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ENTEROTOXIGENICITY OF BOVINE STAPHYLOCOCCI ISOLATED FROM CALIFORNIA MASTITIS TEST-POSITIVE MILK IN JAPAN

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Enterotoxigenic staphylococci (ES) were detected in 363 (34.4%) out of 1,056 coagulase-positive isolates obtained from California Mastitis Test-positive milk from 708 cows on 168 farms scattered throughout Japan. The ES produced enterotoxins C (54.3%), A (31.1%), D (27.0%) and B (10.7%) either alone or together. None produced enterotoxin E. The ES were isolated from 34.0% of the mammary quarter-milk samples. Of the enterotoxigenic isolates, 38.6% were typable by the basic set for typing bovine staphylococci, while 62.3% of the nonenterotoxigenic isolates were typable. Almost all of the enterotoxin A- and D-producing isolates belonged to phage groups III or I/III. Several repeated examinations of the same cows showed that the ES persisted in the mammary glands of the same cow for at least 8 months and that it was transmitted to other cows of the same farm. These results indicated that staphylococcal infections of the bovine mammary glands represent a significant reservoir of ES.

INTRODUCTION

Since 1914 the cow has been one of the common sources of enterotoxigenic staphylococci (ES), and raw milk^{1,4,6)} and milk products^{3,12)}, especially raw milk products such as cheese²¹⁾, have been implicated in food poisoning outbreaks. Several investigations carried out in many countries have shown that staphylococcal isolates from cases of bovine mastitis have usually produced enterotoxins C or D, and that the incidence rates of ES varied from 0 to 41.4%^{2,7,8,16,17,20)}.

The purpose of this investigation was to study the enterotoxigenicity and phage sensitivity of bovine staphylococci isolated from mammary quarter-milk samples which showed a positive reaction to the California Mastitis Test (C. M. T.) in farms scattered throughout Japan.

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MATERIALS AND METHODS

Staphylococci collected A total of 1,098 staphylococcal isolates was collected from 35 prefectures, an administrative division of Japan consists 6 districts including 47 prefectures, during a period from July, 1971 to February, 1973. All of the isolates originated from C. M. T.-positive milk obtained from asymptomatic cows. Most of the milk samples showed high, direct microscopic counts of leucocytes ($\geq 510,000/\text{ml}$), pH (≥ 6.5), and plate counts of *Staphylococcus aureus* ($\geq 1,100/\text{ml}$)¹⁴, indicating that these milk samples were obtained from cows suffering from subclinical mastitis.

Coagulase-positive staphylococci tested Each staphylococcal isolates was transferred to a sheep blood agar plate. From the colonies grown on the blood agar plate, a representative colony was selected and tested for the production of coagulase. A total of 1,056 coagulase-positive staphylococci was selected for further study; these isolates originated from 1,037 mammary quarters of 708 cows from 168 farms in 35 prefectures. All of the coagulase-positive isolates were tested by enterotoxin- and bacteriophage-typing, and some of them were also tested for drug sensitivities.

Coagulase test The production of coagulase was measured by the tube method using fresh rabbit plasma. The rabbit plasma was diluted 1:4 with saline, and 0.5 ml of this was added to 0.5 ml of a culture grown overnight at 37°C in pepton medium (Difco). Readings were made after 24 hours of maintenance at room temperature, as well as after 1, 2, 3, and 4 hours of incubation at 37°C. The resulting solid and fibrinous coagulum was evaluated as positive.

Enterotoxin typing All of the staphylococcal isolates were tested for their ability to produce enterotoxins A, B, C, D, and E. Production of enterotoxins followed the method described previously¹¹. The enterotoxins in a culture supernatant and in a concentrated culture supernatant (40 times) produced by the staphylococcal isolates were identified by the microslide double gel-diffusion technique, as described by KATO et al. (1978). The lowest level of enterotoxins detectable by the test was 1.0 $\mu\text{g}/\text{ml}$ of reference enterotoxins. The reference enterotoxins and their corresponding antisera were supplied by M. S. BERGDOLL of the Food Research Institute, the University of Wisconsin, Madison, U. S. A.

Bacteriophage typing A basic set for typing bovine staphylococci⁹ provided by I. DAVIDSON of the Central Veterinary Laboratory, the Ministry of Agriculture, Fisheries and Food, Weybridge, England, was used. The basic set consisted of 16 phages of the following groups: Group I-29, 52 A; Group II-3 A, 116; Group III-6, 42 E, 53, 75, 84; Group IV-42 D, 102, 107, 117; miscellaneous-78, 118, 119. The propagation and titration of the phages, the typing method of the staphylococcal isolates, and the interpretation of the results were those described in a previous report¹³. The phages were used only at a 100 \times routine test dilution (RTD), except for phage 84, which was

used only at RTD.

Drug sensitivity The sensitivities of the staphylococcal isolates to penicillin, methylchlorophenylisoxazolympenicillin, spiramycin, erythromycin, leucomycin, chloramphenicol, tetracycline, dihydrostreptomycin, kanamycin, sulfisoxazole, and sulfadimethoxine were determined by the disk-diffusion method with Tridisks (Eiken).

RESULTS

A total of 1,056 staphylococcal isolates were tested for enterotoxigenicity (tab. 1). Of these isolates, 363 (34.4 %) produced one or more of enterotoxins A, B, C, and D. No isolates produced enterotoxin E. In a decreasing order of frequency the enterotoxin types of the 363 isolates were C, D, A, B, A + D, A + C, A + C + D, C + D, A + B, B + C and B + D. Of all the toxigenic isolates, 54.3 %, 31.1 %, 27.0 %, and 10.7 % produced enterotoxins C, A, D, and B, respectively; and they produced in either single types or in combination with other types of enterotoxins. The frequencies of toxigenic isolates in bovine staphylococci ranged from 11.7 to 57.4 % in the 6 districts surveyed. The isolates from the Chugoku-Shikoku district were most often the producers of enterotoxin A alone or in combination, while those from the Kinki and Kyushu districts produced enterotoxin D alone or in combination. The enterotoxin C producers were distributed most commonly.

The frequency of ES in farms, cows, or cows' quarters in the 6 districts ranged from 30.8 to 65.2 %, 15.6 to 52.6 %, and 11.9 to 58.0 %, respectively (tab. 2). A total of 55.4 % of the farms, 34.9 % of the cows, and 34.0 % of the cows' quarters were observed for their yields of ES. The number of enterotoxin types found in each farm is shown in table 3. Staphylococci from most of the farms produced one enterotoxin type; there were few which produced more than 3 enterotoxin types.

Of the 1,056 isolates, 576 (54.2 %) were lysed by phages of the basic set for typing bovine staphylococci (tab. 4). It was found that of the 363 toxigenic isolates, 38.6 % were typable, while of the 693 nontoxigenic isolates, 62.3 % were typable. None of the toxigenic isolates belonged to phage group I alone. Almost all of the enterotoxin A- and D-producing isolates belonged to phage groups III or I/III, while most of the enterotoxin B- or C-producing isolates belonged to phage groups II, IV, miscellaneous, or to a mixed group. In addition, 136 (46.9 %) of the 290 single enterotoxin-producing isolates were lysed by the phages, while only 4 (5.5 %) of the 73 multiple enterotoxin producing isolates were lysed.

Table 5 indicates the drug sensitivity of the staphylococcal isolates. Thirty-two percent of the enterotoxigenic isolates were resistant to penicillin, 41 % to sulfisoxazole, and 44 % to sulfadimethoxine. There was no difference between the toxigenic and nontoxigenic isolates in their sensitivity to antimicrobial drugs.

Several repeated examinations of the same quarters of the same cows were per-

TABLE 1 *Enterotoxin production of bovine staphylococci isolated from subclinical mastitis milk*

DISTRICTS	NO. OF ISOLATES TESTED	NO. OF TOXIGENIC ISOLATES	ENTEROTOXIN TYPES DETECTED												
			A	B	C	D	E	AB	AC	AD	BC	BD	CD	ACD	A~E Negative
Hokkaido and Tohoku	248	91 (36.7)	23	4	28	15	·*	1	18	1	·	·	1	·	157
Kanto	191	62 (32.5)	·	6	51	3	·	·	·	1	·	·	·	1	129
Chubu	137	52 (38.0)	8	4	30	4	·	2	·	·	1	·	3	·	85
Kinki	188	53 (28.2)	8	7	15	18	·	·	·	1	·	·	·	4	135
Chugoku and Shikoku	155	89 (57.4)	5	11	29	6	·	·	8	24	·	·	·	6	66
Kyushu	137	16 (11.7)	2	2	2	9	·	·	·	·	·	1	·	·	121
Total	1,056	363 (34.4)	46	34	155	55	·	3	26	27	1	1	4	11	693

* Not detected

TABLE 2 *Frequency of enterotoxigenic staphylococci-positive milk in subclinical mastitis cows in Japan*

DISTRICTS (NO. OF PREFECTURES)	NO. OF PREFECTURES SURVEYED	FREQUENCY WITH WHICH TOXIGENIC ISOLATES WERE FOUND			
		Farm	Cow	Quarter	
Hokkaido and Tohoku	(7)	7	18/ 28* (64.3)	57/161 (35.4)	87/243 (35.8)
Kanto	(7)	6	11/ 22 (50.0)	47/133 (35.3)	60/190 (31.6)
Chubu	(9)	7	22/ 36 (61.1)	44/ 98 (44.9)	49/134 (36.6)
Kinki	(7)	6	19/ 33 (57.6)	35/131 (26.7)	50/185 (27.0)
Chugoku and Shikoku	(9)	4	15/ 23 (65.2)	50/ 95 (52.6)	87/150 (58.0)
Kyushu	(8)	5	8/ 26 (30.8)	14/ 90 (15.6)	16/135 (11.9)
Total		35	93/168 (55.4)	247/708 (34.9)	349/1,037 (34.0)

* Numerator: No. of farms, cows, or quarters in which toxigenic staphylococci were isolated from quarter-milk samples
Denominator: No. of farms, cows, or quarters in which coagulase-positive staphylococci were isolated from quarter-milk samples

TABLE 3 *Number of enterotoxin types found in each farm*

DISTRICTS	NO. OF FARMS IN WHICH TOXIGENIC ISOLATES FOUND	NO. OF ENTEROTOXIN TYPES DETECTED				
		1	2	3	4	5
Hokkaido and Tohoku	18	12	5	. *	1	.
Kanto	11	9	2	.	.	.
Chubu	22	17	4	1	.	.
Kinki	19	16	3	.	.	.
Chugoku and Shikoku	15	12	1	1	.	1
Kyushu	8	7	1	.	.	.
Total (%)	93	73 (78.5)	16 (17.2)	2 (2.2)	1 (1.1)	1 (1.1)

* Means zero

TABLE 4 *Sensitivity to phages of the basic set for typing bovine staphylococci and relation between enterotoxin types and phage groups*

PHAGE GROUPS	NO. OF ISOLATES		ENTEROTOXIN TYPES										
	Nontoxigenic n=693 %	Toxigenic n=363 %	A n=46	B 34	C 155	D 55	AB 3	AC 26	AD 27	BC 1	BD 1	CD 4	ACD 11
I	5 (0.7)	0
II	18 (2.6)	11 (3.0)	. *1	1	10
III	81 (11.7)	57 (15.7)	13	1	19	23	.	.	1
I/III	6 (0.9)	3 (0.8)	1	1	.	1
IV	100 (14.4)	16 (4.4)	.	.	13	1	1	1	.
Mis.*2	4 (0.6)	17 (4.7)	.	8	9
Mixed*3	218 (31.5)	36 (9.9)	.	5	28	2	1
Untypable	261 (37.7)	223 (61.4)	32	18	76	28	2	26	26	1	.	3	11

*1 Means zero

*2 Miscellaneous

*3 Mixed groups except I/III

formed during a period from August, 1971 to May, 1972 in 2 farms from different districts. In farm M a total of 44 coagulase-positive staphylococci were isolated from the quarter-milk samples of 12 cows (tab. 6). Of the 44 isolates, 31 were enterotoxigenic. The enterotoxin C-producing staphylococci belonging to phage group III (phage type 53 or 53/84) were predominant. In this farm the enterotoxin C- and B-producing staphylococci were detected in the first examination in 5 and 2 out of 12 cows, respectively. These ES had inhabited the same mammary quarters from 2 to 4 months. The C-producing staphylococci were transmitted to other quarters of the same cow and also to other cows. In farm G a total of 57 coagulase-positive staphylococci were isolated from the quarter-milk samples of 11 cows (tab. 7). Of the 57 isolates, 55 were enterotoxigenic, 27 were A+D-producers, 10 A+C+D-producers, 8 A-producers, 8 A+C-producers, and 2 B-producers. Only the B-producing isolates were lysed by the phages and belonged to the miscellaneous phage group (118) or to I/miscellaneous (52 A/118). Cow No. 9 of farm G revealed that staphylococci producing the same type of enterotoxin persisted in the mammary glands for 8 months, which was the longest period examined.

TABLE 5 *Comparison of drug sensitivity between toxigenic and nontoxigenic isolates*

DRUGS	HIGHEST DRUG CONCENTRATION	NO. OF RESISTANT ISOLATES	
		Toxigenic n = 223 %	Nontoxigenic n = 495 %
Penicillin	10 u	72 (32.3)	153 (30.9)
Methylchlorophenyl- isoxazolylpenicillin	10 γ	2 (0.9)	2 (0.4)
Spiramycin	300 γ	1 (0.4)	6 (1.2)
Erythromycin	10 γ	1 (0.4)	6 (1.2)
Leucomycin	15 γ	1 (0.4)	2 (0.4)
Chloramphenicol	30 γ	1 (0.4)	0
Tetracycline	30 γ	4 (1.8)	3 (0.6)
Dihydrostreptomycin	50 γ	7 (3.1)	10 (2.0)
Kanamycin	30 γ	1 (0.4)	0
Sulfisoxazole	300 γ	92 (41.3)	201 (40.6)
Sulfadimethoxin	300 γ	97 (43.5)	212 (42.8)

TABLE 6 Several repeated examinations on changes of enterotoxigenic staphylococci in farm M

COW NO.	QUARTER*1	PERIOD OF EXAMINATION				
		Aug., '71	Oct.	Dec.	Feb., '72	Apr.
2	FR	(III)*3	(III)	—	(III)	—
4	FL	—*2	C (III)	—	C (UT)	—
	FR	C (III)	C (III)	(UT)	—	—
	RL	(UT)	C (UT)	(UT)	—	—
	RR	C (III)	C (III)	(UT)	—	—
6	FL	C (III)	—	—	—	—
	FR	C (UT)	—	—	—	—
	RL	C (III)	C (UT)	(UT)	—	—
8	RL	C (III)	C (III)	—	—	—
	RR	B (mis.)	—	—	—	—
9	FL	C (III)	—	—	—	—
	RR	(II)	—	—	—	—
12	FL	—	—	—	—	C (III)
	FR	B (mis.)	—	—	C (UT)	C (III)
	RL	—	—	—	—	C (III)
13	FL	C (III)	C (III)	(UT)	—	—
	FR	—	C (UT)	—	—	—
	RR	C (III)	—	—	—	—
15	FR	(UT)	—	—	—	(II)
	RR	B (mis.)	B (UT)	B (mis.)	—	(II)
16	FL	—	—	—	C (III)	—
17	RL	—	—	—	—	C (III)
19	FR	—	—	—	C (III)	—
22	RR	—	—	—	—	C (III)

*1 FL: Front left FR: Front right RL: Rear left RR: Rear right
Staphylococci were not isolated from undescribed quarters in the cows.

*2 —: Staphylococci were not isolated.

Blank space: Coagulase-positive staphylococci were isolated, but they were not enterotoxigenic.

Capital: Enterotoxin types of staphylococcal isolates

*3 Phage groups are shown in the parentheses.

mis.: miscellaneous UT: Untypable

TABLE 7 *Several repeated examinations on changes of enterotoxigenic staphylococci in farm G*

COW NO.	QUARTER	PERIOD OF EXAMINATION				
		Sep., '71	Nov.	Jan., '72	Mar.	May
2	FL	—	—	AC (UT)	—	—
	FR	—	—	AC (UT)	—	—
	RR	—	AD (UT)	A (UT)	—	—
3	FL	—	—	AD (UT)	(UT)	AD (UT)
	FR	—	—	—	AC (UT)	AC (UT)
	RL	—	—	—	A (UT)	AD (UT)
	RR	A (UT)	—	—	—	AC (UT)
4	FR	AD (UT)	—	—	—	—
	RL	—	—	AD (UT)	—	—
	RR	AD (UT)	(UT)	AD (UT)	—	—
5	FL	—	A (UT)	—	—	—
	RL	—	AD (UT)	—	—	—
7	FR	—	ACD (UT)	—	—	—
	RL	AD (UT)	ACD (UT)	AD (UT)	—	—
	RR	—	AD (UT)	—	—	—
8	FL	—	—	AC (UT)	—	—
	FR	AD (UT)	A (UT)	AD (UT)	—	—
	RL	—	—	AD (UT)	—	—
	RR	—	—	ACD (UT)	—	—
9	FL	AC (UT)	—	AD (UT)	—	A (UT)
	FR	ACD (UT)	—	—	ACD (UT)	ACD (UT)
	RL	—	—	AD (UT)	A (UT)	ACD (UT)
	RR	—	—	AC (UT)	—	—
12	FL	—	—	ACD (UT)	—	—
	FR	—	AD (UT)	—	—	—
14	FL	AD (UT)	—	—	—	—
	FR	AD (UT)	—	—	—	—
	RL	—	B (I/mis.)	—	—	—
	RR	AD (UT)	B (mis.)	—	—	—
15	FL	—	AD (UT)	—	—	—
	RL	—	AD (UT)	—	AD (UT)	—
	RR	—	AD (UT)	—	—	—
16	FL	—	AD (UT)	—	—	AD (UT)
	FR	—	A (UT)	—	—	ACD (UT)
	RR	—	—	ACD (UT)	—	—

DISCUSSION

In the present study 1,056 coagulase-positive staphylococci isolated from C. M. T.-positive quarter-milk cows suffering from subclinical mastitis were collected from areas all over Japan. It was found that 34.4 % of the isolates were enterotoxigenic, and that of the toxigenic isolates, 54.3 % produced enterotoxin C, 31.1 % A, 27.0 % D, and 10.7 % B, either alone or together. The results were somewhat similar to those of NISKANEN & KOIRANEN (1977). In a recent report NISKANEN & KOIRANEN (1977) cited that of the 174 staphylococcal strains isolated from mastitis milk in 5 northern European countries, 41.4 % produced enterotoxins, and of the toxigenic strains, 48.6 % produced enterotoxins A, 5.6 % B, 29.2 % C, and 33.3 % D, either alone or together. From 1967 to 1974, several studies carried out on limited amounts of materials in other countries have shown that staphylococci isolated from cases of bovine mastitis have usually produced enterotoxins C or D, and that the incidence rates of the toxigenic isolates were less than 6 %^{2,7,8,20}, except for one case of 14.6 % reported by OLSON et al. (1970).

The rate of detection (34.9 %) of ES from the subclinical mastitis cows in this study was distinctly higher than that found in stray dogs (5.8 %)¹⁰, chickens (3.8 %)¹⁸, and house rats (3.6 %)¹⁵.

In the present study, ES were isolated from 34.0 % of the quarter-milk samples which showed a positive reaction for C. M. T. A 1969 survey of bovine mastitis performed in 43 prefectures in Japan showed that 26.3 % out of 20,552 quarter-milk samples were positive for C. M. T.⁹ From these two results, it was estimated that ES may be isolated from as much as 9 % of the mammary quarter-milk samples.

The rate of the isolates typable by the basic set for typing bovine staphylococci was 38.6 % in the toxigenic isolates and 62.3 % in the nontoxigenic isolates; the rate was particularly low in the toxigenic isolates. With this in mind, it is necessary to devise a new set of phages which can type the enterotoxigenic strains more efficiently.

Enterotoxins A, C, or D, either alone or together, were the predominant types causing food poisoning in many countries including Japan^{2,16,19,20}. The same enterotoxin types were produced by 89.3 % of the toxigenic isolates used in this investigation. Also, it was estimated that ES was present in 9 % of the mammary quarter-milk. Therefore, the potential for the production of enterotoxins and the development of staphylococcal food poisoning due to poorly refrigerated or processed raw milk containing ES are fairly obvious. The prevention of subclinical mastitis is a major factor, then, in the protection of consumers from staphylococcal food poisoning caused by contaminated raw milk and dairy products.

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