Bacteriology of the Healthy Experimental Animal. (20168)

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The bacterial flora normally resident in the tissues of the abdominal viscera vary considerably among different species. In appropriate circumstances, the interpretation of experimental data should take account of the varying incidence and variety of such resident bacterial flora. For this reason, the bacteriology of the organs and tissues of normal dogs, rabbits, guinea pigs, rats and hamsters was studied and is herewith reported.

Technic. Healthy animals, resident for at least one week in a clean, air-conditioned animal farm, were killed by intramuscular nembutal or ether and autopsied immediately after death. Tissue specimens were taken as follows: The skin was shaved, washed with aqueous zephiran, dried and then burned with a gas flame until carbonized. The burned surface was removed with sterile instruments and a piece of the skin beneath excised for culture. Connective tissue, fascia and muscle were burned and treated similarly. The thoracic and peritoneal cavities were then opened with sterile instruments. All organs to be cultured were flamed *in situ* and cubes of tissue, ½-1 cm in length, were removed, flamed briefly, streaked with some pressure over Endo and blood plates and then plunged into 10 cc of thioglycollate (Brewer) covered with paraffin. The blood plates were incubated in a Brewer jar. Blood, bile, and urine were drawn with sterile syringes after flaming the vessel wall, and cultured similarly. Tubes and plates were observed up to 96 hours, if no growth occurred earlier. Aerobic organisms found were identified by routine bacteriological methods. When smears of growth in thioglycollate showed large gram-positive bacilli, aerobic subcultures on agar and blood plates were made to exclude aerobic bacilli of the subtilis group. These large gram-positive bacilli always proved to be anaerobic. Fifty of these strains were identified by comparison of the detailed test data against those of anaerobic strains classified in Bergey's Manual. One hundred and twenty additional strains of clostridia were recovered and were tentatively classified by responses to the following tests: hanging drop, gram and capsule stains, blood plate, litmus milk, and sugar fermentations. All seemed to belong to the first group described below. The high frequency of clostridia in the dog's tissues led to repeated checking of instruments, test tubes, culture media, etc. These were always found sterile.

Results. Incidence of Bacteria in Different Species. Dogs. See Table I. The aerobic bacteria found were always gram-negative bacilli. They were found only rarely (4% of 228 cultures) and only in subcultures from thioglycollate in very small numbers. Of the 9 positive cultures, 8 were *E. coli*, 1 was *Pseudomonas*. Smears from some 50 other thioglycollate cultures showed gram-negative bacilli in addition to large gram-positive bacilli. But since aerobic subcultures of these were sterile, the gram-negative bacilli may be considered non-viable, or as belonging to the genus *Bacteroides* a normal inhabitant of the intestines. No attempt has been made to identify this species. Of the 228 cultures, 61% were positive for anaerobic, gram-positive spore bearers. If we exclude all cultures of body fluids, which were uniformly sterile, this percentage is 80. *Rabbits.* In 159 cultures from rabbits, 29% of all anaerobic cultures were positive for clostridia, but 40% of cultures from tissues were positive for anaerobic spore-bearers. Only 1 positive aerobic culture was obtained. This was from a liver and showed *E. coli* and *A. aerogenes. Rats, Guinea Pigs, and Hamsters.* 75 cultures from 15 rats, 60 cultures from 10 guinea pigs, and 70 cultures from 10 golden hamsters, both aerobic and anaerobic, were all sterile (Table I).

Comment. Positive clostridial cultures were obtained only from subcultured thioglycollate, never from direct anaerobic blood plates. Hence their numbers in the tissues cultured must have been very small. They were never

TABLE I. Clostridia Recovered from Normal Organs.

Organs	Normal dogs		Normal rabbits		Normal hamsters		Normal guinea pigs		Normal rats	
	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures
Skin	10	80	6	67	10	0	—	—	—	—
Muscle	17	76	13	23	10	0	10	0	15	0
Heart muscle	16	81	6	50	10	0	—	—	—	—
Portal vein	12	17	11	9	—	—	—	—	—	—
Vena cava	12	0	9	0	—	—	—	—	—	—
Lung	20	80	11	45	—	—	—	—	—	—
Pancreas	15	87	15	46	10	0	10	0	15	0
Liver	20	90	15	46	10	0	10	0	15	0
Kidney	16	75	15	20	10	0	10	0	15	0
Urine	6	0	5	0	—	—	—	—	—	—
Adrenal	12	75	7	57	—	—	—	—	—	—
Thyroid	13	77	5	20	—	—	—	—	—	—
Spleen	18	83	13	39	10	0	10	0	—	—
Testis ovary	10	80	6	33	—	—	—	—	—	—
Bile	12	0	7	0	—	—	—	—	—	—
Peritoneum	19	16	15	0	—	—	10	0	15	0

TABLE II. Incidence of Clostridia by Sampling Similar Areas of Different Livers.

Liver	Right lobe near hilus	Right lobe near periphery	Right middle lobe	Left lobe	Quadrangular lobe	Three adjacent samples
1	Many clostridia	S*	S	S	S	Many " "
2	Sterile	Few	M	Many	M	" Few M
3	Sterile	Many	S	M	S	Many Few "
4	Few	M	Many	Few	Few	Many M Few

* S = sterile; M = moderate.

present in bile, urine or vena cava blood. Rarely they were found in peritoneum and in portal blood. A sterile culture from a small sample of an organ does not exclude the presence of bacteria in the entire organ. Table II shows that there is a very irregular distribution of clostridia in the dog's liver. Hence the figures underestimate contamination for large organs which cannot be cultured *in toto*.

Classification of Positive Cultures. Fifty strains of the clostridia isolated could be divided into 3 groups on the basis of a detailed morphological and cultural analysis. The first group of 30 strains showed all the morphological and cultural characteristics of *Cl. welchii* (*perfringens*) as described in Bergey's Manual of Determinative Bacteriology (1948). A second group of 12 strains showed the following characteristics: Clotting of litmus milk delayed (48-96 hours); acid and gas in dextrose, but not in lactose or sucrose; weak or negative hemolysis on anaerobic blood plates. Other morphological and cultural findings were identical with those of group 1. The third group of 8 strains showed the following: Litmus milk unchanged or very slightly acidified, but no clotting after 2 weeks. Dextrose unchanged or slightly acid without gas formation; other sugars not attacked. Liquefaction of gelatin slow. No hemolysis on anaerobic blood plates. H_2S not produced. Other findings similar to group 1.

It was not possible to identify the strains of the second and third groups from the list

in Bergey's Manual (1948). This Manual lists among 60 species of clostridia (excluding those listed in appendix I and II), 8 species which are not motile. Of these only 3 are capsulated (*Cl. welchii, Cl. malenominatum, Cl. angulosum*). Groups 2 and 3 as described above do not show the cultural characteristics of any of the 3 species. Group 3 has some resemblance to *Cl. malenominatum* (no coagulation of milk, lack of sugar fermentation), but this species has been isolated only once from the feces of a diarrheal infant and is said to be very pathogenic to guinea pigs, a property not found in Group 3 strains. Nor are Group 2 or 3 identifiable as the bacillus which Wolbach and Saiki(1) cultured from the normal dog's liver.

Toxicity of the Clostridia. Identification of a strain of *Clostridium* as *Cl. welchii* (*perfringens*) requires the demonstration that it is able to produce a soluble toxin. The ability of these strains to produce exotoxin was investigated as follows. Cultures in thioglycollate or pancreatic digest of beef heart(2) were incubated 2-7 days and filtered through Seitz filters. Filtrates were tested for sterility and then injected in volumes varying from 1-5 cc subcutaneously in one group of guinea pigs, and intramuscularly in a second group. In spite of such large doses, none of the animals died or showed any local reaction at the point of injection up to a period of 3 weeks. Hence the strains examined did not produce exotoxin in the test tube in any demonstrable amount. [Injection of filtrates into the breast muscle of pigeons, as suggested by Logan(2) for demonstration of minimal amounts of exotoxin, has not been done.] But results were different when pure cultures of eight strains, grown in thioglycollate for 3-5 days, were injected intraperitoneally or subcutaneously into healthy guinea pigs. Sterile thioglycollate was injected in similar volumes into other guinea pigs serving as controls. Four of eight animals injected intraperitoneally with 5 cc of the thioglycollate culture died within 6-24 hours; the other 4 survived. Two injected with 4 cc and 3 with 3 cc all survived. The dead guinea pigs showed hyperemia of the peritoneum with a very small volume of bloody fluid from which the injected strain was recovered in pure culture. In 2 of them liver cultures were done and revealed the injected clostridia in pure culture, while normal guinea pig livers always proved sterile.

Of 8 guinea pigs injected subcutaneously with 5 cc of the same cultures, 6 died within 6-24 hours and 2 survived. Of 8 injected with 3 cc two died. Postmortem showed widespread hemorrhagic edema of the subcutaneous tissue. The peritoneum was hyperemic. Cultures of the subcutaneous tissue, peritoneum, and liver revealed pure cultures of the strains injected. No gas bubbles were found even when the autopsy was done a few hours after death. Only guinea pigs injected with strains of the first group, *i.e.*, probably *Cl. welchii*, died. Control guinea pigs for each group, injected with sterile thioglycollate, were unaffected by the injection.

Additional evidence that the clostridia from normal dogs' livers are *Cl. welchii* is the following: An aseptically prepared homogenate of dog's liver was injected intraperitoneally into normal guinea pigs and into guinea pigs which had received 3 cc of polyvalent clostridial antitoxin* subcutaneously daily for 3 days prior to injection. Many more untreated than treated guinea pigs died (Table III). Since the cultures of the strains recovered correspond to those of *Cl. welchii* and to none of the other species, it is clear that the antitoxin protected against *Cl. welchii* and that these bacteria produced exotoxin.

Frequent transfers of cultures in the thioglycollate or cooked meat medium did not encourage toxin production, nor did the strains recovered from dead guinea pigs show any increased toxicity.

Discussion. Earlier bacteriologic studies of normal tissues, beginning in 1885(3,4,5,6) showed no bacteria in the organs of rabbits or guinea pigs, or the mesenteric lymph glands of cattle. The cultures in these studies were observed for only 3 days. Ford(7) found bacteria in rabbit, guinea pig, dog and cat tissue cultures after 5-17 days. But these bacteria were non-hemolytic *Micrococcus pyogenes*, var. aureus and albus, *Bacillus mesen-*

* 15 cc of this polyvalent antitoxin (Lederle) contains antibodies against *Cl. welchii* (10,000 units), *Cl. septicum* (10,000 units) *Cl. novyi* (1500 units), *Cl. bifermentans* (1500 units), *Cl. histolyticum* (3000 units).

TABLE III. Intraperitoneal Injection of Homogenized Dog Liver into Guinea Pigs.

Vol. inj., cc	No antitoxin — No. of animals	No antitoxin — Death, %	Antitoxin — No. of animals	Antitoxin — Death, %
7	4	75	4	25
6	4	100	4	25
5	14	57	12	0
4	14	21	10	0
3	14	30	10	0
Summary	48	46	40	5

tericus, Bacillus cereus, var. mycoides, *Bacillus megatherium,* and *Proteus, i.e.,* non-pathogenic bacteria which were very likely contaminants. Gage(8) found bacteria in nearly all livers of healthy dogs, but did not identify the species. Wolbach and Saiki(1) found clostridia regularly in the livers of healthy dogs, but considered them not to be *Cl. welchii.* Others confirmed these findings(9,10,11). Dragsted *et al.*(12) considered the clostridia which they found regularly in the dog's pancreas to be *Cl. welchii.*

It appears that all organs of the healthy dog, not only liver and pancreas, are more or less regularly contaminated with clostridia. These are presumably invaders from the intestine. That they invade *via* the portal vein is suggested by the finding of clostridia in the portal vein in 2 of 12 specimens, while their incidence in 12 specimens of vena caval blood was zero.

Presumably, the bacteria in the tissues of normal animals are located intracellularly, because the secretions of organs containing clostridia were always sterile. Since absorbable intraintestinal antibiotics can eliminate clostridia from the intestine, but not from the liver of the healthy animal, those in the liver of the healthy animal are not in a stage of multiplication. But the protection afforded by active and passive immunization against clostridial exotoxins and by penicillin and aureomycin in certain experimental conditions which activate these bacteria(15,16) demonstrates the need to consider carefully the role of these bacteria in experiments involving the abdominal viscera of dogs and rabbits.

Two observations of interest are the following: 1) Fifteen liver biopsies from humans taken during operation were sterile for aerobes and anaerobes, thus confirming the findings of Romieu and Brunschwig(13) and Sborov *et al.*(14); 2) The organs of 10 nearly mature dog embryos from 2 mother dogs, which showed the usual contamination, were sterile.

Conclusions. 1) The organs of normal rats, guinea pigs and golden hamsters are sterile. Blood from vena cava, bile and urine of normal dogs and rabbits was always sterile. Most of the tissues of healthy dogs, and rabbits to a lesser degree, harbor clostridia. The embryos of healthy dogs are sterile. 2) The great majority of the strains recovered from the tissues of dogs and rabbits show all the morphological and cultural characteristics of *Cl. welchii* (*perfringens*). Toxin production, though weak, was demonstrated.

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