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AN OUTBREAK OF HEMOLYTIC-STREPTOCOCCAL
INFECTION AMONG CHICKENS OF A FLOCK
II. CHARACTERS OF THE ISOLATED STREPTOCOCCI

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INTRODUCTION

The outbreak of chicken streptococcosis appears to be comparatively rare. So far as the present authors know, no occurrence of the disease has been reported in Asia. They observed an outbreak of hemolytic-streptococcal infection among adult chickens in 1956. In the previous report, USHIJIMA and SATO described some results of investigations on the disease with special reference to the pathological findings of the birds. The present paper gives detailed descriptions of characters of the isolated hemolytic streptococci (*Str. zooepidemicus*).

There is a question whether so-called *Str. gallinarum* should be considered as a definite species in the avian family or not. From this point of view, it is noteworthy that MERCHANT and PACKER state: "Investigators who study avian diseases should take care in the identification of the streptococci and from such reports it will be possible to distinguish the important streptococcus in the avian family".

EXPERIMENTS

History of the outbreak - As detailed descriptions of the outbreak were given in the first paper (in Japanese), its history is given briefly here.

In May of 1956, acute or subacute deaths occurred in a small flock consisting of 56 female White Leghorn chickens (about one-year-old) at a farm in the City of Ebetsu. They were kept in a pen which occupied a corner of a stall during the winter and had been let free several days before the onset of the disease. From the beginning of May, the chickens were not fed regularly. Their egg laying rate was about 70% at the end of April despite malnutrition. The rate lowered under 40% at the peak of the outbreak. From May 3rd, when the first death occurred, to June 6th, 10 birds (about 18%) suffered from the disease and 6 of them (about 11%) died.

Symptoms: The diseased birds showed dulness and crouched first; sometimes, they

appeared sleepy. The birds staggered in walking, but there were no nervous disturbances. They emaciated. Some of them showed diarrhea and labored breathing. Temperature of a chicken was taken. It was 40.9°C at the stage of sleepy appearance. The diseased birds succumbed in 1 to 5 days after the onset of symptoms.

Isolation of β -hemolytic streptococci: Six cases including 2 died and 4 killed birds in the flock were examined bacteriologically. Pure growths of β -hemolytic streptococci were obtained from 5 out of the six as shown in table 1.

TABLE 1. *Isolation of β -hemolytic Streptococci from the Diseased Chickens*

NO. OF BIRDS	TERMINATION	MATERIALS EMPLOYED FOR CULTIVATION											
		Trachea	Lung	Heart Blood	Ascites	Exudates in the Peritoneal Cavity	Spleen	Liver	Kidney	Ovary	Oviduct	Cloaca	Bone Marrow
1	Died	•	∞	∞	•	∞	∞	∞	∞	•	•	•	•
2	Killed	•	∞	—	•	∞	+	—	+	##	+	•	•
3	"	+	+	—	—	•	—	—	—	—	—	•	—
4	"	—	—	—	—	•	—	—	—	—	—	•	—
5	Died	++	∞	##	##	##	##	##	##	•	•	•	•
6	Killed	+	∞	—	+	∞	+	—	∞	∞	∞	+	•

Remark: • indicates the specimens not examined.

From No. 4 streptococci were not cultivated. It remained uncertain whether streptococci could not be isolated because of small number of the organisms in the body materials at early stage of disease or because of other kind of disease. In the histopathological examinations, however, slight changes similar to those observed in the other diseased birds were found.

On May 10th, a few days after the first isolation of the organisms, all 48 apparently healthy chickens of the flock were bled and the blood samples were cultivated onto sheep blood agar after enrichment by horse meat infusion broth containing 1% glucose. One sample showed growth of the hemolytic streptococci.

However, examination for detection of other aerobic bacteria including *Salmonella* organisms, infectious bronchitis virus and its neutralizing antibody, and for demonstration of H.I. antibody to Newcastle disease resulted negative in this flock.

Pathological findings: Six cases including 2 died and 4 killed birds were examined pathologically. Macroscopical changes were found such as swelling of internal organs, subcapsular white flecks on the liver, ovaritis and large amount of fibrinous exudate in the peritoneal cavity. Some of the carcasses showed salpingitis accompanied by cheese-like exudate in the oviduct resembling the findings in chronic streptococcosis described by EDWARDS and HULL. However, in the histopathological examinations, exudative pneumonia and bronchitis, degeneration of parenchyma, circulatory disturbances and activity of reticulo-endothelial cells were found. Peritonitis or ovaritis seemed not primary changes in these

cases, but salpingitis was significant.

From the above-described findings, the disease seemed to be subacute septicemia in nature which was caused by primary infection with the hemolytic streptococci in the respiratory tract or the oviduct.

BACTERIOLOGICAL FINDINGS

Fourteen cultures isolated from the lung and exudate in the peritoneal cavity of Nos. 1, 2, 5, and 6 in listed table 1 were examined bacteriologically. Several controlled strains such as *Str. pyogenes*, a human strain of *Str. equisimilis*, *Str. equi*, *Str. zooepidemicus*, *Str. zymogenes* and a rabbit strain of Group B were employed. For these strains the present authors are indebted to Dr. IMAIZUMI of the National Institute of Health, Tokyo and Assist. Prof. SHIMIZU of this faculty. Strains of *Str. zooepidemicus* which were isolated from horse, cattle, sheep or guinea pig were also employed for controls. Biochemical characteristics were examined according to the method described by SHERMAN, OCHI and HIRAO (1942) and BERGEY'S Manual. Serological identification was made according to LANCEFIELD.

Morphology and staining: In the tissue smears such as blood, exudate or organs, Gram-positive cocci were observed. These cocci occurred in diplococoid form or in short chain as well as on blood agar. In broth culture, moderately long chain was observed. Capsular formation was found by BENIAN'S method in some strains forming moist colonies on blood agar.

Growth appearance: In broth culture, many strains examined formed moderate amounts of sediment on the bottom of tube with fine granules or without granules in supernatant fluid. Moist colonies appeared to show heavy sediment.

Though the appearance of the colonies was various, they seemed to be divided into following three types on the primary culture from the birds: (1) a small, grayish white, round, smooth and glistening colony, (2) a round, grayish white, slightly flat, moderate-sized colony with raised centrum and (3) a larger, round, moist, somewhat transparent and flat colony with raised centrum brown-colored often showing confluent growth. The organisms developing into a colony of the 3rd type formed capsules on blood agar or in animal body and their colonies became dry and wrinkled as they aged. The above-mentioned types were observed in different percentages on primary cultures according to individuals or organs examined. It is of interest that the moist colony of the 3rd type was found predominantly on primary culture from the diseased birds which died at the last stage of the outbreak. In subcultured strains, a small, round, convex, grayish white and glistening colony and a larger, flat, grayish white and round colony with a raised centrum appeared to be dominant. In these strains, the moist colony was often found on blood agar containing glucose.

On pour plate of horse or sheep blood agar, the strains formed lenticular colonies about 1 mm in diameter after incubation for 48 hours.

Hemolytic activity: On blood agar, the isolated strains produced β -hemolytic zone showing most clear after incubation for 48 hours. The diameter of hemolytic zone produced by the isolated strains were about 1.5~5 mm after 2 days of incubation on pour plate of horse or sheep blood agar with or without added glucose. It is an interesting fact that slightly hemolytic colonies developed from a culture which had indicated activity against blood cells on primary

isolation, and had been subcultured for several months on blood agar.

Fibrinolysis: Among nine chicken strains only one, slightly hemolytic strain, proved to be moderately positive by the method of TILLET and GARNER. Two human strains (*Str. pyogenes* & *Str. equisimilis*) were strongly positive, while controlled strains such as Groups B, C and D of other animal origin were all negative.

Other biochemical characteristics: Results obtained from carbohydrate fermentation tests and other examinations for biochemical characteristics are shown in table 2.

TABLE 2. *Biochemical Characteristics of the Isolated 14 Strains*

TEST	RESULT	NOTE
Hemolysis	β	
Fibrinolysis	—	One of 9 strains examined was moderately positive
Growth at 45°C	—	
" 10°C	—	
Growth in presence of 6.5% NaCl	—	
pH 9.6	—	
0.1% methylen blue	—	
Strong reduction	—	
Survival at 60°C for 30 minutes	—	
NH ₃ from peptone	+	
Sodium hippurate split	—	
Starch hydrolysed	+	
Esculin split	—	One strain indicated weakly positive reaction
Growth on 40% bile-blood agar	—	
" 10% "	—	Six strains showed faint growth
Gelatin liquefied	—	
Litmus milk	Acid	
Final pH in glucose broth	4.6 ~ 4.8	
Acid production from		
Lactose	+	Four strains indicated weak activity against lactose on repeated tests
Trehalose	—	
Sorbitol	+	
Maltose, Sucrose & Salicin	+	
Raffinose, Inulin, Glycerol, & Mannitol	—	

Serological identification: Bacterial suspensions of 2 chicken strains which were treated with pepsin were employed to prepare immune sera. Antigens of test strains were prepared by hot-HCl extraction. Four known grouping sera were given to the authors by Dr. SHIMIZU

of this faculty.

From table 3, it will be seen that the isolated strains belong to Group C of LANCEFIELD.

On the basis of the above-described bacteriological and serological characters, it is obvious that the strains isolated by the present authors are identical to *Str. zooepidemicus* described in BERGEY's Manual.

TABLE 3. Serological Group of the Isolated Strains

ANTI-SERUM	ANTIGEN				
	<i>Str. equi</i> (Hokudai)	<i>Str. zooepidemicus</i> (G-36)	<i>Str. equisimilis</i> (Yukimoto)	Isolated Strain (D 1)	Isolated Strain (D 2)
Group A (Gr-13)	—	—	—	—	—
" B (Rf)	—	—	—	—	—
" C (G-36)	+	+	+	+	+
" D (C6D)	—	—	—	—	—
Isolated Strain (D 1)	+	+	+	+	+
" (D 2)	+	+	+	+	+

INFECTION EXPERIMENTS

The inocula were prepared from 24-hour cultures respectively of the organisms. Media were horse meat infusion broth added 1% glucose. The seed streptococci were isolated freshly from naturally infected materials of organs or of exudates which had been preserved at -40°C in order to maintain the virulence. Mice, guinea pigs, rabbits and adult chickens were used for experiments.

Mice: Eight strains of chicken hemolytic streptococci were used for experiments. A dose of 0.2 or 0.5 ml containing 10^{-1} ~ 10^{-8} ml of original broth culture of the streptococci was inoculated intraperitoneally into 3 or 5 albino mice (ddN strain) aged several weeks. The mice were observed for 3 weeks. One hundred percent lethal doses of the strains were as follows: One strain was 10^{-2} ml, three 10^{-3} , two 10^{-4} , one 10^{-5} and one 10^{-7} respectively. However, colony forms of the streptococci which were employed for preparation of inocula and their effects on virulence for mice were not investigated.

Rabbits: These animals seemed to be susceptible to the organism so far as strains were employed. Each dose of 0.1, 1 and 5 ml of broth culture of moist or not moist strain was injected intravenously into one adult rabbits. All the rabbits died of septicemia within 2 days.

Guinea pigs: They were refractory to intraperitoneal injection with each dose of 0.01, 0.1 and 1 ml of broth culture of moist or not moist strain. Post mortem cultivation of the streptococci made 3 weeks after the injection showed positive in 2 animals inoculated with the moist colony strain.

Chickens: Seven White Leghorn and 6 New Hampshire adult chickens were injected with fresh strains from frozen infected materials, strains subcultured for 1.5 years and saline suspension of naturally infected yolk material. Results obtained from three series of experiments are shown in tables 4-1 and 4-2.

TABLE 4-1. *Infection Experiments in Adult Chickens*

EXPERI- MENT	NO. OF CHICKEN	BREED	AGE	SEX	STRAIN	DOSE	ROUTE	TERMINA- TION	DAYS TO DEATH	CHIEF SYMPTOMS
			Month			ml				
I	1	WL	10	♀	Fresh	1	i. v.	killed	28	
	2	"	"	"	"	"	"	"	"	
II	3	NH	5.5	"	"	0.1	"	died	19	febrile reaction, diarrhea, emaciation and sleepy appearance
	4	"	"	"	"	1	"	killed	34	almost normal
	5	"	"	"	"	"	"	"	"	febrile reaction and lameness
	6	"	"	"	"	0.1	"	died	28	febrile reaction and emaciation
	7	"	"	♂	"	0.5	i. p.	"	35	febrile reaction, emaciation and sleepy appearance
	8	"	"	♀	Yolk material	1.5*	"	killed	"	febrile reaction, diarrhea and ruffled feather
III	9	WL	12	♂	Fresh	1	i. v.	killed	34	almost normal
	10	"	"	♀	"	"	"	"	37	febrile reaction and emaciation
	11	"	"	"	"	"	"	died	5	dulness
	12	"	"	"	Sub- cultured	"	"	"	1	sudden death
	13	"	"	"	"	"	"	"	10	febrile reaction, diarrhea and dulness

WL: White Leghorn breed

NH: New Hampshire breed

* : Unit in gram

TABLE 4-2. *Infection Experiments in Adult Chickens*
(Pathological and Bacteriological Findings)

EXPERIMENT	NO. OF CHICKEN	MACROSCOPICAL FINDINGS	ISOLATION OF HEMOLYTIC STREPTOCOCCI
I	1	petechiae on the tracheal membrane, swelling of the spleen and atrophic ova	lungs and spleen
	2	no changes	non
II	3	emaciation, increase of ascites and acute sero-fibrinous peritonitis	septicemia
	4	no remarkable changes	non
	5	emaciation and abscess of hip-joint and hip-bone	oviduct, hip-joint and hip-bone
	6	emaciation, peritonitis, abscess of hock-joint and coagulated blood in the peritoneal cavity	parenchymatous organs other than the heart and bone marrow
	7	emaciation, a large amount of fibrinous exudate in the peritoneal cavity	parenchymatous organs other than the heart
	8	emaciation, swelling of the oviduct, peritonitis and changes in the lungs	parenchymatous organs other than the liver and heart
III	9	no remarkable changes	non
	10	emaciation, suppurative peritonitis and concreted yolks in the oviducts	eyes, lungs, kidneys, ovary and oviduct
	11	emaciation and acute sero-fibrinous peritonitis	septicemia
	12	anemia and rupture of the liver	lungs and spleen
	13	emaciation, anemia and changes in the lungs	septicemia

Two birds which died showed severe febrile reaction (Nos. 7 & 13). A total of 6 among 13 injected intravenously or intraperitoneally died within a period from 1 day to 5 weeks after inoculation. Chickens which died under chronic course showed systemic infection. It does not always appear that deaths of the chickens correspond to the applied doses.

All birds examined gave pathological findings such as bronchitis, pneumonia and parenchymatous degeneration. These findings are similar to those of the natural cases observed by the present authors.

From the above-noted findings it may be concluded that the present streptococci are able to cause a disease in the chicken, though natural route of infection or focus of primary growth of the organisms was not clarified experimentally.

DISCUSSION

Regarding the portal of entry of the causative agent in chicken streptococcosis, a number of experiments have been carried out by several workers. Among the routes of infection such as alimentary, genital, and respiratory tracts, the last described may be the most possible portal of entry as emphasized by HUDSON. However experimental clarification of this problem remains unaccomplished.

Experimental results of previous investigators were variable in respect of reproduction of chicken streptococcosis similar to natural cases. Also in the present experiment, chickens did not always die even when they received the large doses of streptococcal culture. It is well known that animal streptococcosis occurs under some unknown conditions, though, of course, streptococci play the most important part in the etiology of the disease.

Occurrence of chicken streptococcosis seems to be rare. OCHI and HIRAO (1942) examined hemolytic streptococci on normal mucous membrane of many healthy animals of different species such as horse, cattle, sheep, dog, rabbit, guinea pig, albino rat and mouse. On the basis of their results, they stated that many strains of the organisms were isolated only from the animal species such as horse, in which streptococcal infections occur most frequently. The above-mentioned opinion suggests that occurrence of hemolytic streptococci on healthy mucous membrane of chicken may be uncommon.

No experiment to clarify the portal of entry of the streptococci was undertaken by the present authors. However, in the histopathological examination on natural cases, bronchitis or pneumonia was recognized as remarkable changes. These findings suggest that primary infection might occur in respiratory tract.

It is difficult to determine whether the organisms described as *Str. gallinarum* in the early literatures belong to the same species or not, because of lack of complete investigations with special reference to fermenting reactions and serological characteristics and because of variable descriptions of the data. Moreover, the organisms do not correspond to any species of animal hemolytic streptococcus

which is classified by modern methods. Thus *Str. gallinarum* is described as a definite species or a subgroup of *Str. zooepidemicus* in some text-books, though it is supposed that the streptococci may be classified as *Str. zooepidemicus* or as *Str. equisimilis*. EDWARDS was the first to classify avian hemolytic streptococci by modern methods. He investigated a strain of the streptococci isolated by HUDSON from appoplectiform septicemia of chicken. This strain had previously appeared to be of the same species of *Str. gallinarum*. It fermented sorbitol actively and also belonged to the serological group of *Str. zooepidemicus*. From EDWARDS' investigations, HUDSON's strain was identified as *Str. zooepidemicus*. On the other hand, HUDSON had previously reported that the organism did not attack sorbitol. The above-described contradictory reports seem to be very interesting. However, it is impossible to determine whether the discrepancy between the reports of HUDSON and of EDWARDS was due to the difference of methods employed by them or due to biochemical variation as described by OCHI and HIRAO (1941) in equine hemolytic streptococci. OCHI and HIRAO reported that characters of *Str. equi* which had been subcultured for a long time tended to change into those of *Str. zooepidemicus*, resulting in acquirement of ability of fermenting lactose and sorbitol. They also reported that stock culture of *Str. zooepidemicus* lost ability of fermenting sorbitol and acquired ability to ferment trehalose. Therefore, in avian hemolytic streptococci, the same kind of variation may occur.

From the above-mentioned facts, the present authors cannot deny completely the possibility of the existence of *Str. gallinarum*. However, they believe that the most common species of hemolytic streptococci in the avian family is *Str. zooepidemicus*, because almost all of the hemolytic streptococci of chicken origin which were described in the literatures later than the report of EDWARDS have been identified as *Str. zooepidemicus*. Namely *Str. zooepidemicus* was isolated by EDWARDS and HULL in U.S.A., GENEST and NADEAU in Canada, PACKER in U.S.A., BUXTON in England, AGRIMI in Italy and GENEST from bone marrow of 6 birds in Canada. Moreover, the streptococci isolated by the present authors belonged to the same species. Concerning species other than *Str. zooepidemicus*, the streptococci isolated by GIBBS from bronchitis of baby chicks were identified as *Str. equisimilis* by EVANS and strains of β -hemolytic streptococci (*Str. faecalis*) were isolated by AGRIMI from septicemia. Unidentified hemolytic streptococci were recovered by MOORE and MARTEN from the oviduct of a hen showing reproductive disorder. From the above-described facts that have been proved during the past quarter of a century, the most important species of hemolytic streptococci in the avian family is *Str. zooepidemicus* and it seems necessary to reexamine whether *Str. gallinarum* is a definite species of streptococci inde-

pendent of *Str. zooepidemicus*.

SUMMARY

The hemolytic streptococci derived from diseased adult chickens of a small flock (56 birds), 11% of which died of septicemia were identified as *Str. zooepidemicus*. Mice and rabbits were susceptible to the streptococci, but guinea pigs were refractory. The half of adult chickens injected with broth culture of the organisms died of septicemia generally in chronic form. The chickens infected experimentally indicated histopathological findings (bronchitis, pneumonia and parenchymatous degeneration) similar to those of the natural cases.

The most important species of chicken hemolytic streptococci was discussed.

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