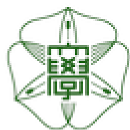
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GUANOFURACIN MEDIA FOR ISOLATION OF *L. MONOCYTOGENES* AND ITS PRACTICAL APPLICATION

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I. INTRODUCTION

Since 1950 when M. TAJIMA first suggested the existence of listeriosis in this country, several incidences of the disease have been reported in various districts. Its occurrence in Japan seems to be increasing year by year.

HIRATO and his associates have published in this journal on a small epizootic outbreak of listeriosis in a sheep-herd in the vicinity of Sapporo. In the course of this observation, they experienced technical difficulties in the isolation of the organism especially from nasal discharge of the infected animals.

Naturally, it is very important to clarify the source of infection from the view-point of disease control. OLSON and his colleagues suggested the existence of some inapparent infection which shows slight febrile and leucocytic reactions without any nervous symptom. However, they neglected to make bacteriological tests of the suspected cases.

The present authors tried to detect some healthy carrier which would discharge or excrete the bacilli for its spread. However it is very difficult to detect the causal agents from contaminated materials such as nasal discharges or feces. Accordingly, it is first of all necessary to find some suitable selective media.

Then the authors made several attempts to find such media by using various dyes and chemicals as inhibitors for contaminating organisms other than *Listeria*. Finally they found effective media containing 5 Nitro-2 Furfurylidene-Aminoguanidine Hydrochloride (hereafter called guanofuracin, the commercial name in Japan). Provisionally the authors called them "Guanofuracin Media".

Moreover, using these media, they succeeded in detecting one healthy carrier of *Listeria* organism among the individual animals in

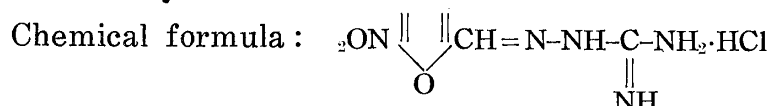
the sheep flocks where an epizootic listeriosis occurred in May~June, 1952.

In the present communication, several experiments for the above-mentioned selective media and experiments on its practical use are described as follows.

II. EXPERIMENTS ON THE SELECTIVE MEDIA

1. The growth inhibitory power of guanofuracin

Chemical formula :



The growth inhibitory power of guanofuracin for several species of bacteria were studied by using both solid and flulid media. In these experiments, 5% sheep-blood nutrient agar with 1% glucose were employed as a solid medium and 1% glucose nutrient broth as a fluid enrichment medium. Bacteria species and guanofuracin concentrations tested are indicated in table 1. One loopful of twenty hours nutrient broth culture of each organism was diluted with 4 cc of nutrient broth and one loopful of these were cultivated on solid media. Data in table 1 indicate the growth of each organism after 48 hours' incubation at 37°C.

Listeria organisms are able to grow on 1 : 6,000 concentration of guanofuracin, but their growth is highly suppressed. At the dilution of 1 : 8,000, the number of colonies are almost same but their sizes are smaller than in the control. However, in the concentration less than 1 : 10,000, the growth of *Listeria* are not at all influenced. On the contrary, 1 : 10,000 concentration was sufficient to inhibit the growth of *B. subtilis*, *E. coli*, other Gram-negative organisms and staphylococci. However, α type streptococci alone grow well in this concentration as also do *Listeria*.

Inhibitory effects of the fluid media which are added to the various concentrations of guanofuracin are similar to the solid media, i. e. the 1 : 8,000 concentration is suffucient for growth. For the enriching purpose, 12~18 hours incubation showed the best result. Over 28 hours incubation causes the development of mold.

2. Comparative studies on the basic media

From the above-mentioned results, the effectiveness of guanofuracin

TABLE 1. *Growth Inhibiting Power of Guanofuracin on the Nutrient Agar (with 1% Glucose and 5% Sheep-blood)*

TESTED ORGANISMS		CONCENTRATION OF GUANOFURACIN											
		1:2000	1:4000	1:6000	1:8000	1:10000	1:15000	1:20000	1:50000	1:100000	1:150000	1:200000	Control
B. subtilis		–	–	–	–	–	–	–	–	–	+	++	+++
E. coli		–	–	–	–	–	–	–	–	–	+++	+++	+++
S. abortus equi		–	–	–	–	–	–	–	–	–	+	++	+++
S. senftenberg		–	–	–	–	–	–	–	–	+++	+++	+++	+++
Sta. aureus		–	–	–	–	–	–	–	–	+	+++	+++	+++
α type Str.		–	–	+	++	++	+++	+++	+++	+++	+++	+++	+++
Str. pyogenes		–	–	–	–	–	–	–	–	–	+	+	+++
Enterococcus		–	–	–	–	–	–	–	+++	+++	+++	+++	+++
Listeria	L 1	–	+	+	++	+++	+++	+++	+++	+++	+++	+++	+++
	L 3	–	–	+	++	+++	+++	+++	+++	+++	+++	+++	+++
	L 6	–	–	+	++	+++	+++	+++	+++	+++	+++	+++	+++

Notes:

+++ Full growth (equal to the control).
++ Not sufficient growth.
+ Slight growth.
– No growth.
The numbers of growing colonies are almost same in +, ++, +++ and control.

seems to be favorable as the isolating media, however, the increasing selectiveness resulting from various basic media must be studied.

The authors made comparative studies using brain agar, liver agar and ordinary nutrient agar to which are added 1% glucose, 5% sheep-blood and graded concentrations of guanofuracin. Inoculated doses of each organism are the same as above. As indicated in table 2, compared with the ordinary nutrient agar, excellent growths of *Listeria* were observed on the brain and liver agar; however, in accordance with the good growth of *Listeria*, α type streptococci also showed favorable growth in these media. In the examination of the contaminated materials, the growth of α type streptococci and other related organisms are especially encouraged. Consequently, the brain and liver agar would somewhat disturb the detection of *Listeria* from the fully contaminated materials, such as feces, nasal and eye discharges.

TABLE 2. *Comparative Studies on Several Media for Differential Use*

BACTERIA INOCULATED	MEDIA TESTED	CONCENTRATION OF GUANOFURACIN								
		1:3000	1:5000	1:8000	1:10000	1:15000	1:20000	1:25000	1:30000	Control
Str. pyogenes	A	−	−	−	−	−	−	−	−	+++
	B	−	−	−	−	−	−	−	−	+++
	C	−	−	−	−	−	−	−	−	+++
E. coli	A	−	−	−	−	−	−	−	−	+++
	B	−	−	−	−	−	−	−	−	+++
	C	−	−	−	−	−	−	−	−	+++
Sta. aureus	A	−	−	−	−	−	−	−	−	+++
	B	−	−	−	−	−	−	−	−	+++
	C	−	−	−	−	−	−	−	−	+++
S. aborutus equi	A	−	−	−	−	−	−	−	−	+++
	B	−	−	−	−	−	−	−	−	+++
	C	−	−	−	−	−	−	−	−	+++
α type Str.	A	−	+	+++	+++	+++	+++	+++	+++	+++
	B	−	++	+++	+++	+++	+++	+++	+++	+++
	C	−	−	−	+	++	+++	+++	+++	+++
Listeria L 6	A	−	++	+++	+++	+++	+++	+++	+++	+++
	B	+	+++	+++	+++	+++	+++	+++	+++	+++
	C	−	+	++	+++	+++	+++	+++	+++	+++
Listeria L 8	A	−	++	+++	+++	+++	+++	+++	+++	+++
	B	+	+++	+++	+++	+++	+++	+++	+++	+++
	C	−	+	++	+++	+++	+++	+++	+++	+++

Above results were read after 48 hours' cultivation.

A: Brain agar B: Liver agar
C: Oridinary nutrient agar } with 1% glucose and 5% sheep-blood.

+++ : Full growth (equal to the control). + : Slight growth.
++ : Not sufficient growth. − : No growth.

The numbers of growing colonies are almost the same in +, ++, +++ and control.

After all, the use of the ordinary nutrient agar which are added with 1:8,000∼1:15,000 concentration of guanofuracin are the most favorable for this purpose as is shown in table 2.

3. Experiments on the contaminated materials

Above experiments with pure cultures showed excellent results; however, for practical purposes, the guanofuracin concentration in solid and fluid media is still under investigation.

Nasal swabs from the supposedly healthy sheep were firstly inoculted into the fluid media with *Listeria* organisms, at 37°C for 20 hours. The inoculted doses are the same as above. After this, one drop of these was transplanted respectively to the media with graded

TABLE 3. *Showing Results of Various Combinations of Concentration of Guanofuracin Enrichment Media and Solid Media Cultures*

MATER.	THE GROWTH OF *Listeria* IN ENRICHMENT MEDIA							NOTES: Preceding Enrichment Cultures were Seeded on the Solid Media at the Following Concentrations of Guanofuracin
	1	2	3	4	5	6	Control.	
A	−	−	−	−	−	−	+++	
B	−	−	−	−	−	−	+++	Not added
C	−	−	−	−	−	−	+++	
A	−	+++	−	−	+	+	+++	
B	−	+	−	++	++	−	+++	1 : 15,000
C	−	+++	+++	+	+	−	+++	
A	+++	+++	+++	+	−	++	+++	
B	−	+	+	+++	+++	−	+++	1 : 10,000
C	+	+++	+++	+	+++	−	+++	
A	+++	+++	+++	+	−	+	+++	
B	−	+++	+++	+++	+++	+	+++	1 : 8,000
C	−	+	+++	+++	+++	+	+++	

Notes:

1. Figures in the first columns indicate the guanofuracin concentration in enrichment media.
   1 = 1 : 0 2 = 1 : 15000 3 = 1 : 10000 4 = 1 : 8000 5 = 1 : 6000 6 = 1 : 4000.
2. A, B and C indicate the nasal swabs mixed with strains L 1, L 3 and L 6 respectively.
3. − *Listeria* could not be detected, being disturbed by mold and other bacteria.
   \+ 1–100 colonies of *Listeria*.
   ++ 101–500 colonies of *Listeria*.
   +++ Over 500 colonies of *Listeria*.

guanofuracin concentration and the growth of *Listeria* was examined. Table 3 indicates these results.

From this experiment, it is obvious that the 1 : 10,000~1 : 15,000 guanofuracin concentrations are desirable for enrichment fluid media and 1 : 8,000~1 : 10,000 for solid media in order to detect *Listeria* from the contaminated materials.

In the next place, it was tested how a minute number of *Listeria* in the contaminated materials could be detected by using these selective media. From this experiment, it was found that these media generally have the ability to detect four hundred organisms and in one case only forty (table 4).

TABLE 4. *Experiments on Detection of Listeria from Contaminated Materials*

MATERIALS	NUMBER OF *Listeria* ORGANISMS MIXED	GUANOFURACIN CONCENTRATION IN ENRICHMENT MEDIA			
		0	12000	10000	8000
A	4×10^5	+	+	+	+
	4×10^4	+	+	+	+
	4×10^3	−	+	+	+
	4×10^2	−	+	−	+
	4×10	−	−	−	−
B	4×10^5	−	+	+	+
	4×10^4	−	+	+	−
	4×10^3	−	+	+	−
	4×10^2	−	+	+	−
	4×10	−	−	−	−
C	4×10^5	−	+	+	+
	4×10^4	−	+	+	−
	4×10^3	−	+	+	−
	4×10^2	−	+	+	−
	4×10	−	+	−	−

Notes:

+ *Listeria* were detected.
− *Listeria* were not detected.
A: Nasal swab mixed with strain L 1.
B: Nasal swab mixed with strain L 3.
C: Nasal swab mixed with strain L 6.

From the above data, the authors suppose that these selective media are very suitable for their puposes. However, in actual practice, some resistant unknown streptococci, micrococci and Gram-positive bacilli frequently appear in the culture.

For the purpose of the differential diagnosis from *Listeria*, the addition of potassium tellurite in the proportion of 0.01% will bring good result. On this media, *Listeria* colonies present black color with greyish-green periphery while streptococci and micrococci pinkish-grey and pinkish-yellow respectively. Colonies of other bacilli are large in size and show greyish-blue color.

III. Result of Practical Application

As already mentioned, it is a very important problem to examine whether there can be a healthy carrier or not. G. Pallaske in Germany has already isolated the *Listeria* from nasal cavity in two cases of naturally infected cases. He emphasized the possibility of the nasal infection because the catarrhal purulent inflammations of the nasal cavity could be frequently observed in the natural cases of sheep and artificially inoculated rabbits.

The authors planned to trace out the presence of some healthy carrier. This experiment was carried out on the nasal specimens from sheep in the Hokkaido Agricultural Experiment Station near Sapporo where an about twenty cases of listeriosis occurred in May, 1952.

One hundred and eighty-three nasal swabs were taken from healthy sheep in this farm and the authors could isolate several doubtful bacterial strains resembling *Listeria.* However, after several biochemical and serological examinations, only one strain N 29 is to be identified with *Listeria monocytogenes* as is indicated in tables 5 and 6.

Lactose, sucrose and sorbitol fermenting activity are variable by different strains as previously reported. This isolated strain was serologically determined to be identical with our serotype B which corresponds to Paterson's LS-2 strain.

From the above efforts at tracing, the present writers got only one positive detection of *Listeria* organism from the nasal swab of a healthy sheep. However, this result seems to be very important because it would suggest the existence of a healthy carrier of *Listeria monocytogenes* even though it is very rare.

TABLE 5. *Biochemical Characteristics of the Isolated Strain*

	ISOLATED STRAIN (N 29)	KNOWN *Listeria* STRAIN (L 6)
STARCH, GALACTOSE, MANNITOL, GLYCOGEN, INOSITOL, INULIN, RAFFINOSE, DULCITOL, XYLOSE, ARABINOSE, ADONITOL	−	−
LACTOSE	···	$+_{4}$
SUCROSE	−	$+_{11}$
SORBITOL	···	−
GLYCEROL	$+_{20}$	$+_{3}$
RHAMNOSE	$+_{2}$	$+_{1}$
GLUCOSE, SALICIN, LEVULOSE, TREHALOSE, MALTOSE, DEXTRIN, AMYGDARIN, MANNOSE	$+_{1}$	$+_{1}$
GELATIN LIQUEFACTION	−	−
H_2S FORMATION	−	−
INDOL FORMATION	−	−
MOTILITY	+	+
HEMOLYSIS	+	+

TABLE 6. *Serological Characteristics of the Isolated Strain (N 29)*

ANTIGEN	SERA (TYPE*)	SERUM DILUTION 100	200	400	800	1600	3200	6400
N 29	8615 (A)	+	+	−	−	−	−	−
8615 (Serotype A)		++	++	++	++	+	−	−
L 3 (" B)		+	+	−	−	−	−	−
L 4 (" C)		++	++	++	++	+	−	−
N 29	L 3 (B)	+++	+++	+++	++	+	+	−
8615		−	−	−	−	−	−	−
L 3		+++	+++	+++	+++	+	+	−
L 4		++	++	++	++	+	+	−
N 29	L 4 (C)	++	++	++	++	++	+	±
8615		+	−	−	−	−	−	−
L 3		++	++	++	++	++	+	±
L 4		++	++	++	++	++	+	−

* HIRATO et al (1954): Vet. Res., **1**, 191.
Type A is identical with Paterson's serotype 1.
Type B is identical with Paterson's LS–2 strain.
Type C is identical with Paterson's serotype 4.

IV. Consideration

Several workers recommended liver or brain agar as favorite media for *L. monocytogenes.* However, the best growing media for *Listeria* are not always the best selective ones, because these are also favorable for the growth of other organisms which are also present in the materials.

From the present investigation, ordinary nutrient agar was determined to be rather suitable basic media for this purpose. M. L. Gray et al employed potassium tellurite as the inhibitor and indicator, however, the present authors utilized this chemical only as a colony indicator and recognized a considerably good result as a differential diagnostic agent. The authors compared the effectiveness of these guanofuracin media with M. L. Gray et al's potassium tellurite media on the contaminated materials which contained graded numbers of *Listeria.* The authors'media proved to be highly excellent in comparison with the latter.

By the practical use of these media, the authors could detect one healthy carrier of *Listeria monocytogenes.* Up to the present, the natural mode of infection and the source of this disease are not yet clarified, however, it will easily be supposed that such carrier may play some important role in the occurrence of this disease.

V. Summary

In the present report, it is the selective and enrichment media for *Listeria monocytogenes* and their practical application that are mainly described.

1. One per cent glucose nutrient broth with 1:12,000 concentration of guanofuracin will be recommened as an enrichment medium, and 1% glucose nutrient agar with 5% sheep-blood and 1 : 10,000 concentration of guanofuracin as a selective solid medium.

In the latter medium, addition of 0.01% potassium tellurite is beneficial for the differentiation by the coloring of the colony.

2. By using these selective media, *Listeria monocytogenes* was detected in one nasal swab from a total 183 sheep. From the viewpoint of epidemiology of this disease, this finding will suggest the existence of a healthy carrier of the organism.

The authors wish gratefully to acknowledge the encouragement given to this work by Professor Hirato, the Chief of this Laboratory, and also would express their thanks to the members of the Department of Animal Husbandry, Hokkaido Agricultural Experiment Station for their willingly supplying materials.

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