On Direct Cultivation of Bacterium Tularensis.

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During the fourteen months from November, 1949 to February, 1951, 53 cases of Yato-byo, or tularemia of Japan, were reported to have broken out throughout the whole area of Fukushima Prefecture and in parts of Miyagi, Tochigi and Yamagata Prefectures, all of which are situated in the north-east of Japan. Table I shows the number of the patients in each district; Table II shows the frequency of each clinical type.

TABLE I.

Number of Cases in North-east
of Japan during 14 Months
Ending February, 1951.

Name of prefecture	Number of cases
Fukushima	39
Miyagi	10
Tochigi	3
Yamagata	1
Total	53

TABLE II.

Frequency of Clinical Types:

Clinical types	Number of cases
Ulceroglandular	11
Oculoglandular	3
Glandular	39
Typhoidal	0
Total	53

40 cases out of all were treated surgically with the total removal of the pathologic lymph nodes; in 6 cases of which streptomycin was given to the patients in combination with surgical operation, with the fairly satisfactory result of their recovery in about two weeks.

We have tried to cultivate directly the Bacterium tularensis taken from the lymph nodes of the above forty patients.

The following is to make clear that the isolation of the organism from man has become easier in this country than it was before.

Materials and Methods.

Patients: 39 cases were Glandular or Ulceroglandular; only one was Oculoglandular. 10 out of them were treated in other hospitals and their removed lymph nodes, with blood, were sent to us for examination.

Contact with wild rabbits (Japanese hares and Echigo hares) was, with no exception, seen in the histories of all. Diagnosis was based mainly on the clinical symptoms; in our hospital, intradermal test with phenolised 0.01% bacterial suspension, as well as serum agglutination and slide agglutination, was employed.

Appearance of materials and their clinical examination: The materials used were small pieces of tissue punctate or pus of the axillary, elbow or cervical nodes excised 3 to 48 days after the onsets, except one case in which the interval was about two months. They contained multiple grayish and yellow-white necrotic foci partly abscessed. The pus was highly mucous and our simple staining could not prove the existence of pyogenic cocci in it; however, minute cocci of a sort, less than 0.5μ in diameter and negative to Gram's staining, were recognizable.

In several cases we studied the motility of the minute cocci in the pus by dark-field examination and could observe their peculiar motion among leucocytes. As a control material we used pus taken from five cases of acute lymphadenitis, in which staphylococci showed the unmistakable Brownian motion. The former's characteristic motion differed markedly from the latter's Brownian motion.

Methods: Two kinds of solid media were used. One was coagulated hen's egg-yolk medium which has been popularly known to be the best medium obtainable for first isolation of *Bacterium tularensis* from pathologic sources. The other was pig-liver medium (Hiwatashi); it is an improved blood-glucose-cystine-agar (Francis).

The former was a mixture of 40% sterile saline solution and 60% pure egg-yolk, coagulated.

To get the latter, a filtered extract was made from pig-liver cut into small pieces and mashed, and this extract served as substitute for beef infusion in getting blood-glucose-cystine-agar by ordinary procedure.

The materials were rubbed onto these media immediately, as a rule, after operation. When the materials were those sent from other hospitals, the process had to be delayed by 24 to 72 hours after that. The temperature in the incubator was 34°C .

Results of Experiments.

As shown in Table III, when yolk medium was used, direct isolation of the organism was successful in 9 out of 20 cases.

Compared with this, however, a far better result was reached through the use of pig-liver medium, where 14 out of 20 cases turned out to be successful.

Much has already been discussed as to cultivation on the former

TABLE III.
Results of Direct Cultivation.

Kind of medium	Number of tested cases	Number of successful cases
Egg-yolk	20	9
Pig-liver	20	14
Total	40	23

medium, so here our statement will naturally be restricted to what we have discovered on the latter.

48 hours were the minimum necessary time before the growth started; in some cases where the growth was slow, it took as long as 7 days for the first colonies to be formed. The colonies, small and circular at first, were seen, like drops of water, to stand isolated one time and become confluent another, coming finally to take on a slightly bluish, opalescent tinge. When a small bit of the culture was taken out with a loop and shaken on a slide in a drop of saline solution, it became colloidal and mucous.

All these cultivated germs had a conspicuous tendency to agglutinate with the patients' serum.

Getting subcultures on the pig-liver medium was possible even after three months' lapse of time at the room temperature of about 28°C. in summer. It usually took 24 hours for the subculture germs to start growth.

Virulence of the organism: A 750 g. guinea pig died nine days after it had been subcutaneously inoculated with 0.002 mg. of the oldest strain (Ebina) of 48-hour cultivation. From its pathologic liver the same organism could be detected by cultivation.

Morphological features: Our observation on the morphology and motility of the germ in question is in process at present; we are using those fourteen strains (No. 1 to No. 14) listed in Table IV as well as seven others cultivated on egg-yolk medium and the heart blood of infected mice.

Its motility is being studied by dark-field examination in saline solution. The study of its morphological structure is being done through the simple staining (carbolic gentian violet), Gram's, and the flagella staining (Saizawa-Sugawara's, Kobayashi's, and Gray's) method.

At the present stage of our study, we have succeeded only in recognizing that the organism from these Yato-byo strains reveals polymorphism of high degree, its forms changing with the changes in the cultivating conditions, and that certain forms of the cultivated organisms and of those in the heart blood of infected mice occasionally reveal an obvious active motion which cannot be found in the case of the organisms in human pus.

On the six cases in which cultivation was unsuccessful: We failed to isolate

TABLE IV.
Results of Cultivation by Pig-liver Medium.

No.	Designation of culture	Pathologic source	Interval between onset and test	Days required before formation of first colonies	Agglutination title
1	Ebinr	tissue and pus	20 days	4	1280
2	Oura	tissue and pus	20 "	3	640
3	Nagasawa	tissue and pus	20 "	7	40
4	Wataume	tissue and pus	10 "	5	160
5	Momma	tissue and pus	8 "	6	80
6	Orikasa	punctate and tissue	13 "	2	160
7	Shiraiwa	tissue	3 "	3	(—)
8	Kusakari	punctate and tissue	4 "	4	(—)
9	Yamada	tissue and pus	18 "	3	80
10	Namatame	punctate	16 "	7	1280
11	Kaneko	tissue and punctate	10 "	5	(—)
12	Samago	tissue	9 "	4	(—)
13	Sayu	tissue	9 "	3	(—)
14	Takano	tissue	11 "	5	160
15	Saharu	punctate (pus)	48 "	—	80
16	Kanna	punctate (pus) and tissue	48 "	—	80
17	Komatsu	punctate (pus)	30 "	—	160
18	Watatome	tissue	ca. one month	—	80
19	Oka	tissue	44 days	—	320
20	Satatsu	tissue	ca. two months	—	80

directly the organism in six cases where the material was human pus in three and tissue of lymph nodes in the other cases. In all of these the intervals between the onsets and cultivations were about a month or more, during which period the patients were given such drugs as penicillin, sulfonamides, antipyretics, etc.

In one of these cases the patient experienced no other subjective symptoms than adenopathy in both axillary fossae, which was found, even after a month, to contain many necrotic foci but showing no sign of suppuration.

We also tried to isolate the germ through inoculated mice and failed. A possible explanation for our failure to isolate it may be that, in these unsuccessful cases, the organisms in the examined materials were not strong enough or utterly dead.

SUMMARY.

Since McCoy and Chopin succeeded in 1912 in isolating *Bacterium*

tularensis using egg-yolk as medium, many contributions have been made towards securing better media for cultivating this organism. Among them, Foshay^{1,2)} and his associates, Gibby, Nicholes and Tamura³⁾, succeeded recently in getting more suitable liquid media. They are at present making further efforts to elucidate the effects of the components of the media upon the growths.

In this country, though some amount of contribution has been made in this field of medicine, it is still difficult to directly cultivate *Bacterium tularensis* from any given pathologic source, and egg-yolk has been considered to be the most suitable medium for this purpose.

Pig-liver medium was devised by Hiwatashi a short time ago and has hitherto been valued as suitable for subculture; but, as the one to be used for first isolation of the organisms got from human pathologic materials, it has not been given much credit.

T. Ota,⁴⁾ after an attempt to isolate *Bacterium tularensis* using the Hiwatashi medium in nine cases out of more than forty of tularemia which occurred 1949 in Chiba Prefecture, had to confess his failure.

In the latest outbreak of Yato-byo in this part of Japan, we at first used egg-yolk medium for direct cultivation. But, since the spring of 1950, we have come to find a far better medium in pig-liver. It certainly enables us to make further study of the morphology of the organism more easily, of which the present work is only a part.

And it may well be stated that the direct isolation of *Bacterium tularensis* from human lymph nodes presents a considerably good result and has its diagnostical value in case the materials can be secured within about a month after onsets and inoculated onto suitable media; though it will be difficult to do so after the lapse of over a month of the clinical course, even if animal examination is performed immediately after operation.

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