Jap. J. vet. Res., **20**, 91~110 (1972)

CLASSIFICATION OF CHICKEN COAGULASE-POSITIVE STAPHYLOCOCCI INTO FOUR BIOLOGICAL TYPES AND RELATION OF THE TYPES TO ADDITIONAL CHARACTERISTICS INCLUDING COAGULASE-ANTIGENIC TYPE*[1]

Gihei SATO, Shiro MIURA and Nobuyuki TERAKADO*[2]

Department of Epizootiology
Faculty of Veterinary Medicine
Hokkaido University, Sapporo, Japan

(Received for publication, August 24, 1972)

A total of 1,021 (98.4%) of 1,038 chicken coagulase-positive staphylococcal strains obtained from 27 farms were classified into 4 biological types (types 1~4) on the basis of properties such as pigmentation, hemolysis on sheep blood agar plate, Voges-Proskauer reaction and fermentation of lactose, galactose or mannitol. Only type 2 strains gave no pigment and were non-hemolytic. Voges-Proskauer reaction was negative, except for type 3, lactose- and galactose-fermentation was negative only in type 1, and mannitol fermentation was positive in all but type 2. DNase production was observed in all the biological types and egg-yolk factor in types 3 and 4. Caseinase was produced in about 80% of strains of each type. Fibrinolysin production was of low frequency, with the exception of type 2 strains. Only type 3 strains were phage-typed (about 38% of the type). Types 1 and 4 strains were classified into coagulase-antigenic type V, and type 3 strains into VII, VI and II of the coagulase type. Type 2 strains could not be coagulase-typed because of insufficient coagulase production, and indicated low virulence for mice and chickens.

INTRODUCTION

In the last decade, a large number of reports have been published on avian staphylococcosis and the characteristics of avian staphylococci. This seems to have been due to the worldwide increase in number of outbreaks of avian staphylococcosis in the remarkable development of poultry industry. In addition, attention has been paid to the characters of the organisms of avian origin from

*1 Presented in part at the 63rd Meeting of the Japanese Society of Veterinary Science (1966), *Jap. J. vet. Sci.*, **28**, Suppl. 337 (summary in Japanese) and the 42nd Meeting of the Japan Bacteriological Society (1969), *Jap. J. Bact.*, **24**, 537 (summary in Japanese)

*2 Department of Veterinary Public Health of this faculty
Present address: Second Assay Division, National Veterinary Assay Laboratory, Kokubunji, Tokyo, Japan

the viewpoint of rather academic interest in the host-specific parasitism of *Staphylococcus aureus*[4,15,17,26,27].

On the other hand, there are few reports on the ecological study of avian *S. aureus* because of the absence of adequate typing methods including phage typing. The present authors have been making a long-term study on the ecology of chicken coagulase-positive staphylococci in certain farms. During the study, attempts were made to classify the organisms into certain biological types.

This paper deals with the biological types and their additional characteristics including phage susceptibility and antigenic types of coagulase[28~30].

MATERIALS AND METHODS

Source of coagulase-positive staphylococci examined

A total of 1,038 strains were obtained from 27 farms in Japan (Hokkaido–17 farms, Osaka–9 and Tokyo–1) during the period from 1959 to 1968. Of these strains, 881 were isolated from 470 diseased or normal chickens of 101 flocks. Seven hundred and five of the 881 strains were obtained from 2 farms in which long-term investigations (5~7 years) on coagulase-positive staphylococci had been made. With the exception of the 6 farms from each of which only one strain was obtained, 2 or more strains from each farm were tested. The remaining 157 strains were isolated from air samples in 4 farms in Hokkaido. The strains were preserved in a cooked meat medium.

Isolation of coagulase-positive staphylococci

Except for the strains from Osaka and Tokyo, the organisms were isolated by the procedure described below, and representative strains were preserved in a cooked meat medium for further tests.

Isolation from lesions or visceral organs Duplicate colonies of staphylococci grown on a sheep blood agar plate were checked for coagulase production by the method given later.

Isolation from the skin, oral cavity, eyes, cloaca or intestines All or parts of the sites described above were rubbed with a sterile cotton swab in normal or diseased chickens. The swabs were cultured in nutrient broth containing 7.5% NaCl. After overnight incubation, the broth cultures were subcultured onto the sheep blood agar plate. Several staphylococcal colonies were checked for the coagulase production.

Isolation from the air Sheep blood agar plates were exposed in chicken houses for half a minute to a few minutes. Several staphylococcal colonies grown were employed for further examination.

Examination for biological characteristics

Coagulase production Citrated rabbit plasma was used for the tube test. A loopful of overnight agar culture of staphylococci was inoculated into 0.5 ml of the 10% plasma and incubated at 37°C. Readings were made 1, 2, 4, and 24 hrs after incubation.

Pigment production The production on 10% skimmed milk agar plate was recorded after the plate had been left 3 days at room temperature following overnight incubation at 37°C.

Hemolysin production Initially, hemolysis on a sheep blood agar plate was recorded.

Then, hemolysin types were checked according to the previous description[16]. Two or three % blood agar plates prepared from rabbit, sheep and horse red blood cells were applied.

Voges-Proskauer (V-P) reaction Barrit's method was used. A two-day culture was used for the test.

Fermentation of carbohydrates Modified Barsiekow's media containing 1% of each carbohydrate were inoculated and incubated. Fermentation within 7 days was regarded as positive.

DNase production Strains to be tested were inoculated onto a DNA (deoxyribonucleic acid) agar (Eiken) plate. The DNase producer indicated a clear zone around the growth with the addition of 1.5 N HCl.

Caseinase production The test was made according to SHIMIZU et al[19]. Test strains were inoculated onto a nutrient agar plate, with 2% of skimmed milk added. After a one-day incubation at 37°C, the plate was observed for a transparent zone around the colony.

Egg-yolk factor A basal agar with 7% NaCl and 1% mannitol for egg-yolk factor (Nissan) added was used. Into the medium, an egg-yolk solution (one egg yolk+30 ml of 10% NaCl solution) was mixed at the rate of 10%. Needle-culture of the test strains was made on the agar plate. After a 2-day incubation at 37°C, the plate was observed for a turbid ring around the growth.

Production of fibrinolysin A nutrient agar plate containing rabbit plasma at 1 : 5, which had been heated for 20 minutes at 60°C, was used for needle culture. Transparency around the growth after a 2-day incubation was regarded as positive.

Antibiotic sensitivity Only strains isolated in Hokkaido were tested for sensitivity to penicillin (PC) and oxytetracycline (OTC). In the early stage of this study, discs (Eiken) of 3 concentrations of the antibiotics were used. Then the nutrient agar plate dilution method was employed as described in table 5.

Pathogenicity test Mice—The test was made according to KURAMASU et al[11]. A dose of 0.2 ml of 18-hr nutrient broth culture of the isolates was inoculated subcutaneously into the back of 3 mice (gpc strain, 4 weeks old, body weight-14 g). After 3 days, pathological changes (dermatitis or suppurative foci) were observed. Chickens—A dose of 0.5 ml of overnight broth culture of the isolates were injected subcutaneously into the outside thigh of each of 5 chickens (5 weeks old, White Leghorn). Deaths and pathological changes in the inoculation site were observed for 4 days and the birds were killed for cultivation.

Phage typing Phages and procedures were the same as NAKAGAWA's description[13] except for the additional use of phages 80 and 81. A phage concentration of the routine test dilution (RTD)×100 was used. Grouping of the 22 phages from the National Collection of Type Culture (NCTC) was as follows:

Group I: 29, 52, 52A, 79 and 80;
Group II: 3A, 3B, 3C, 55 and 71;
Group III: 70, 42E, 6, 7, 73, 47, 54, 75, 18 and 19;
Group IV: 42D;
Miscellaneous: 81.

Serological typing of staphylococcal coagulase The coagulase typing was made according to the descriptions of ZEN-YOJI et al.[28~30] with some modifications. Seven standard anticoagulase rabbit sera were commercial products (Eiken). The typing procedure was as follows. Strains to be tested were inoculated into 10 ml of heart infusion broth (Eiken) and incubated for 5 days. Then the broth cultures were centrifuged at 3,000 rpm for 30 minutes. The supernates were preserved in a refrigerator and used as coagulase. At first, the minimal clotting dose of coagulase per hr (MCD/hr) was determined in the following way: to a tube containing 0.1 ml each of 2-fold serially diluted coagulase, 0.1 ml of a 1 : 5 diluted normal rabbit serum was added and incubated in a water bath at 37°C for 30 minutes. Then, 0.2 ml of a 1 : 10 diluted rabbit plasma was added to each tube and the tubes were reincubated at 37°C for 1 hr. The coagulase dilution that was just sufficient to allow minimal clotting was designated as one MCD/1 hr. When the coagulase activity was weak, the tubes were incubated for 3 or 24 hrs. In this case, MCD/3 hrs or MCD/24 hrs was recorded.

A dose of 0.1 ml of coagulase to be typed, containing MCD/1 hr or MCD/3~24 hrs was dispensed into 8 tubes (Nos. 1~8). To each tube, 0.1 ml of the standard anticoagulase serum of I~VII types respectively was added. That is, to No. 1 tube, type I antiserum was added and to No. 2 tube, type II antiserum, etc. To No. 8 tube, the same amount of 1 : 5 diluted normal rabbit serum. After incubation in a water bath at 37°C for 30 minutes, 0.2 ml of a 1 : 10 diluted rabbit plasma was added to each tube. Then the tubes were incubated for a further 1~24 hrs depending on the times at which MCD was recorded. The coagulase type was determined when the coagulase of a test strain was specifically neutralized with the standard anticoagulase serum and did not coagulate the plasma.

RESULTS

Detection of coagulase-positive staphylococci from diseased or normal chickens

In this study, mainly broiler chickens (1~70 days) were examined for the staphylococci. Occasionally semiadult or adult breeders were checked. Normal chickens gave coagulase-positive staphylococci in different sites of the body with variable frequencies (0~100%) depending on the degree of environmental contamination. Dead birds with dermatitis had the organisms in the intestines with high frequency (72% or more). Data in the ecological study of coagulase-positive staphylococci in chickens will be published in another paper.

Subdivision of chicken coagulase-positive staphylococci into 4 biological types

Several thousand cultures of staphylococci were tested for coagulase production, and representative strains of coagulase positive were employed for further tests. As shown in table 1, 1,021 of 1,038 strains were divided into 4 types on the basis of the properties described. The remaining 17 untypable strains were isolated mainly from normal birds or from the air with a very low frequency. The numbers of strains in each type do not show the actual distribution, because type 1 included strains from 2 farms on which long-term investigations were conducted.

Table 2 indicated that type 3 strains were isolated from 21 of the 27 farms examined and distributed most widely in Japan. Primary staphylococcal infections (dermatitis,

TABLE 1 *Biological types of chicken coagulase-positive staphylococci*

BIOLOGICAL TYPE	NO. STRAINS (NO. ISOLATES FROM AIR)	PIGMENT	HEMOLYSIS ON SHEEP BLOOD AGAR	V-P REACTION	FERMENTAION *1 OF		
					lactose	galactose	mannitol
1	699 (121)	yellow	+	−	−*2	−*2	+
2	65 (12)	white	−	−	+	+	−
3	251 (21)	yellow	+	+	+	+	+
4	6	yellow	+	−	+	+	+
	1,021 (154)…98.4 %						
Untypable a	2	yellow	+	+	+	−*2	+
b	3	yellow	−	+	+	+	+
c	4	lemon yellow	+	+	+	+	+
d	2 (1)	white	−	+	+	+	+
e	4 (1)	white	$+_2$*3	$+_1$	$+_3$	$+_2$	+
f	2 (1)	yellow	−	$+_1$	+	+	$+_1$
Total	1,038 (158)						

*1 Fermentation within a week was recorded as positive.

*2 Sometimes fermented after prolonged incubation

*3 Figures indicate No. of positive strains.

TABLE 2 *Sources and distribution of chicken coagulase-positive staphylococci of each biological type*

BIOLOGICAL TYPE	NO. STRAINS	NO. FARMS*1 GIVING THE ORGANISMS	LOCALITY OF FARMS	DISEASE STATUS OF CHICKENS HARBORING THE ORGANISMS			Air
				primary infection	mixed infection	normal	
1	699	9	Hokkaido Osaka	4*2	5	4	2
2	65	4	Hokkaido	•	4	2	2
3	251	21	Hokkaido Osaka Tokyo	11	3	8	3
4	6	2	Hokkaido	1	1	•	•
Untypable a	2	1	Tokyo	1	•	•	•
b	3	1	Osaka	1	•	•	•
c	4	1	Hokkaido	•	•	1	•
d	2	2	Hokkaido	•	•	1	1
e	4	3	Hokkaido	•	1	1	1
f	2	2	Hokkaido	•	•	1	1

*1 A total of 27 farms were investigated.

*2 No. farms

septicemia or arthritis) occurred in 17 of the 27 farms, and mixed infection with fowl pox or other diseases occurred in 5 farms. The remaining 5 farms gave coagulase-positive staphylococci only from normal chickens. Strains from Osaka and Tokyo were isolated only in diseased chickens.

Type 2 strains were not obtained from the primary staphylococcal lesions. Among untypable strains, 5 of a and b shown in table 2 were detected from a farm in Osaka and in Tokyo respectively. A farm which had been observed for 7 years gave predominantly type 1 strains. Another farm gave predominantly type 1 strains in the early stage of 5-year period and later both types 1 and 3 equally. Both farms yielded occasionally also type 2 staphylococci. Generally speaking, a uniformity of biological types was observed among strains derived from single flock.

Regarding the stability of the properties of the 4 biological types, strains which were tested again after more than 5 years indicated almost the same properties, although some yellow strains contained decolorized colonies or hemolysin patterns changed occasionally after the storage.

Biological characteristics of coagulase-positive staphylococci

Representative strains were tested for hemolysin patterns and the additional characteristics described below.

Coagulase activity It should be noted that type 2 strains did not give coagulase in the supernates of broth culture. Moreover, strains of the type indicated often weak and delayed coagulation in the test. Plasmas of animal species other than rabbit were not used for the coagulase test.

TABLE 3 *Hemolysin types of chicken coagulase-positive staphylococci*

BIOLOGICAL TYPE	NO. STRAINS TESTED	α	$\alpha\delta$	$\alpha\beta$	$\alpha\beta\delta$	$\beta\delta$	β	δ	WEAK
1	375 (8)*	215 (7)	15 (2)	18 (2)	121 (2)	5 (1)	•	1	•
3	113 (18)	50 (9)	21 (3)	2 (1)	33 (3)	2 (2)	•	2 (2)	3 (1)
4	6 (2)	1	5 (1)	•	•	•	•	•	•
Untypable a	2 (1)	2	•	•	•	•	•	•	•
Total	496 (23)	268 (14)	41 (5)	20 (3)	154 (4)	7 (2)	•	3 (3)	3 (1)

* Figures in parentheses indicate No. farms from which test strains were isolated.

Hemolysin type In both types 1 and 3, the number of strains giving only alpha-lysin occurred most frequently as shown in table 3. Moreover, in both types, the strains having alpha-lysin were 369 (98.4%) and 106 (93.8%) respectively. On the other hand, those having beta-lysin were 144 (38.4%) and 37 (32.7%). Consequently, there was no difference in the hemolysin types between both biological types. Generally speaking, the strains from a single flock gave the same hemolysin type.

TABLE 4 *Additional characteristics of each biological type of chicken coagulase-positive staphylococci*

BIOLOGICAL TYPE	PRODUCTION OF			
	DNase	Caseinase	Fibrinolysin	Egg-yolk factor
1	497/497 (9)*-100 %	380 (6)/495 (7)-76.8 %	41 (4)/157 (6)-26.1 %	0/520 (9)
2	47/ 47 (4)-100 %	41 (4)/ 47 (4)-87.2 %	35 (2)/ 37 (2)-97.3 %	0/ 54 (4)
3	125/125 (21)-100 %	77 (10)/104 (13) -74 %	15 (8)/ 77 (15)-19.4 %	125/125 (21)-100 %
4	5/ 5 (1)-100 %	5/5 (1) -100 %	0/5 (1)	5/5 (1) -100 %
Untypable a	2/2 (1)	2/2 (1)	2/2 (1)	2/2 (1)
b	3/3 (1)	Not done	3/3 (1)	3/3 (1)
c	4/4 (1)	4/4 (1)	Not done	0/4 (1)

* No. positives/No. strains tested; Figures in parentheses indicate No. farms giving strains or positives.

DNase production Table 4 indicates that all strains tested produced the enzyme.

Production of caseinase In each type, the frequency of the strong caseinase producer was as follows: Two hundred and thirty strains (60.5% of total positives) in type 1, 35 (85.4%) in type 2, 22 (28.6%) in type 3 and 5 (100%) in type 4. As described above, the frequency of the strong caseinase producer was lowest in type 3.

Production of fibrinolysin Type 2 strains gave the highest frequency of enzyme production, as seen in table 4.

Egg-yolk factor It was interesting that all of types 1 and 2 strains did not produce the factor, while types 3 and 4 were positive, as in table 4.

Antibiotic sensitivity Table 5 indicates that half of type 1 strains was highly resistant to OTC, and in other types the same resistance was observed with decreased frequencies. One farm gave *S. aureus*, being resistant to OTC from 1959, and another farm from 1961. Only a few type 3 strains were weakly resistant to PC.

Pathogenicity Table 6 shows that type 2 strains were of low virulence for mice. All of the mice inoculated with the strains gave only localized suppuration. The strains from lesions or caseinase producers did not always indicated a high virulence. Table 7 indicates that type 2 was low virulent also for chickens. Moreover, caseinase production and pathological changes in the site of inoculation were not always parallel.

Phage typing Of 330 strains, 29 (8.8%) were typable as shown in table 8. The typable strains belonged only to biological type 3. They were isolated from 4 farms. One of the 4 farms at which staphylococcal septicemia occurred gave the organisms being susceptible to phage 75 (4 strains). In the other 3 farms, normal chickens gave typable strains. In one of them, *S. aureus* resistant to PC of 2 units indicated phage patterns 70/7/54 (5 strains) and 70/54 (1 strain). In the remaining 2 farms, phage patterns 70/42E/54/53/77/73 (1 strain), 70/42E/54/53/77 (2 strains), 70/42E/54/53/73 (1 strain), 70/42E/54/53 (6 strains), 70/42E/50 (1 strain), 70/42E/54 (1 strain), 70/73 (1 strain), 42E/53 (1 strain), 42E (3 strains), 42E/70 (1 strain) and 70 (1 strain) were observed. A part of chickens of the same flock gave *S. aureus* of completely identical phage pattern. Of the 29 typable strains, one was a fibrinolysin producer.

Antigenic types of staphylococcal coagulase As indicated in table 9, a total of 194 strains (85.8%) were typable. Most of untypable strains were those which had been stored for long time. Type 2 strains could not be tested for coagulase typing because of the absence of coagulase in the supernates. Type V was found most frequently (122/194-62.9%). However, type VII distributed most widely (in 12 of 21 farms). It was interesting that all of the strains of biological types 1 and 4 were coagulase type V, and the coagulase type did not occur in biological type 3. Twenty two of 26 strains typed with the phages were classified into coagulase types II (6 strains), VI (1 strain) and VII (15 strains). Uniformity of coagulase types was observed among strains from single outbreak or the same flock. However, different coagulase types occurred occasionally in a single bird. The following was one of the examples. Five strains supplied by Dr. S. KURAMASU had been isolated in a diseased bird in an outbreak of staphylococcal dermatitis[11]. These were designated as follows: CH84 was isolated in the lung, CH85 in the liver, CH88 in the kidney, CH90 in the oral cavity and CH91 in the skin lesion. Except for strains CH85 and CH91 which

TABLE 5 *Incidence of antibiotic-resistant strains of each biological types*

BIOLOGICAL TYPE	NO. STRAINS TESTED	PENICILLIN				OXYTETRACYCLINE			
		0.5 u	2	10	20	5 mcg	10	30	40
	208*1 (2)*2	1	•	•	ND*4	107 (2)	106 (2)	99 (2)	ND
1	402*3 (7)	3 (2)	•	•	•	227 (6)	227 (6)	226 (6)	225 (6)
	610 (7)	4 (2) 0.7%	•	•	•	334 (6)	333 (6)	325 (6) 53.3%	225 (6)
	2*1 (1)	•	•	•	ND	2 (1)	2 (1)	2 (1)	ND
2	58*3 (4)	1	•	•	•	4 (1)	4 (1)	4 (1)	4 (1)
	60 (4)	1	•	•	•	6 (2)	6	6 10%	4 (1)
	35*1 (6)	1	•	•	ND	33 (4)	30 (4)	30 (4)	ND
3	196*3 (9)	11 (2)	9 (2)	1	•	39 (6)	35 (6)	33 (6)	33 (6)
	231 (14)	12 (3)	9 (2) 3.9%	1	•	72 (10)	65 (10)	63 (10) 27.7%	33 (6)
4	6*1 (2)	•	•	•	ND	2 (2)	1	1	ND
Untypable a	2*1 (1)	1	•	•	ND	•	•	•	ND
c	4*3 (1)	•	•	•	•	•	•	•	•
d	2*3 (2)	1	1	•	•	2 (2)	2	2	2
e	4*3 (3)	•	•	•	•	4 (3)	4	3 (3)	3
f	2*3 (2)	•	•	•	•	•	•	•	•
	14 (6)	2 (2)	1	•	•	6 (5)	6	5 (4) 35.7%	5

*1 Tested by the disc method

*2 Figures in parentheses indicate No. farms from which test strains originated.

*3 Tested by the agar plate dilution method

*4 Not done

TABLE 6 *Mouse virulence of staphylococci of each biological type*

SOURCE OF STRAINS	NAME OF STRAIN	BIOLOGICAL TYPE	CASEINASE PRODUCTION	SKIN LESION IN INOCULATION SITE				DIED
				necrosis	edema	localized suppuration	non lesion	
Lesions	K 171	1	−	•	3*	•	•	•
	U 111	1	+	•	•	•	3	•
	F 3	4	++	•	1	2	•	•
	F 4	4	++	•	3	•	•	•
Normal chickens	K 197	1	Not tested	1	2	•	•	•
	H 2-4	2	Not tested	•	•	3	•	•
	H 2-6	2	+	•	•	3	•	•
	H 7-33	3	+	2	1	•	•	•
	H 7-35	3	+	1	2	•	•	1
Air	K 176	1	Not tested	3	•	•	•	•
	U 116	1	+	3	•	•	•	•

* No. mice

TABLE 7 *Pathogenicity test for chickens with chicken staphylococci of each biological type*

NAME OF STRAIN	BIOLOGICAL TYPE (CASEINASE PRODUCTION)	SEPTICEMIC DEATH WITHIN 4 DAYS POST INOC. (CHANGES IN INOC. SITE)	PATHOLOGICAL CHANGES IN INOCULATION SITE OF SURVIVORS		DETECTION OF STAPHYLOCOCCI AT NECROPSY OF SURVIVORS		
			non	edema or swelling	inoculation site only	visceral organs & inoculation site	bacteriemia
K 171	1(−)	1/5*(necrosis)	2/4	2/4	•	2/4	1/4
U 116	1(+)	5/5 (necrosis & edema)	•	•	•	•	•
H 2-171	2(卄)	0/5	5/5	•	1/5	2/5	•
H 2-173	2(−)	0/5	4/5	1/5	1/5	1/5	•
H 7-33	3(+)	3/5 (necrosis-1, swelling-2)	•	2/2	•	•	2/2
H 7-35	3(+)	1/4 (swelling)	1/3	2/3	•	•	3/3
F 2	4(卄)	2/5 (necrosis & edema)	1/3	2/3	•	1/3	2/3
F 4	4(卄)	3/5 (necrosis & edema)	•	2/2	•	•	2/2

* No. positives/No. chickens inoculated

TABLE 8 *Phage typing of chicken coagulase-positive staphylococci of the biological types*

BIOLOGICAL TYPE	NO. STRAINS TESTED	NO. TYPABLE STRAINS	PERCENT	PHAGE GROUP
1	218 (6)*	•	•	•
2	28 (2)	•	•	•
3	76 (10)	29 (4)	38.2	III
4	5 (1)	•	•	•
Untypable a	2 (1)	•	•	•
c	1	•	•	•
Total	330 (15)	29 (4)	8.8	III

* Figures in parentheses indicate No. farms from which test strains originated.

TABLE 9 *Relationship between biological types of chicken coagulase-positive staphylococci and their antigenic types of coagulase*

BIOLOGICAL TYPE	NO. STRAINS GIVING SUFFICIENT COAGULASE FOR COAGULASE TYPING	NO. TYPABLE STRAINS	COAGULASE-ANTIGENIC TYPE*1				
			I	II	V	VI	VII
1	135 (7)*2	118 (7)	•	•	118 (7)	•	•
3	77 (16)	67 (15)	•	8 (3)	•	6 (2)	53 (12)
4	5 (1)	3 (1)	•	•	3 (1)	•	•
Untypable	9 (3)	6 (3)	2 (1)	•	1 (1)	•	3 (1)
Total	226 (22)	194 (21) 85.8 %	2 (1)	8 (3)	122 (9)	6 (2)	56 (12)

*1 No strain was classified into the types III and IV.
Biological type 2 strains did not give coagulase in the supernates.

*2 Figures in parentheses indicate No. farms.

were untypable by the biological properties (untypable a in table 1), the 3 strains belonged to biological type 3 and coagulase type II. Strain CH 91 was coagulase type V and CH 85 was untypable because it was a weak producer of coagulase.

Regarding the geographical distribution of coagulase types, biological type 3 strains from 6 of 7 farms in Osaka were classed as type VII. In Hokkaido, the *S. aureus* strains of biological type 3 from 5 farms having staphylococcosis belonged to type VII. The organisms of the same biological type from healthy flocks of 3 farms were coagulase types VII, II and VI respectively. Thus, types I (1 farm), II (2 farms), V (6 farms), VI (2 farms) and VII (6 farms) were isolated in Hokkaido, types V (2 farms) and VII (6 farms) in Osaka, and types II and V from a farm in Tokyo.

Tabel 10 indicates that coagulase type VII was detected most frequently in the farms with staphylococcosis. On the other hand, coagulase types I and VI were obtained only from normal birds.

TABLE 10 *Relationship between coagulase types and sources of test strains*

SOURCE OF STRAINS	NO. TYPABLE STRAINS	COAGULASE-ANTIGENIC TYPE*1				
		I	II	V	VI	VII
Diseased chickens	90 (18)*2	•	3 (1)	48 (7)	•	39 (11)
Normal ckickens	80 (5)	2 (1)	4 (1)	61 (4)	6 (2)	7 (1)
Air	24 (3)	•	1	13 (1)	•	10 (2)
Total	194 (21)	2 (1)	8 (3)	122 (9)	6 (2)	56 (12)

*1 No strain was classified into the types III and IV.
*2 Figures in parentheses indicate No. farms.

TABLE 11 *General properties of 4 biological types of chicken coagulase-positive staphylococci*

PROPERTY	BIOLOGICAL TYPE			
	1	2	3	4
Pigment	yellow	white	yellow	yellow
Hemolysis on sheep blood agar	+*2	−	+	+
V-P reaction	−*2	−	+	−
Fermentation*1 of				
Lactose & galoctose	−	+	+	+
Mannitol	+	−	+	+
DNase	+	+	+	+
Egg-yolk factor	−	−	+	+
Growth at 45°C*3	−	+	(+)	−
Caseinase	v+*2	(+)*2	v+	+
Fibrinolysin	v−*2	(+)	(−)*2	−
Typability with NCTC phages	−	−	v−	−
Coagulase-antigenic type	V	Not*4 tested	VII, VI, II	V

*1 Within a week
*2 +: All strains were positive.
−: All strains were negative.
(+): More than 80% of strains tested were positive.
(−): More than 80% of strains tested were negative.
v: Variable (v+ and v− indicate predominance of positive and negative strains respectively.)
*3 TERAKADO & SATO[25]
*4 Not tested because of the absence of coagulase in the supernates

DISCUSSION

It has been described that a single strain of *S. aureus* was present or predominated in birds of each farm[6,17]. The fact indicates that isolation frequency or distribution of chicken *S. aureus* of certain properties should be discussed on the basis of not only number of strains, but also on that of farms or flocks giving the organisms. Therefore, the number of farms was always given in the results of this paper.

The biological types given here were devised for the conducting an ecological study on the organisms at certain farms. During and after the study, the types were adopted also for isolates from staphylococcal infections or normal chickens in other farms. Further, the relationship between the biological types and some additional properties has been studied.

A number of reports[2,5,6,8,11] indicated that there was no difference of characteristics between non-lesion strains of *S. aureus* and those from lesions in birds. In this study, the same findings were obtained, except for the incidence of type 2 strains which were not isolated from the primary infections.

The isolation of coagulase-positive staphylococci of white colonies in chickens has been described before[7,16,26,27]. In this study, biological type 2 strains of white were isolated only in normal chickens or in those having mixed infection.

The data in this study indicate that *S. aureus* strains possessing alpha-lysin predominated, as shown by previous studies[2,3,5,6,8,11,26,27].

V-P reaction has been applied as one of the key properties for the host-specificity of *S. aureus*[17,26,27], though it was variable among avian strains. SATO et al. also indicated the same variability in chicken strains. However, the reaction was evidently one of the very important properties in this study.

JYLLING, HARRY[5,6] and DEVRIESE et al. described that all of their poultry strains fermented both lactose and galactose. On the other hand, KURAMASU et al.[11] reported that 47 of 67 chicken strains of *S. aureus* did not attack galactose and 10 of them did not ferment lactose. KITA & IWATA also described that a part of their strains did not ferment galactose or lactose, and both. It should be noted that there will be untypale strains (non-fermenter of lactose and fermenter of galactose, or fermenter of lactose and non-fermenter of galactose) as seen in reports[8,11]. A few strains of the charactristics were included in this study.

Although biological type 2 strains did not ferment mannitol, they were regarded as *S. aureus* of unusual characterisitics on the basis of coagulase and DNase production[4]. A report[27] indicated variable production of DNase in chicken coagulase-positive staphylococci, but all the organisms were the enzyme

producer in this study.

Caseinase (protease) production was observed in about 80% of the strains used. This high frequency of caseinase production agrees with the results of other investigators[2,9,24,27]. A necrotic dermatitis in chickens, mice and rabbits injected subcutaneously with chicken *S. aureus* has been described[10,11]. The injection with mammalian *S. aureus* did not induce the pathological changes. TADOKORO[22] found that some strains of chicken *S. aureus* were strong caseinase producers. He[23] indicated further that a cutaneous abscess formation in mice injected subcutaneously with *S. aureus* had relation to its coagulase activity, but the severe necrosis or tissue liquefaction in the cutaneous lesions had an relationship with protease (caseinase) activity of the organism. Moreover, there were 2 types of alpha-lysin production in chicken *S. aureus*. That is, some strains produced the hemolysin persistently during the incubation (type A) and others indicated rapid fall of the production (type B)[9]. In addition, the latter strains indicated strong caseinase production and gave more severe cutaneous lesions including wide tissue liquefaction than those of the former. Human *S. aureus* did not give cutaneous lesion and showed the same character as that of the former.

SHIMIZU et al.[19] could not obtain any evidence indicating that there might be any direct relationship between the ability to produce caseinase and the virulence for mice in the case of canine *S. aureus*. The present data indicated that biological type 2 strains produced caseinase with high frequency, but they were of low virulence for mice and chickens. In addition, caseinase producers of other biological types did not always give the severe cutaneous lesion in the animals. This lack of direct correlation of the enzyme production with the virulence appeared to be partially due to the test procedure applied for caseinase production. TADOKORO[23] pointed out that, of his strains, only strain (CH91) of chicken *S. aureus*, strong caseinase producer, gave caseinase in the supernates of broth cultures or positive for the enzyme on agar containing 8% skimmed milk, while *S. aureus* from other sources was positive only on the media containing 2% skimmed milk. In this study, only the 2% skimmed milk agar was used. Moreover, some of the strains used for the virulence study might be of the type A described by KURAMASU et al.[9]

It has been reported that fibrinolysin production is variable in chicken strains, and its frequency is low or zero[2,4,15,17,21,26,27]. However, it was interesting that biological type 2 strains in this study produced the enzyme more often than other types.

The egg-yolk factor has been described as variable property in poultry strains of *S. aureus*[2,3,5,6,15,17,21,26,27]. YOSHIMURA indicated that his chicken-specific *S.*

aureus was negative for the enzyme production. HARRY[5] noted that 75% of alpha-hemolysin-positive strains were positive for the egg-yolk factor, while strains lacking the lysin were negative. In this study all of the type 1 strains having alpha-lysin did not produce the enzyme. On the other hand, all of the type 3 strains possessing the lysin were enzyme producers.

More than half of the strains tested were highly resistant to OTC, but most of them very sensitive to PC. KURAMASU et al.[11] indicated that 6 of 43 strains of chicken *S. aureus* isolated in 1960 in Tokyo were resistant to PC (10 units). On the other hand, SHIMIZU et al.[18] showed that a half of their strains obtained in 1963~1964 in Tokyo district were resistant to PC and CTC. These data indicate an increase of antibiotic resistant strains in Japan. KITA & IWATA gave results indicating that 42.5% of their chicken strains isolated in 1964 in a western district of Japan were resistant to PC and 4.6% to TC. The discrepancy between the results of the present authors and those of KITA & IWATA and others appeared to be due to the difference in the districts in which the strains were isolated.

A number of reports[2,3,5,8,15,16] indicated that stronger phage suspension (RTD×100~1,000) gave more typable strains of chicken *S. aureus* than RTD suspension did. However, in Japan, untypability or low percentage of typable strains with NCTC phages have been reported[8,9,16]. Also in this study, only some of biological type 3 strains were typed with group III phages.

ZEN-YOJI et al.[30] reported coagulase typing of *S. aureus* strains from human foci, the throats of healthy children, food poisoning and milk. NAKAMURA described that coagulase type V strains were isolated frequently (46.4%) from human suppurative lesions. The frequencies of typable strains of *S. aureus* by the coagulase typing were 82.5%[30] and 92.3%[14] respectively. However, no description of coagulase typing of chicken *S. aureus* strains has been given.

From the present data, it may be concluded that coagulase type VII predominated in infected chickens. In this study, biological types 1 and 4 strains gave only type V of coagulase as far as the test was carried out. This coagulase type was also detected in a strain (CH91), of untypable by the biological properties, from the skin lesion. Biological type 3 strains of chicken origin did not give coagulase type V, but an attendant of a farm had the organisms of biological type 3 and of coagulase type V.

Although the frequency of typable strains of chicken *S. aureus* by the coagulase typing was 85.8%, higher compared with that by the phage typing, uniformity of coagulase type seen in biological types 1 and 4 strains appears to indicate a limitation of the typing method in its application in epizootiological studies on chicken coagulase-positive staphylococci.

TERAKADO & SATO reported that all strains of the biological types 1 and 4 of chicken *S. aureus* did not grow at 45°C incubation, but strains of types 2 and 3 and those of other sources such as canaries, cows, pigs, horses, dogs, and humans did with a high frequency. Thus they stated that the growth ability at 45°C of chicken *S. aureus* appears to be a valuable marker in the characterization of the organisms.

HARRY[5] suggested that the higher body temperature of birds would be expected to be of significance in some degree for the selection of human strains of *S. aureus* introduced to the host. However, according to the description of TERAKADO & SATO, some of chicken pathogenic staphylococci are more sensitive to 45°C than those of humans. Therefore, HARRY's speculation appears to be not always acceptable.

All the examined characteristics of the biological types are summarized in table 11.

A number of reports indicated that *S. aureus* of chicken origin had specific properties. For example, the following properties have been described: Rapid fermentation of salicin[20], low frequency of galactose fermentation[11], severe dermatitis with tissue liquefection in animals injected subcutaneously with chicken *S. aureus*[10,11], a mode of caseinase production[9,23], uniform antigenicity of protease[24], high incidence of strains possessing delta-hemolysin only[5], lysis of sheep fibrin[26,27], etc. However, of these properties, rapid fermentation of salicin, low frequency of galactose fermentation, or lysis of sheep fibrin was not always accepted[2,5~8,11,16,17].

The host-specific parasitism of *S. aureus* has been confirmed in the strains from humans, cattle and dogs[4,12,15,17,26,27]. Moreover, there are a few reports[2,4,15,26,27] indicating chicken *S. aureus* has host-specific characters of biological or serological. On the other hand, SHIMIZU[17] stated that chicken *S. aureus* had not host specificity from the viewpoint of biological properties as well as serology. In this study, the key properties, such as coagulation of bovine plasma[4,12], tellurite reduction[4], clumping factor[4,17], growth on crystal violet agar[4,12,26,27], or lysis of sheep fibrin[26,27], which had been applied to demonstrate the host-specific parasitism by the previous researchers, were not tested. Therefore, the relationship between the biological types in the present study and the properties described by the previous investigators could not be ascertained.

There are many descriptions indicating that *S. aureus* strains of chicken origin resembled, in their properties, or contained those of human origin[3,5,17,26,27]. Alpha-hemolysin production and low ability of beta-lysin production, egg-yolk factor, positive V-P reaction, fibrinolysin production, susceptibility to the international set of phages and antigenic structure have been regarded as the proper-

ties of human *S. aureus*[4,12,15,17,26,27]. Biological type 3 strains used in this study had all or some of the properties described above with or without fibrinolysin production. A part of biological type 3 strains were typable with the phages, but most of the typable strains did not produce fibrinolysin. On a poultry farm, attendants were sampled for *S. aureus* in the throat. Most of the human isolates belonged to biological type 3 and the remainder to type 1. Type 1 strains were unsusceptible to the typing phages, but belonged to coagulase type V. Most of the strains of biological type 3 were phage-typed into group II, III or mixed, and were coagulase types II, IV and V. The human strains phage-typed were often fibrinolytic and all of them resistant to PC (unpublished data). As described above, there seems to be some difference between human *S. aureus* and that of chicken of biological type 3.

It is difficult to decide which biological type is the chicken-specific *S. aureus*. Except for biological type 2 of low virulence, type 1 strains differ much from human *S. aureus*, though their coagulase type V has been described as a predominating type also in human strains.

Consequently, it may be stated that chicken *S. aureus* has a variety of characters, but it will be able to classify almost all isolates by the biological types given in this paper. Moreover, the types were useful for the ecological study of the organisms, as will be shown in another paper.

Acknowledgments

The authors are indebted to Dr. S. KURAMASU (Nippon Institute for Biological Sciences, Tachikawa, Tokyo), Dr. S. YOSHIMURA (former address: Osaka Municipal Poultry Experiment Station, Osaka), Dr. S. SHIMAKURA (Faculty of Agriculture, Gifu University, Kagamihara) and Dr. T. SHIMIZU (National Institute of Animal Health, Kodaira, Tokyo) for supplying staphylococcal strains. Moreover, the data of the mouse virulence test were prepared with the help of Dr. S. KURAMASU. The authors wish to thank Drs. H. ZEN-YOJI and T. TERAYAMA, Tokyo-to Laboratories for Medical Sciences, Tokyo, for their assistance in coagulase typing. The authors also are grateful to Drs. T. MIYAMAE and S. ANDO, Department of Epizootiology, for their help during this study, and Dr. E. KATO, Department of Veterinary Public Health of this faculty, for his helpful advice.

References

1) BARRIT, M. M. (1936): *J. Path. Bact.*, **42**, 1441
2) DEVRIESE, L. A., DEVOS, A. H., BEUMER, J. & MAES, R. (1972): *Poult. Sci.*, **51**, 389
3) GENIGEORGIS, C., & SADLER, W. W. (1966): *Ibid.*, **45**, 973
4) HÁJEK, V. & MARŠÁLEK, E. (1971): *Zentbl. Bakt. ParasitKde I Orig.*, **217**, 176
5) HARRY, E. G. (1967): *Res. vet. Sci.*, **8**, 479

6) HARRY, E. G. (1967): *Ibid.*, **8**, 490
7) JYLLING, G. (1962): Proceedings of the 9th Nordic Veterinary Congress (Section H, No. 13)
8) KITA, E. & IWATA, A. (1967): *Bull. nat. Inst. Anim. Hlth*, No. 55, 8 (in Japanese; English summary, *Nat. Inst. Anim. Hlth Quart.*, **7**, 232, 1967)
9) KURAMASU, S., IMAMURA, Y., TADOKORO, I., MORITA, S. & TAJIMA, Y. (1964): Proceedings of the 37th Meeting of the Japan Bacteriological Society, *Jap. J. Bact.*, **19**, 281 (summary in Japanese)
10) KURAMASU, S., IMAMURA, Y. & TAJIMA, Y. (1968): *Zentbl. VetMed.*, B, **15**, 640
11) KURAMASU, S., IMAMURA, Y., TAKIZAWA, T., OGUCHI, F. & TAJIMA, Y. (1967): *Ibid.*, **14**, 646
12) MEYER, W. (1967): *Int. J. syst. Bact.*, **17**, 387
13) NAKAGAWA, M. (1958): *Jap. J. vet. Res.*, **6**, 19
14) NAKAMURA, T. (1965): *Yonago Med. J.*, **16**, 186 (in Japanese)
15) PULVERER, G. (1966): *Postepy Mikrobiol.*, **5**, 429
16) SATO, G., MIURA, S., MIYAMAE, T., NAKAGAWA, M. & ITO, A. (1961): *Jap. J. vet. Res.*, **9**, 1
17) SHIMIZU, Takamasa (1968): *Mem. Fac. Agric., Univ. Miyazaki*, **5**, 1
18) SHIMIZU, Takeshi, TAKIZAWA, T. & SHIBATA, S. (1964): *Nat. Inst. Anim. Hlth Quart.*, **4**, 214
19) SHIMIZU, Takeshi, TAKIZAWA, T. & SHIBATA, S. (1965): *Ibid.*, **5**, 162
20) SMITH, H. W. (1947): *J. comp. Path.*, **57**, 98
21) SMITH, H. W. (1954): *J. Path. Bact.*, **67**, 73
22) TADOKORO, I. (1963): *Saishin-Igaku*, **14**, 940 (summary in Japanese)
23) TADOKORO, I. (1967): *Mod. Media*, **13**, 110 (in Japanese)
24) TAKEUCHI, S. & SUTO, K. (1971): Proceedings of the 72nd Meeting of the Japanese Society of Veterinary Science, *Jap. J. vet. Sci.*, **33**, Suppl. 176 (summary in Japanese)
25) TERAKADO, N. & SATO, G. (1972): *Jap. J. vet. Res.*, **20**, 31
26) YOSHIMURA, H. (1970): *Jap. J. vet. Sci.*, **32**, 263
27) YOSHIMURA, H. & NISHI, T. (1970): *Res. Bull. Obihiro Univ.*, **6**, 217
28) ZEN-YOJI, H., TERAYAMA, T., BENOKI, M. & KUWAHARA, S. (1961): *Jap. J. Microbiol.*, **5**, 237
29) ZEN-YOJI, H., TERAYAMA, T., BENOKI, M. & KUWAHARA, S. (1961): *Ibid.*, **5**, 367
30) ZEN-YOJI, H., TERAYAMA, T., BENOKI, M. & KUWAHARA, S. (1962): *Ibid.*, **6**, 59