BACTERIOLOGICAL STUDIES ON STREPTOCOCCI FROM BOVINE UDDER I.

SEROLOGICAL AND BIOCHEMICAL OBSERVATIONS ON GROUP-B STREPTOCOCCI AND A GENERAL DESCRIPTION OF STREPTOCOCCI FROM BOVINE MILK IN HOKKAIDO

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(Received for publication, Oct. 2, 1958)

I. INTRODUCTION

In comparison with the many countries where dairy farming is prevailing widely since hundreds of years ago, in this country, not so much attention has been paid to the bovine mastitis especially to its causal agents until several years ago. However, since 1954 when a pretty large scale food poisoning occurred among school children due to their consumption of powdered skim milk, the problems concerning bovine milk are attracting serious attention. The research work on bovine mastitis also necessarily has become one of the most important problems in the field of veterinary medicine in Japan. Pertaining to the causal agents of the bovine mastitis, in foreign countries, it is generally accepted that more than 85% of the chronic bovine mastitis is streptococcal in nature and that about 80% of the streptococcal mastitis is caused by *Str. agalactiae.*

In Japan, as regards the causal agents of the bovine mastitis, there was no available bacteriological data till quite recently. However, in 1951, the present author made small scale bacteriological observations on 48 mastitis milk samples; he detected streptococci in pure or mixed form in 54.0%. At that time, the occurrence of *Str. agalactiae* and *Str. dysgalactiae* in Hokkaido had also been clarified, although the serological identification had not been carried out. However the prevalence of these organisms in Hokkaido had remained in question.

In 1955, a bacteriological survey of 3,176 quarters from 801 cows of the dairy farmers was executed by this Department in four districts where dairy farming is extensively carried on (Yakumo, Hayakita, Obihiro and Okoppe). The outline of this survey has already been reported by HIRATO et al.

In that survey, the present author could recognize the occurrence of streptococcal mastitis mostly due to *Str. agalactiae* in high rate as in other countries

abroad. After this, bacteriological observations have been also expanded on the materials from scattered farms in the neighborhood of Sapporo.

In the present paper, serological and biochemical observations on *Str. agalactiae* isolated in Hokkaido will be especially described, including data which were obtained on streptococcal mastitis.

II. MATERIALS AND METHODS

1. Strains Examined

One loopful each of aseptically drawn milk samples which were cooled in ice-box were transferred onto 5% horse or sheep blood agar less than 2~3 hours after sampling; examination was made after 48 hours at 37°C. Total 319 strains from 418 quarters of 239 cows, as listed in table 3, were examined.

They were biochemically identified by routine methods chiefly following BERGEY's Manual of Determinative Bacteriology and SHERMAN. Splitting of esculin and sodium hippurate was tested following SLAVIN except the recognition of ammonia from arginine, which was carried out by using the micro-diffusion method in the room temperature.

2. Serological Grouping and Typing by Precipitation

Immune rabbit sera Method of preparation and absorption of sera chiefly followed laboratory notes "Technical procedures employed in the production and use of streptococcus grouping sera" kindly supplied by Dr. LANCEFIELD, and PATTISON et al.

Antigen The method of preparation of antigen seems to be the most important. BRUNER and TUCKER's method seems to be more practical for testing a great number of the strains, however, it is not so sensitive as LANCEFIELD's method as listed in table 1, probably due to the small quantity of the culture used. FULLER's method and SHATTOCK's method following the text book by SEELEMANN are somewhat complicated. In case of the use of FULLER's method, group specific carbohydrate antigen of group-B tends to be destroyed by prolongation of heating time. Also the antigens by SHATTOCK, and by LANCEFIELD always indicated the same results as far as group-B streptococci are concerned. Accordingly, LANCEFIELD's method appears the most suitable for routine work. In this case, the pH range of acid extraction is very important. Under pH values of below 1.2 or over 3.8, group specific carbohydrate antigen will be destroyed or diminished, in contrast

TABLE 1. *Comparison of Sensitivities of Antigens Following LANCEFIELD, and BRUNWR and TUCKER*

NO. OF *STR. AGALACTIAE* TESTED	ANTIGEN A*				ANTIGEN B**		
	++++	+++	++	−	++++	+++~±	−
327	327	0	0	0	300	7	20

Notes: * following LANCEFIELD; ** following BRUNER and TUCKER.

TABLE 2. *Influence of pH in Acid-Extraction upon Group and Type Antigens of Str. agalactiae*

PH	SERA					
	H 36 B		V 8		D 136 C	
	Type	Group	Type	Group	Type	Group
Under 1.0	+	−	+	−	++	−
1.2	+	±	+	±	+++	−
2.6 ~ 3.0	+++	+++	+++	+++	+++	+++
3.8 ~ 4.0	+++	−	+++	±~−	++'	+
5.6	+++	−	+++	−	−	−

FIG. 1. *Preparation of Antigen following* LANCEFIELD

Streptococcal broth culture of 40 ml at 37°C 24 hours
↓
Centrifugation
↓
Washing with physiological saline 1 time
↓
Bacterial sediment plus 0.4 ml of N/5 HCl saline
↓
Adjustment of pH 2.6~3.0 with N·NaOH and N/5 HCl saline
↓
Extraction in boiling water, 10 minutes
↓
Cooling
↓
Adjustment of pH to 7.0 with N·NaOH
↓
Centrifugation
↓
Clear supernatant fluid (Antigen)

to the stable characteristic of the type specific carbohydrate. The scheme of the technics of preparation of antigen which was employed in the present report is shown in fig. 1.

III. RESULTS

1. The Incidence of Streptococci in Milk Samples

The data listed in table 3 revealed the presence of streptococci in high percentage – namely an average 27.0% of the cows or almost 12% of the quarters inspected, harboured streptococci in pure or in mixed state with other organisms.

Relationships of these streptococci to the disease are summarized in table 4.

TABLE 3. *Incidence of Streptococci in Milk Samples*

COWS FROM	NO. OF COWS EXAMINED (No. of Quarters)	STREPTOCOCCUS-POSITIVE MILK SAMPLES	
		No. of Cows (%)	No. of Quarters (%)
Individual farmers in 4 areas	801 (3,176)	191 (23.8)	298 (9.4)
Farm A	23 (86)	13 (56.5)	33 (38.4)
" B	24 (95)	20 (83.3)	49 (51.6)
" C	7 (28)	7 (100.0)	22 (78.6)
" D	14 (55)	6 (42.9)	12 (21.8)
" E	17 (68)	2 (11.8)	4 (5.9)
Total	886 (3,508)	239 (27.0)	418 (11.9)

TABLE 4. *Incidence of Streptococci in Mastitis or Abnormal Milk*

MATERIAL FROM	THE ISOLATION OF STREPTOCOCCI (No. of Positive/No. of Examined)						AVERAGE PERCENT
	4 Different Areas	Farm A	Farm B	Farm C	Farm D	Farm E	
Mastitis*	23.1% (39/169)	84.6% (11/13)	100% (4/4)	—	—	—	69.2
Abnormal milk**	16.8% (127/756)	91.7% (11/12)	65.9% (29/44)	84.0% (21/25)	41.4% (12/29)	8.7% (2/23)	51.4
Normal	6.4% (107/1678)	25.6% (11/43)	43.2% (16/37)	33.3% (1/3)	0% (0/26)	4.4% (2/45)	18.8

* means the cow which shows clinical symptoms or positive strip cup test.
** means the milk which shows one of the signs: higher than pH 6.6 by B.T.B. test, more than half million cell count.

Although there could be seen many differences of percentage among the data, it will be accepted that almost 70% of mastitis and 50% of the abnormal milk are caused by streptococci. On the other hand, about 19% of normal milk contained this organism; however, it is not clear whether these organisms have pathogenic significance or not, because of the lack of further observation.

From these data of tables 3 and 4, it is clear that the role of streptococci in mastitis or abnormal milk in Hokkaido is also important as in many dairy countries abroad.

2. Identification of the Strains Isolated

Total 319 strains were identified biochemically. Except for 31 strains, they could be classified into 10 species as is in table 5.

It is a serious problem that *Str. agalactiae* ranked first among them, corresponding to about 40% of the strains isolated. Some of these species were serologically identified. All strains of *Str. agalactiae* and *Str. dysgalactiae* reacted to the group-B and -C antisera

TABLE 5. *Identification of the Strains Isolated*

Str. agalactiae	126 (39.5%)
" *uberis*	80 (25.1)
" *bovis*	34 (10.7)
" *fecalis*	17 (5.3)
" *lactis*	12 (3.8)
" *dysgalactiae*	8 (2.5)
" *acidominimus*	5 (1.6)
" *pyogenes*	4 (1.3)
" *durans*	1 (0.3)
" *faecalis* var. *zymogenes*	1 (0.3)
Not identified	31 (9.7)

respectively; one of the *Str. pyogenes* to group-A, the others to group-C; a small number of *Str. uberis* and almost all strains of *Str. faecalis* reacted with group-D antisera in various degrees. However, none of *Str. lactis* reacted with group-N antiserum.

From the data in table 6 showing the relationship between the streptococcal species and milk abnormalities, it is clear that almost 80% of each, *Str. agalactiae* and *dysgalactiae*, and 70% of *Str. uberis* were derived from mastitis and abnormal milk.

Comparatively high incidence of *Str. lactis* in mastitis and abnormal milk must be especially kept in mind. On the other hand, streptococcal species such as *Str. faecalis*, *Str. faecalis* var. *zymogenes*, *Str. durans* and *Str. bovis* tended to be found more frequently in normal milk.

As to the pathogenic significance of *Str. agalactiae* in normal milk, long-term observation will give answer in future.

From the data briefly mentioned above, the incidence of various species of streptococci in bovine milk in Hokkaido will be understood.

TABLE 6. *Relationship of Streptococcal Species among Mastitis, Abnormal and Normal Milk Samples*

SPECIES	NO. EXAMINED	NO. OF SPECIES DERIVED FROM			PERCENT FROM NORMAL MILK
		Mastitis Milk	Abnormal Milk	Normal Milk	
Str. agalactiae	101	11	69	21	20.8 %
" *dysgalactiae*	5	2	2	1	20.0 %
" *uberis*	63	7	36	20	31.7 %
" *bovis*	30	5	8	17	56.7 %
" *faecalis, durans,* " *faecalis* var. *zymogenes*	17	1	7	9	52.9 %
" *lactis*	9	2	6	1	11.1 %

3. Serological Typing of *Str. agalactiae*

1) Observations by precipitation

From the different quarter milk samples 145 strains of *Str. agalactiae* were serologically typed by using precipitation. The data in table 7 indicate that in Hokkaido, the distribution of the strains of type II is the most predominant among them, whilst the strains of types Ia and Ib were never detected. Type III organism was recognized only in 1 case. The

TABLE 7. *Type Differentiation of Str. agalactiae Isolated in Hokkaido*

STRAINS EXAMINED	NO. OF TYPES				NO-POLYSACCHARIDE TYPE
	Ia	Ib	II	III	
145	0	0	114 (78.6%)	1 (0.7%)	30 (20.7%)

remaining organisms were those which apparently lack any type specific carbohydrate antigen.

Recently PATTISON et al. reported the type classification by LANCEFIELD's precipitation method of 236 human and bovine strains of group-B streptococci isolated in Britain. According to those workers, the total 170 bovine strains were divided into type Ia, 0; type Ib, 0; type II, 105 (61.8%); type III, 22 (12.9%) and no-polysaccharide, 43 (25.3%). These reults were almost the same as those obtained by the present author except for the high incidence of the strains of type III.

The serological type distributions of *Str. agalactiae* were studied on the cows from several dairy farms near Sapporo. From table 8, it will be seen that the cows in Farm A were all attacked by type II, however, in Farm D, the infections were caused only the strains of no-polysaccharide type. Moreover, in Farms B and C two different sero-types were demonstrated. In addition to this, the type distributions in each quarter were investigated. These results are indicated in table 9. In Farm C, no-polysaccharide type of *Str. agalactiae* was detected only in 3 quarters of cow No. 1 and the other quarters examined were all affected by the strains of type II. In Farm B, cows Nos. 47 and 55 were affected by different types or species in each quarter respectively, namely in cow No. 55, left anterior by *Str. dysgalactiae*, right anterior by no-polysaccharide type, left posterior by type II and right posterior by no-polysaccharide type.

TABLE 8. *Distribution of Serological Types of Str. agalactiae*

HERD	TYPE II	TYPE III	NO-POLYSACCHARIDE TPYE	TOTAL
Farm A	44	0	0	44
Farm B	39	0	3	42
Farm C	11	0	3	14
Farm D	0	0	11	11
				111

Owing to the lack of serological studies on individual colonies from an infected quarter, the author is unable to say whether there are mixed infections of a quarter by several species or types or not.

2) Some observations on agglutination test

Concerning the type differentiation of *Str. agalactiae* by using agglutination and precipitation, STABLEFORTH has made great contributions; he reports six main types including 16 subtypes. Recently PATTISON et al. examined the relationships between the types of STABLEFORTH and of LANCEFIELD and recognized that 11 out of 16 subtypes of STABLEFORTH fall into 4 types of LANCEFIELD whilst the remaining 5 subtypes of STABLEFORTH were not detected to have any type specific carbohydrate antigens corresponding to LANCEFIELD's sero-types.

With respect to the typing of the present strains by agglutination, the author could

TABLE 9. *Distribution of 2 Serotypes of Str. agalactiae in Each Quarter*

SOURCE	NO. OF STRAIN	COWS No.	COWS Quarter*	SEROTYPES DEMONSTRATED
Farm B	132, 133, 134	51	1, 2, 4	II, ", "
	33	52	2	No-polysaccharide
	303	47	1	II
	304		2	No-polysaccharide
	292	55	1	*Str. dysgalactiae*
	142		2	II
	143		3	"
	144		4	No-polysaccharide
	29	43	1	II
	114		4	"
	118	46	1	II
	119		2	"
	152		3	"
	121		4	"
	.	.	.	.
	.	.	.	.
	.	.	.	.
	.	.	.	.
Farm C	G 1	1	1	No-polysaccharide
	G 2		2	"
	G 3		3	"
	G 4	2	1	II
	G 5	3	1	"
	G 6		2	"
	G 7	4	1	"
	G 8		2	"
	G 9		3	"
	G 10		4	"
	G 11	5	1	"
	G 13		2	"
	G 15	6	1	"
	G 16		2	"

* Numerals in this column indicate;
1—left anterior, 2—right anterior,
3—left posterior, 4—right posterior.

make no examination because of non-availability of the type cultures other than 3d, 4a and 4b of STABLEFORTH. However, many complicated agglutination types were found among the present strains.

By the use of the sera of group-B, types Ia, Ib, II and III of LANCEFIELD's sero-types total 11 agglutination types were recognized among only 94 strains tested, as listed in table

10. In addition, the author also observed spontaneously agglutinable strains in some high percentage; moreover he observed cross agglutinations with some other species of streptococci such as *Str. faecalis* and varied agglutinability of the same strain from test to test. Although these examinations are only preliminary ones, these cross agglutinations and the varied agglutinability which are very troublesome in sero-typing, have already been mentioned by LANCEFIELD (1934) and PATTISON et al. The author considers that additional studies will be required making use of the agglutination test for sero-typing.

TABLE 10. *Slide Agglutination Tests*

TYPE OF AGGL.	SERA EMPLOYED (Strain No.) Group (RF)	Type Ia (O-90)	Ib (H 36B)	II (V 8)	III (D 136C)	NO. OF STRAIN	LANCEFIELD'S TYPE II	III	No-polysa.
A	−	−	−	+	−	31	26	0	5
B	+	−	−	+	−	26	22	0	4
C	+	+	+	+	+	14	5	0	8
D	+	−	−	−	−	9	5	0	4
E	+	−	−	+	+	4	3	0	1
F	+	+	−	+	+	3	0	0	3
G	+	+	−	+	−	2	0	0	2
H	+	+	+	+	−	2	1	0	1
I	−	+	−	+	−	1	1	0	0
J	−	+	−	−	−	1	0	0	1
K	−	−	−	−	−	1	1	0	0
Total						94		94	

3) Observations on agar gel diffusion method

Whether the agar gel diffusion method can be useful for sero-typing of *Str. agalactiae* was examined. The technic chiefly followed was that of MANSI.

Media were prepared of 1.5 g Bacto-agar, 1.5 g of sodium chloride, 7.5 ml of 10% phenol saline and 150 ml of distilled water. Of the above media 25 ml were poured into a petri-dish (diameter 90 mm).

From the preliminary tests, the distance of each basin (8 mm diameter) was decided to be 6 mm. One set of basins comprised from 7—6 basins surrounding the central one. Two drops of undiluted immune rabbit serum were poured into the central basins and the same volume of each antigen into the surrounding basins. These petri-dishes were incubated at 37°C for 3 days and after this, they were kept at room temperature 1 week in sealed container. Reading was made once a day.

Four kinds of antigens were tested: saline suspension of living cells, broth culture of 24 hours at 37°C and its clear supernatant fluid, and acid-extract following LANCEFIELD. The best reaction was observed in the HCL-extracted antigen as is indicated in table 11. Lines usually appeared during 24~72 hours. No group reaction was observed between the

sera which contained high titre of group-B antibody and HCl-extracted antigen; type reaction only occurred. These relationships will be seen clearly from table 11. These facts seem to be the most characteristic phenomenon in case of agar gel diffusion method. In comparison with the precipitation by the small test tubes of 3~4 mm inside diameter, several doubtful cross reactions were recognized between types Ib antigen (H 36 B) and group-B serum which contained also type Ia antibody (O-90), and type II antigen (V 8) and group-B serum containing type III antibody (D 136C). Moreover, repeated test sometimes result differently.

Accordingly, for serological diagnosis, this method does not seem to have superior points over the precipitation test, as far as *Str. agalactiae* is concerned. However, the author tried to see how the no-polysaccharide type strains of group-B react with sera of various types or of no-polysaccharide type strains by this method.

As is listed in table 12, twelve strains out of 19 do not reveal any positive reactions against group- or type-sera and sera by no-polysaccharide type strains. Strains Nos. 6, 34, 683 and 9 sometimes indicated unstable reactions, though the reason is not understood. However, only 3 strains, K 6, K 7 and 568, revealed clear positive reactions with 3 sera of

TABLE 11. *Results obtained by Use of Various Kinds of Antigens in Agar Gel Diffusion Method*

ANTIGEN		SERA CONTAINING GROUP AND TYPE ANTIBODY				TYPE SERA (Strain No.)			
		B, Ia (O-90)	B, Ib (H36B)	B, II (V 8)	B, III (D 136C)	Ia (O-90)	Ib (H36B)	II (V 8)	III (D136C)
O-90	1	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−
	4	+	−	−	−	+	−	−	−
H 36 B	1	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−
	3	−	++	−	−	++	−	−	−
	4	±	++	−	−	++	−	−	−
V 8	1	−	−	−	−	−	−	−	−
	2	−	−	±	−	−	−	−	−
	3	−	−	+	±	−	−	+	−
	4	−	−	++	+	−	−	++	±
D 136C	1	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−
	4	−	−	−	++	−	−	−	++

Notes: 1—Broth culture. 2—Supernatant of broth culture.
3—Saline suspension of live organisms.
4—Acid-extract following LANCEFIELD.

TABLE 12. *Relations of No Polysaccharide Type Strains in Agar Gel Diffusion Method*

	ANTIGEN	GROUP SERA (O-90)	GROUP SERA (H36B)	GROUP SERA (V 8)	GROUP SERA (D136C)	TYPE SERA (STRAINS USED) (O-90)	TYPE SERA (STRAINS USED) (H36B)	TYPE SERA (STRAINS USED) (V 8)	TYPE SERA (STRAINS USED) (D136C)	UNABSORBED SERA PREPARED FROM NO-POLYSACCHARIDE TYPE STRAIN No. 1* (6, 34)	No. 2* (40, B9, 683)	No. 3 (9)	No. 4 (568)
No-Polysaccharide type strains	G 3, Y 9, B 9, N 33, 40, 138, 142, N144, 144 139, 685, 686	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	+**	−
	34	−	−	−	−	−	−	−	+	+	−	−	−
	683	−	−	−	−	−	−	−	−	−	+**	−	−
	9	−	−	−	−	−	−	−	−	−	−	+**	+**
	K 6	−	−	−	−	−	−	−	−	+**	+	+	+
	K 7	−	−	−	−	−	−	−	−	−	+	+	+
	568	−	−	+	−	−	−	+	−	−	+	+	+
Control	Type II (Isolated)	−	−	+	−	−	−	+	−	−	−	+	+
	Type II (V 8)	−	−	+	−	−	−	+	−	+	+	+	+
	Type III (D 136C)	−	−	−	+	−	−	−	+	+	−	−	−
	Type Ia (O-90)	+	−	−	−	+	−	−	−	±~−	−	±~−	−
	Type Ib (H 36B)	+	+	−	−	−	+	−	−	±~−	−	±~−	−

Notes: * Polyvalent sera. ** Negative reactions were obtained in repeated tests.

no-polysaccharide type strains and in addition, strain 568 reacted positively with type II serum (V 8), similarly to the reaction with V 8 antigen.

It will be supposed that these reactions may be due to some unknown type specific antigenic substance which could not yet be demonstrated by small test tube precipitation reaction. Although these findings naturally need additional elaborations, the author considers that the data suggest the possibility of some of no-polysaccharide type strains for further sero-typing.

4. Biochemical Characters of *Str. agalactiae*, Especially on Its Sugar Fermentations

With respect to the biochemical characteristics of *Str. agalactiae*, on several principal points such as abilities of hydrolyzing sodium hippurate, splitting of esculin, production of ammonia from arginine, no growth at 10°C and 45°C also in broth of pH 9.6 and 6.5% NaCl, there seems to be no controversy amongst the various workers. However, descriptions hitherto published on its power to ferment salicin, glycerol, lactose etc. have not always coincided with each other. For instance, SLAVIN stated that *Str. agalactiae* fermented salicin, however following the descriptions in 7th Edition of BERGEY's Manual, it is said that salicin may or may not be fermented. On glycerol fermentation, SHERMAN and 6th Edition of BERGEY's Manual reported negative but it was changed to positive in 7th Edition of BERGEY's. Also on lactose, it was described as positive or negative by SHERMAN. However in 7th Edition of BERGEY's Manual, it was reported that nearly all strains ferment lactose, although an occasional strain may fail to do so, contrary to the description in 6th Edition of having lactose fermenting ability without exception. As mentioned above, statements by the several authors about fermentation reactions of *Str. agalactiae* on several sugars are not yet clearly in agreement.

The present author studied these characters on several hundreds strains from bovine milk samples of different quarters or on the strains isolated at various intervals of time. All of these strains used indicated strong positive reactions with group-B serum by precipitation. Among them, the behaviours of 99 strains from different quarters and quarters in the various districts, are listed in table 13,

From the table, hydrolysis of sodium hippurate, splitting of esculin and production of ammonia from arginine were the most characteristic features of *Str. agalactiae* as SLAVIN has already reported. However, it did become clear that trehalose was fermented only by the strains less than 50%, contrary to the data hitherto reported as all positive. As to glycerol fermentation, about half of the strains fermented it but the other half did not. Arabinose, inositol and glycogen were very rarely fermented. Maltose and lactose are usually fermented by almost all strains, however, there seem to be some strains which do not attack them.

Accordingly, the following table briefly illustrates the most reliable fermentation reactions of *Str. agalactiae*.

Sodium hippurate	Esculin	Ammonia	Mannitol	Sorbitol	Inulin	Raffinose
+	−	+	−	−	−	−

TABLE 13. *Fermentation Reactions of Group-B Streptococci*

NO. EXAMINED		0.01% METHYLENE BLUE	LITMUS MILK	SODIUM HIPPURATE	ESCULIN	AMMONIA FROM ARGININE	SALICIN	GLYCEROL	GALACTOSE	MANNOSE	LEVULOSE	SUCROSE	MALTOSE	LACTOSE	XYLOSE	ARABINOSE	RHAMNOSE	AMYGDALIN	INOSITOL	GLYCOGEN	RAFFINOSE	INULIN	SORBITOL	MANNITOL	DEXTRIN	TREHALOSE
99	C.	$+_{40}$	A.C. +	−	+	$+_{73}$	$+_{41}$	+	+	+	+	$+_{98}$	$+_{96}$	−	$+_{1}$	−	−	$+_{1}$	$+_{2}$	−	−	−	−	$+_{33}$	$+_{37}$
	C.R.	$-_{59}$					$-_{26}$	$-_{58}$					$-_{1}$	$-_{3}$		$-_{98}$			$-_{98}$	$-_{97}$					$-_{56}$	$-_{52}$

Notes: C.···Cogaulation, C.R.···Coagulation and reduction, A.C.···Acid and coagulation.
Numerals indicate the number of strains.

These differences in fermentation reactions were studied on several strains derived from various herds. The data obtained, are presented in table 14. From these data it will be seen that, as far as the author examined, there seem to be 3 types of *Str. agalactiae* which show different biochemical reactions. They are the salicin-negative- and trehalose-positive, the salicin-positive- and trehalose-negative and the both positive type respectively. The author never encountered the both negative type. These biochemical types do not seem to have any correlations with their sero-types. So the author examined the relations of these biochemical types and their origin. The data obtained are recorded in table 14.

TABLE 14. *Differences of Biochemical Reactions among Strains from Separate Farms*

SOURCE	NO. OF STRAINS	SEROTYPE	SOD. HIPPURATE	ESCULIN	AMMONIA	SALICIN	GLYCEROL	TREHALOSE	DEXTRIN	MANNITOL	INULIN	SORBITOL	RAFFINOSE	INOSITOL	ARABINOSE	GLYCOGEN	LACTOSE	MALTOSE	SUCROSE
Farm A	32	II	+	−	+	+	$+_{29}$	$+_{3}$ $-_{29}$	$+_{11}$	−	−	−	−	−	−	−	+	+	+
Farm B	28	II	+	−	+	+	$+_{12}$	−	$+_{3}$	−	−	−	−	−	−	$+_{1}$	+	+	+
	1	No-Poly-saccharide	+	−	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+
Farm C	3	"	+	−	+	−	−	+	$+_{1}$	−	−	−	−	−	−	−	+	+	+
	10	II	+	−	+	−	−	+	$+_{3}$	−	−	−	−	−	−	−	+	+	+
Farm D	10	No-Poly-saccharide	+	−	+	−	−	+	−	−	−	−	−	−	−	−	+	+	+
Control	O-90	I a	+	−	+	+	−	+	+	−	−	−	−	−	−	−	+	+	+
	H 36 B	I b	+	−	+	+	−	+	+	−	−	−	−	−	−	−	−	+	+
	V 8	II	+	−	+	−	−	+	+	−	−	−	−	−	−	−	+	+	+
	D 136 C	III	+	−	+	+	−	+	+	−	−	−	−	−	−	−	+	+	+
	B 125	No-Poly-saccharide	+	−	+	−	−	+	+	−	−	−	−	−	−	−	+	+	+

Notes: Numerals under the signs of + or − indicate the number of strains.

Following this table, in Farms C and D, the infection was caused only by the salicin-negative- and trehalose-positive strain, however in Farm B, all strains which were examined, were salicin-positive- and trehalose-negative ones. In Farm A, mixed infections by the salicin-positive- and trehalose-negative strains and the both positive strains were demonstrated.

As mentioned above, the strains which were originated from one farm, generally tend to show the same biochemical type. The author considers that hereafter such biochemical types among *Str. agalactiae* may have some significance in epidemiological research, like

the serological types of them.

IV. DISCUSSION

In Japan, serological grouping and typing of *Str. agalactiae* derived from bovine sources were carried out by the author for the first time, though until now the detection of 2 strains of group-B streptococci from human throat swabs was already reported by KODAMA and also by IKUTA and SAITO, a case of infection of rabbit.

From the present work, it has become clear that the prevalence of type II of group-B streptococci is dominant in Hokkaido and the type which lacks any type-specific carbohydrate ranks next. These results almost coincide with the data from PATTISON et al. in England, though the percentages of the isolations of these types are something different in detail, namely in Hokkaido the distribution of type II is dominant and type III is very rare in comparison with the data from England. Through the two sets of data, the fact that types Ia and Ib were not demonstrated at all, seems to be somewhat interesting. Whether these types of group-B streptococci do not have any very intimate relationship with the bovine mastitis may be of interest; it remains an unsolved problem which must be studied soon.

As to the strains lacking the type specific carbohydrate, the present author also made repeated tests by various extraction methods in different pH ranges after successive cultures for more than 10 generations in glucose or glycerol serum broth. However, by capillary tube method of precipitation, any type specific antigen could not be demonstrated in them. In the course of examination, the author experienced the disappearance of type specific antigen in 2 strains of type III and type Ia respectively. Moreover, by using the agar gel diffusion method, the author observed that 2 strains examined which are lacking type specific carbohydrate, showed the clear positive reactions probably due to the type antigens with the sera containing known antibody of type II and type III respectively and also with some sera prepared from the strains lacking type specific carbohydrate in capillary tube method.

The above-stated facts may suggest that there may be some possibilities to demonstrate the type antigen among the strains in question by using the appropriate technics. It was very interesting that in agar gel diffusion method, the group reactions which always appeared promptly in capillary tube method, could not be demonstrated at all. The author does not know the reason why only the type reaction had appeared.

As to the slide agglutination of *Str. agalactiae*, it was found that there are considerably many strains which showed autoagglutinable character; in some

strains the results were not always constant from test to test. Accordingly, this method seems to be not a very good one.

The precipitation test following LANCEFIELD gave always good reactions. In this case, it must be emphasized that the pH range in acid extraction is very important and the group specific antigens could not be demonstrated by the acid extraction in pH range of below 1.2 or over 3.8, contrary to the case of the type specific antigens which were stable in the above-mentioned pH ranges.

Concerning the biochemical characteristics of *Str. agalactiae*, the author could recognize that galactose, mannose, levulose, and sucrose were always fermented, however, xylose, rhamnose, raffinose, mannitol, sorbitol and inulin were not attacked. The above-stated fermentation reactions seem to be the invariable characteristics of this organism, though there are some exceptions on inositol, glycogen, arabinose, maltose and lactose. As to glycerol, it presents different reactions according to the strain.

From the survey, it may be stated that there seem to be 3 biochemical types. One biochemical type of *Str. agalactiae* tends to become prevalent among cows in 1 farm. Accordingly the author supposes that the different results of fermentation reactions of *Str. agalactiae* on salicin, trehalose and glycerol by authors such as SLAVIN, SHERMAN, DUBOS or BERGEY'S MANUAL, may be due to the differences of herd or districts from which the strains were derived.

From these data, the fermentation reaction of *Str. agalactiae* on salicin, trehalose or glycerol may not constitute a reliable feature differentiating it from other species. However, with the negative reactions to xylose, rhamnose, raffinose, mannitol, inulin and sorbitol, the author is confident that hydrolysis of sodium hippurate and esculin, and production of ammonia from arginine are unquestionably main characteristics of group-B streptococci, as SLAVIN has already emphasized.

V. SUMMARY

Bacteriological studies on streptococci, especially on *Str. agalactiae* isolated from bovine milk in Hokkaido during 1955 and 1956 were described. The data obtained are summarized as follows:

1. A total 3508 quarter milk samples derived from 886 cows were examined from the view point of a study of streptococci. Incidence of streptococci in bovine milk in Hokkaido was 27.0% in cows (239 out of 886), and 11.9% in quarters (418 quarters out of 3508).

2. These streptococci were demonstrated from average 69.2% of mastitis quarters and 51.4% of quarters which secreted the abnormal milk, though it was demonstrated from normal milk in 18.8%. These percentages are widely diverse by farms.

3. Isolated strains of streptococci to the number of 319 were divided into *Str. agalactiae*; 126 (39.5%), *Str. uberis*; 80 (25.1%), *Str. bovis*; 34 (10.7%), *Str. faecalis*; 17 (5.3%), *Str. lactis*; 12 (3.8%), *Str. dysgalactiae*; 8 (2.5%), *Str. acidominimus*; 5 (1.6%), *Str. pyogenes*; 4 (1.3%) and others.

4. Eighty percent of *Str. agalactiae* and *Str. dysgalactiae* strain, 70% of *Str. uberis* strain and about 90% of *Str. lactis* strain were derived from mastitis or abnormal milk. Contrary to this, more than 50% of *Str. bovis* or *Str. faecalis* were isolated from normal milk.

5. One hundred forty-five strains which reacted with group-B serum were serologically typed into type II; 114 (78.6%) and type III; 1 (0.7%) by precipitation reaction following LANCEFIELD. The author could not demonstrate any type specific carbohydrate antigens in the remaining 30 strains (20.7%) examined.

6. Some experiments were performed by agar gel diffusion method on the strains lacking type specific carbohydrate. Discussion was offered.

7. Survey on the distributions of these sero-types among cows in 4 farms showed interesting results characteristic to each farm.

8. Biochemical properties, especially sugar fermentation reaction were examined. According to this there seem to be 3 types; the salicin-negative- and trehalose-positive type, the salicin-positive and trehalose-negative type and the both-positive type. One biochemical type of *Str. agalactiae* tends to be detected in 1 farm.

9. *Str. agalactiae* produce acid from galactose, mannose, levulose, sucrose but not from xylose, rhamnose amygdalin, raffinose, inulin, sorbitol and mannitol without exception. With the characters of hydrolysis of sodium hippurate, non-splitting of esculin and production of ammonia from arginine, these fermentation reactions seem to be invariable by strain.

10. The strains which manifested the above mentioned biochemical characters all reacted promptly strong positive with group-B sera by precipitation.

The author wishes to express his sincere thanks to Prof. HIRATO, chief of the Department of Hygiene and Microbiology, for his kind direction and review. The author is indebted to Dr. Rebecca C. LANCEFIELD for strains O-90, H 36B, V 8 and D 136C, representative of types Ia, Ib, II and III respectively in her system. The author's sincere thanks are also due to Dr. Rebecca C. LANCEFIELD, Dr. IMAIZUMI, National Institute of Health, Tokyo, Prof. KUDO, Institute for Infectious Diseases, Tokyo University and Communicable Disease Center, Georgia, U.S.A. for the other type strains.

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