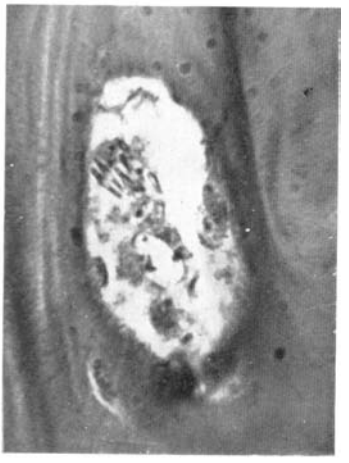


Fig 5 Haversian canal. Spore bearing rods (×860).

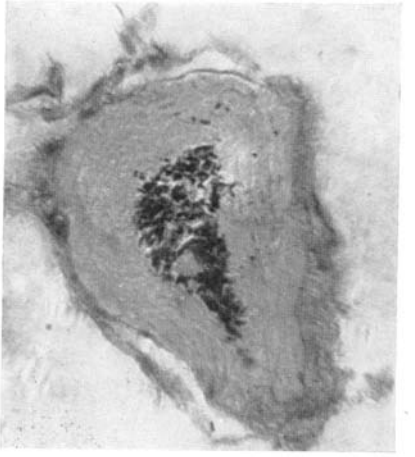


Fig. 6 Small blood vessel (×330) Showing densely packed organisms

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mannose were the only sugars readily fermented, the *p*H being reduced from 7·4 to about 5·0 within 48 hr. Sucrose and levulose were less easily attacked, each giving a final *p*H from 5·5 to 6·0. Galactose and maltose were apparently fermented to a very slight extent, the acid produced being insufficient to make the reaction acid to bromthymol blue. Lactose, glycerol, mannitol and salicin were not attacked.

PATHOGENICITY

We are indebted to Prof. T. Dalling of the Institute of Animal Pathology, Cambridge, and Dr E. T. C. Spooner of the Pathological Laboratory, Cambridge, respectively for the following observations:

(1) The organism has so far failed to produce toxin of any kind and is completely non-pathogenic for the usual laboratory animals.

(2) The intramuscular injection of 0·1 ml. of a 4-day culture in Robertson's medium into the thighs of two mice failed to produce any evidence of disease in either.

PROBABLE IDENTITY OF THE ORGANISM

The organism is undoubtedly a *Clostridium* having saccharolytic and relatively feeble proteolytic properties. Apart from certain sugar fermentations its cultural characters agree closely with those described in detail by Turner & Davesne (1927) for a strain of *Clostridium oedematiens*. Comparison with other Clostridia showed the bacillus to resemble a strain of *Cl. oedematiens* [*B-novyi* No. 3264] obtained from the Lister Institute. In particular it may be mentioned that both organisms exhibited the characteristic banded appearance, and both produced a characteristic and apparently similar odour in culture. Our organism was not pathogenic by intramuscular inoculation into mice, but, when tested, it had been in culture for five months. Loss of pathogenicity by *Cl. oedematiens* has been described by Weinberg (1937), and he also gives details of several related strains non-pathogenic to laboratory animals, amongst which is an organism isolated by Kraneveld in the Dutch East Indies from the bone-marrow of native cattle suffering from osteo-myelitis. Nevertheless we have so far been unable to obtain any decisive evidence as to the identity of the organism.

EXAMINATION OF THE HIP JOINT IN NORMAL CARCASES

In an attempt to throw some light on the possible mode of infection in cases of bone-taint it was considered desirable to examine the hip joints of apparently normal quarters of beef for their bacterial contents. Hip joints from home-killed beef, protected by at least 5 cm. of muscle, were taken 2–3 days after slaughter, and transported entire to the laboratory. (In cutting up a carcase the butcher divides the pelvic girdle along the pubic symphysis and then makes a transverse saw cut through the shaft of the ilium about 2–3 cm. from the acetabulum.)

The superficial tissues were seared with a bunsen flame and heavy searing irons and the joint opened aseptically in an inoculating chamber. Four samples of the overlying tissues were transferred to nutrient broth to test the efficiency of the sterilizing process, two being incubated at 37° C. and two at 20° C. The joint was then opened and the synovial fluid withdrawn by a Pasteur pipette. The yield of the fluid was usually about 3 to 4 ml. but varied between 0·5 and 10 ml. It was sometimes clear and straw coloured, but was frequently so mixed with blood as to have a deep red colour. Its approximate *p*H, as determined by indicator immediately on withdrawal, was from 6·8 to 8·0, with a mean of *p*H 7·4. Samples of the synovial fluid were inoculated into Robertson's medium, deep Veillon agar, semi-solid liver infusion agar (0·1 %) and bulk samples of the fluid alone incubated anaerobically. Direct examination by dark ground illumination was made at the time of withdrawal. Samples of connective tissue and cartilage round the acetabulum, about six from each specimen, were also inoculated into the above media. All tubes were examined daily for 3–4 days. Although deep tubes etc., were incubated aerobically at 37° C. it was shown that conditions in all three media were sufficiently reduced to allow of growth of strict anaerobes. Some forty joints have been examined. The results of the examination of forty joints are shown in Table I.

Table I. *Cultural examination of the hip joint in normal carcases*

Sample	Incubation temp. °C.	Tubes showing growth	Tubes remaining sterile	% tubes with growth
Surface tissue	20	17	59	22
Surface tissue	37	24	52	32
Acetabular* tissue:				
In Robertson's medium	37	32	54	37
In Veillon agar	—	10	76	12
In liver infusion	—	5	19	21
In all media	—	47	149	24
Synovial fluid:				
In Robertson's medium	—	4	31	11
In Veillon agar	—	2	40	5
In liver infusion	—	2	26	7
In all media	—	8	97	8

* Acetabular tissue = cartilage, connective tissue etc. removed aseptically from the socket.

In no case was there any bone-taint, nor were any lesions or abnormalities, other than in one case, a somewhat higher attachment of the *vastus intermedius* than is normal, observed. Two joints were found to be infected by organisms in pure culture according to the cultural tests and in one of these the organism was observed directly in the synovial fluid to the extent of approximately 10^9/ml. In one the organism appeared to be a *Streptococcus*, in the other a *Proteus*: they are described later. Of the other joints 2 or 3 were of doubtful sterility, but the remainder were almost certainly sterile. No sporing anaerobe was found resembling that from the case of "bone-taint", nor indeed any other obligate anaerobe. Six of the joints came from animals of similar breed and on the same diets as the one giving "bone-taint", but collected from Sprowston some ten months later.

The searing was apparently insufficient to destroy all the micro-organisms contaminating the exposed tissues as roughly one-quarter of the tubes inoculated with superficial tissue gave growths. Superficial samples generally included tissue to a depth of 2–3 mm. and at that depth the temperature may not have been high enough to destroy all bacteria. It is also evident that the percentage of growths obtained from the acetabular tissue was three times as great as that obtained from inoculations of synovial fluid. This is interpreted to be due to the greater difficulty of removing the tissue asceptically. The organisms found in the various growths were always aerobes or facultative anaerobes and it is considered that they were probably laboratory contaminants, or obtained from the insufficiently sterilized surface of the tissues which were heavily infected before reaching us. For example, of twenty-eight tubes of Robertson's medium, fifteen contained micrococci alone, nine a mixture of non-sporing rods and cocci and four non-sporing rods alone. Moreover, most of the growths were obtained in liquid media in which convection currents favoured the growth of aerobes.

It seems probable therefore that in the ox the joint of the head of the femur and the acetabulum is usually sterile 2–3 days after slaughter.

ORGANISMS FROM INFECTED JOINTS

The two infected joints appeared to contain pure cultures, the first a coccus, the second a small rod. Both organisms were purified by repeated replating.

Organism A.

Morphology. Gram-positive *Streptococcus* usually in pairs from agar culture, often lanceolate and resembling the *Pneumococcus*. Some rod-like forms, in short chains of 6–8 elements, occurred.

Culture. Grows sparsely on ordinary nutrient agar but better growth is obtained on Veillon agar and blood agar. Produces green coloration and α-haemolysis on horse blood agar. Broth uniformly turbid, slight sediment easily resuspended. Organism not motile. Not bile-soluble. Gelatin not liquefied. Grows on agar containing 10 and 40 % ox bile, long chains of some hundreds of elements being obtained on the last medium. Milk is acidified and clotted in 3 days at 37° C. with reduction of litmus and methylene blue to within 1·5 cm. of the surface. Nitrite was not formed in Veillon broth nor was nitrate destroyed. Grows at 20° and 37° C. but not at 45° C. Broth cultures do not survive heating for 30 min. at 60° C. Acid, but no gas, is formed in dextrose, sucrose, levulose, maltose, mannitol, trehalose, sorbitol and salicin but not in raffinose and inulin. Dextrose broth (1 %) is acidified to *p*H 4·5. Sodium hippurate is not hydrolized in 3 days. Nitrates are not reduced.

This organism thus appears to be a *Streptococcus* of intestinal origin (*faecalis-lactis* group) but it is not altogether typical in that it is not heat resistant (Topley & Wilson, 1936).

Organism B.

Morphology. Small gram-negative non-motile rod, about $2\mu \times 0{\cdot}5\mu$, occurring singly and in pairs.

Culture. Luxuriant growth on plain agar slant at 37° C. Colonies up to 5 mm. diameter, thick, convex, creamish-white and glistening with entire edge. Facultative anaerobe. Acid and gas in dextrose, sucrose, galactose, levulose, mannitol, sorbitol, mannose, xylose, salicin and acid, without gas, in glycerol and dextrin. Lactose and dulcitol are not attacked. Gelatin is liquefied with napiform liquefaction in 15 hr. at 20° C., the liquefaction becoming stratiform. Nitrates are reduced. Litmus milk is clotted in 2 days at 37° C.

This organism thus appears to belong to the *Proteus* group, and in fact, except for its non-motility, closely resembles certain strains isolated previously from rotten eggs (Haines, 1938).

Discussion

In the past "bone-taint" in beef has been a widespread source of spoilage. It has, however, been virtually eliminated where the dressed sides can be rapidly cooled in refrigerated hanging rooms (Moran & Smith, 1929). Cases of taint still occur sporadically in this country in beef prepared without the aid of refrigeration. Under these circumstances the temperature at the os pubis usually exceeds 20° C. 24 hr. after slaughter (Moran & Smith, 1929), conditions at this site thus being favourable for anaerobic multiplication. The limited number of readings we were able to obtain in the present case show that the temperature of the deep portions of the hind-quarter probably remained above 18° C. for at least 40 hr. after slaughter (Table II).

Table II. *Internal and external temperatures during cooling*[1]

Hours after slaughter	Air temp. °C.	Surface of tissues, °C.	Deep in hind-quarters, °C.
2	19·5	22·9	38·6
2½	18·7	22·8	38·0
3	18·5	21·9	37·6
6	17·7	19·6	34·7
9	17·8	19·2	31·1
12	16·7	18·4	28·4
21½	14·1	15·8	24·2

We have no information on the rate of growth in beef tissues of the *Clostridium* isolated from the tainted quarter, but its rate of growth in liver infusion medium suggests that it may have passed through twenty-five to thirty generations during cooling of the beef. The tainted quarter probably contained 10^{11} bacteria ($=2^{37}$ approximately). If the taint had been caused by a single bacillus germinating *post-mortem* the required number of generations would therefore be thirty-seven, a number somewhat in excess of the twenty-five to thirty mentioned above. Although this estimate is only a rough approximation, it is considered likely that the initial infection was considerably greater than unity.

[1] We are indebted to Mr Owers for the temperature readings.

There is no direct evidence as to how these organisms, presumably derived from the alimentary canal, reached the neighbourhood of the joint. Sometimes such organisms appear to enter the blood stream in small numbers during life (Cobbett & Graham-Smith, 1910; Haines, 1937), particularly in debilitating diseases. Since the great majority of the joints examined were bacteriologically sterile, six of the forty being derived from cattle at the Norfolk Agricultural Research Station reared under identical conditions to the tainted ones but slaughtered 10 months later, and since even in the tainted quarters there was no evidence of any inflammatory reaction, it seems that there cannot have been any great infection of the joint during life.

SUMMARY

1. A *Clostridium*, non-pathogenic to laboratory animals and not producing toxin, but in some ways resembling *Cl. oedematiens*, was isolated from the hip joint in a case of "bone-taint" in beef.

2. It was demonstrated in the synovial fluid, the periosteum, the Haversian canals, the bone marrow of the femur, and in associated muscular and connective tissue and blood vessels.

3. Examination of forty hip joints from normal carcases indicated that thirty-eight were sterile, one heavily infected with a *Streptococcus*, possibly of the *faecalis* type, and one with a rod, probably a member of the *Proteus* group.

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