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Further Studies on Tamaoki's Antigen Factor S_1 of Cholera Vibrio ; Appearance in El Tor Vibrios and Comments on This Factor

Tsuneo TSUTSUMI

Department of Bacteriology, Nagasaki University School of Medicine

(Director : Prof. Yoshio AOKI)

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Abstract

The antigen factor S_1 could be demonstrated in laboratory strains of El Tor vibrios as well, though in a low frequency as compared with that of classical cholera vibrios. The rate of its occurrence in all the laboratory strains appeared to be related to the preservation period or age of them, but not to the mentioned categories of cholera vibrio or the serotypes. The strains possessing S_1 were agglutinated with anti-R-serum in parallel and were sensitive to the killing effect of the complement. Although they were not rough variants by the conventional criteria, it may be said that they were in a precursor stage of the smooth-to-rough variation. This antigen as well as R antigen was detectable also in a part of newly isolated El Tor strains, but these strains were not sensitive to the complement as expected. To this case, the mentioned "age relation" in the laboratory strains is not applicable. The possibility of early development of the serological R variant in the epidemic strains was discussed on the data of the literature.

In the studies by Horikawa[6], a fellow student, on the antigen factor S_1 advocated by Tamaoki and his coworkers, 53 strains of classical cholera vibrio (CV), 29 strains of El Tor vibrio (ET), and 12 strains of non-agglutinating vibrios (NAG) were used, and the appearance of S_1 was testified only in 34 strains of CV. His first statement above seemed to attach an important significance to the serology of *Vibrio cholerae*, as no antigen factor has been known yet for the distinction between CV and ET. But there appears the question point in consideration of his second

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statement that the S_1-carrying strains were sensitive to the killing effect of the complement, because the sensitivity to the complement has been believed by some workers to be a manifestation of the potential rough variation —— called "semirough" by him —— of bacteria, and this type of variation can occure in ET as well. The question point above, after all, was put off to be answered to the day of the completion of further studies, as his study on the relation between S_1 antigen and "semirough" has been centered around CV, especially Inaba type of it.

The present study was what was designed to reinvestigate his experiments using all the stock laboratory cultures including ET and numbers of newly isolated ET culture in foreign countries, with some enlarged means for serological determination of roughness in *V. cholerae*.

Materials and Methods

Strains : They were numbered to 454 in all, and divided into two groups, namely the stock cultures of this department, 73 CV, 29 ET, and 12 NAG strains, and 340 ET strains isolated newly in foreign countries in 1969 and 1970. In the second group, strains isolated in South Korea, 1969 formed the greater part of it (320 out of 340 strains) ; the remainder consisted of 11 strains from Philippines, 6 from Hong Kong, 2 from Africa, and one from Jordan. The Korean strains were confirmed by the present author as non-hemolytic ET of Ogawa type by serological and biochemical means including Colistin sensitivity test. All the laboratory strains have been preserved by cultivating successively at intervals of 5-6 weeks in soft agar containing Bacto-Tryptone. When the cultures were transplanted, colonies were not selected intensionally, but the selection of smooth colonies were made occasionally or when they were prepared to be used for experiments like this.

Demonstration of S_1 factor : Ogawa type serum prepared by immunization with OS-antigen of strain "558", a S_1-carrying strain received from Tokuyama Quarantine Station in 1966, was absorbed with heat-killed (at 100°C for 3 hr) Ogawa type culture "41" (Tokyo Q. S., 1968) lacking in S_1, and this monovalent S_1 serum with titer 1: 320 was submitted to agglutination on plastic trays for micro-titration. The agglutination test was performed by incubating antigen-antibody-mixtures at 37°C for 2 hr and then by placeing them in a refrigerator overnight. The positive reaction of S_1 was given when a test-strain represented the agglutination titer 1:80 or more, showing at the same time no spontaneous agglutination in the medium without serum.

Sensitivity to complement : Four doubling dilutions from 1:20 to 1:160 of the dried complement (Kyokuto-Seiyaku) and two dilutions ($1: 10^5$ and $1 : 10^6$) with saline containing 0.1% peptone of an overnight heart infusion culture of test-strain were prepared. An equivalent dose (0.5ml) of the complement and the cell-suspension mentioned above were mixed, incubated at 37°C for one hr, 0.5ml of the mixture was poured into Petri dishes with melted

soft agar at a temperature slightly below 40°C, allowed to harden, incubated overnight, and population size on and in the medium was determined roughly by comparison with the results obtained by the control experiment using inactivated complement.

Serological determination of rough variation : Two particular strains, Tokuyama 7 (NAG) and Hikojima 3 (CV), developing constantly rough colonies (Rs and Sr, as mentioned in the latter part) were used for preparation of the immune sera containing the antibody peculiar to rough variant. Preparation of the antigen and immunization with it was done by the same method that the author used for preparation of the serum anti-S_1. The absorption of the serum with cholera vibrio cultures of Inaba and Ogawa types were also carried out by way of precaution, though it was obvious that Tokuyama 7 had carried originally no cholera-specific antigens and Hikojima 3 had varied so intensely that it was defective in these antigens.

Results

Distribution of S_1 among test-strains : Before the presentation of the results on distribution of S_1, it will be necessary to make a brief account of the observation on rough variation in a popular sense of the strains. According to the morphological appearances of the colonies, most of the strains, particularly the newly isolated strains, seemed to be smooth type (S) so far as one-day agar plate cultures were concerned. It was remarkable here that exceptional strains developing smooth-to-rough colonies of slightly transformed appearance (Sr) or of intensely (Rs) were found mainly in the laboratory strains of Ogawa and Hikojima types, both CV and ET. As for the first step negation of rough variation, thermo-precipitation (at 100°C for one hr) of cell-suspension in normol saline were taken into consideration together with the mentioned colonial variation, and as shown in Table 1, about one-third or a half of the examined strains were excluded from the strains to be tested serologically. When the strains thus screened were used for S_1-agglutination and also for agglutination with anti-R-sera, there could be found 12 strains in total showing spontaneous agglutination in control tubes without antisera, which were also excluded by this second screening. Among fresh isolates obtained from Korea there were 20 strains causing themo-precipitation, but the degree of precipitation of those strains was too weak to be compared with that of laboratory strains.

The antigen factor S_1 was demonstrated as expected in the laboratory strain of CV in 50 percent (21 out of 42 strains), but it was found, as might have been unexpected from the study of Horikawa, that even ET, both old laboratory strains and fresh isolates, could carry this antigen at a low rate (2 of 22 strains, 9.1%) in the former and 14 of 320 strains, 4.4% in the latter). The occurrence of S_1 seemed to be limited to the serotype Ogawa, but the present author is diffident of this serotype relation because of a decided minority of test-strains other than Ogawa type.

Table 1. Distribution of S_1-antigen, inclusive of data for screening strains suitable for agglutination

Categories (No. of str.)		Serotype	No. of str. tested	No. of strains excluded		No. of str. qualified	No. of str. S_1(+)
				by 1st step screening	by 2nd step screening		
Laboratory strains (114)	CV (73)	Inaba	23	2	1	20	9
		Ogawa	48	18	9	21	11
		Hikojima	2	1	0	1	1
	ET (29)	Inaba	1	0	0	1	0
		Ogawa	26	12	3	11	2
		Hikojima	2	2	0	0	0
	NAG (12)		12	5	1	6	0
Fresh isolates (340)	ET (340)	Inaba	7	0	0	7	0
		Ogawa	333	20	0	313	14

In the laboratory strains two or three subcultures of the same designation but of different supplier have been included. Examples are : WLL, 761, 95 and 35A3 of Inaba type CV, and Maya 3 and 558 of Ogawa type CV.

Table 2. Agglutinability to S_1 and R factors and sensitivity to complement (CV Inaba type)

Strain and source	Agglutination titer			Sensitivity to C′			
	S_1	R(T.7)	R(H.3)	1 : 40	80	160	320
Inaba, Original, Denken	320(320)	80	320	0	0	1	4
H218, SM resistant, Kyudai	160(160)	160	160	1	2	3	4
Nakagawa, Denken	160(160)	40	320	2	2	4	4
375, isolated in 1946	80(160)	80	320	0	1	2	4
95, NIH, Japan, 1968	80 (80)	80	320	1	1	3	3
761, Tokuyama QS, 1966	80(160)	40	160	0	0	3	4
761, Tokyo QS, 1961	80(80)	80	160	0	0	4	4
761, Tokuyama QS, 1968	80(−)	160	160	0	0	2	4
WLL, NIH, Japan, 1968	80(−)	−	80	4	4	4	4
95, Tokyo QS, 1963	−(−)	−	−	1	2	2	3
95, NIH, Japan, 1966	−(−)	−	−	2	2	3	4
87, isolated in 1946	−(−)	−	−	1	2	2	3
35A3, Tokuyama QS, 1968	−(−)	−	−	3	4	4	4
VC-13, Tokyo QS, 1968	−(−)	−	−	3	4	4	4
WLL, Nagasaki QS, 1655	−(−)	−	−	4	4	4	4
WLL, NIH, Japan, 1966	−(−)	−	−	4	4	4	4
H218, Kyudai	−(−)	−	−	4	4	4	4
35A3, Tokuyama QS, 1966	−(−)	−	−	4	4	4	4
35A3, Tokyo QS, 1968	−(−)	−	−	4	4	4	4
Yanagihara, Biken	−(−)	−	−	4	4	4	4

Agglutination titer in parentheses was estimated by using heated antigen. Tokuyama 7 and Hikojima 3 have been abbreviated. Estimation of round number of colonies in the sensitivity test : 4= approximately equal to number of coloinies in controls ; 3= approximately 75%; 2=50%; 1=25%; 0=less than 25%.

Table 3. Agglutinability to S_1 and R factors and sensitivity to complement (CV Ogawa type)

Strain and source	Agglutination titer			Sensitivity to C'			
	S_1	R(T.7)	R(H.3)	1 : 40	80	160	320
558, Tokuyama QS, 1966	320(320)	40	320	2	3	3	4
Maya 3, Tokyo QS, 1961	160(160)	40	160	1	2	4	4
69, isolated in Japan, 1946	160(160)	40	80	0	0	2	3
161, isol. in Japan, 1946	160(160)	80	160	0	0	2	4
Maya 3, Tokyo QS, 1968	80(160)	80	160	0	1	2	4
205, isol. in Japan, 1946	80(160)	—	160	0	0	2	2
8, ditto	80(160)	—	160	0	2	2	3
7, ditto	80(80)	40	80	0	2	3	4
14-S, ditto	80(—)	40	160	3	4	4	4
A-I, isol.in Bangkok, 1958	80(—)	40	160	0	2	3	4
4, isol.in Japan,1946	80(—)	40	160	0	1	4	4
Kamata, isol. in Japan, 1946	—(—)	—	80	1	2	3	4
Maya 3, Tokuyama QS, 1968	—(—)	—	—	0	1	2	4
A-I-M,isol. in Bangkok, 1958	—(—)	—	—	1	2	2	4
16, isol. in Japan, 1946	—(—)	—	—	0	0	2	3
A-II, isol. in Bangkok, 1958	—(—)	—	—	3	4	4	4
B-II, ditto	—(—)	—	—	4	4	4	4
41, Tokyo QS, 1968	—(—)	—	—	4	4	4	4
90, unknown (from India)	—(—)	—	—	4	4	4	4
VC-12, Tokyo QS, 1968	—(—)	—	—	4	4	4	4
1192, isol. in Bangkok, 1957	—(—)	—	—	4	4	4	4

Serological R-reaction and complement sensitivity (Laboratory strains of CV group) : All the qualified laboratory strains of CV, totalling 42, were tested for their agglutinability to S_1 and R sera in parallel with their sensitivity to the complement. Results obtained are shown separately in Table 2 (Inaba type) and in Table 3 (Ogawa type), on which the author can afford a commentary in common to both of the types. In these data the positive reaction of S_1 and of the R factor (Hikojima 3) has been given when a test-strain represented the agglutination titer 1 : 80 or more, but the least titer was set up at 1 : 40 in the case of another R (Tokuyama 7). The minimum titer of S_1 was adopted so as to correspond to one-fourth of the end titer of the anti-serum. As for the R sera, on account of spontaneous agglutination it was impossible to determine their end titers using respective homologous antigens, but possible to estimate them at 1 : 160 (T.7) and 1 : 320 (H.3) using heterologous antigens, for examples strains H218 (SM resistant, Kyudai) and Inaba (Original, Denken) in Table 2.

As shown in these tables, 9 out of 20 strains (Inaba type) and 11 out of 21 strains (Ogawa type) were determined as S_1 positive, respectively. The positive reaction of the test-cultures with the two R sera, especially that with R (H.3), was observed on the whole in the same sphere of the strains, though there was not always the definite ratio among the three titers in every strain. Taking a large view of data, it is unquestionable that the existence of

Table 4. Agglutinability to S_1 and R factors and sensitivity to complement (ET and NAG)

Strain and source	Agglutination titer			Sensitivity to C'			
	S_1	R(T.7)	R(H.3)	1 : 40	80	160	320
ET Inaba type :							
T-58, Taiwan, 1961	−(−)	−	−	2	3	4	4
ET Ogawa type :							
SE-2, Sarawak, 1961	80(160)	80	160	0	3	3	4
PE-20, Philippines, 1961	80(80)	40	160	0	2	4	4
JE-1, Java, 1961	−(−)	−	−	2	2	3	4
T-40, Taiwan, 1961-62	−(−)	−	−	2	2	3	4
T-54, ditto	−(−)	−	−	4	4	4	4
T-67, ditto	−(−)	−	−	4	4	4	4
T-68, ditto	−(−)	−	−	4	4	4	4
JE-10, Java, 1961	−(−)	−	−	4	4	4	4
C-3, Celebes, 1961	−(−)	−	−	4	4	4	4
PE-1, Philippines, 1961	−(−)	−	−	4	4	4	4
PE-21, ditto	−(−)	−	−	4	4	4	4
NAG :							
4716, G.-V.*	−(−)	−	−	0	0	4	4
V. metschnikovii	−(−)	−	−	1	1	2	4
V. proteus	−(−)	−	−	0	1	2	4
Heiberg I, 1774	−(−)	40	−	0	1	3	4
Heiberg II, 1776	−(−)	−	−	0	1	4	4
Heiberg III, 1777	−(−)	−	−	4	4	4	4

* Gardner and Venkatraman.

S_1 and of R (H.3) of a given strain is concerned in its sensitivity to the complement ; In fact, 8 out of 9 strains (Inaba) and 10 out of 11 strains (Ogawa) carrying S_1 and R (H.3) were sensitive to the complement strongly or moderately, while most of the remainder were firmly resistant to it. Exceptions were each one S_1-positive strain of Inaba and Ogawa type (WLL, NIH, Japan, 1968 and 14-S isolated in Japan in 1946) which showed a high resistance to the complement (sensitivity pattern : 3/4/4/4 or 4/4/4/4), and there were some other strains showing no S_1-reaction but a moderate resistance to the complement (1/2/3/4 or the like).

The reactions of two strains of Hikojima type had no peculiarities to refer to. A qualified strain from them, Hikojima (Original, Denken), reacted with both the R sera and presented a sensitivity pattern 0/0/2/3.

Results obtained from laboratory strains of ET and NAG : The results of S_1 and R agglutination and of complement sensitivity test of the qualified 12 ET strains isolated in Southeast Asia in 1961 and 1962 and on 6 NAG strains stocked long in this department are as shown in Table 4. The antigen factor S_1 could be demonstrated for the first time in ET strains named SE-2 (Sarawak, 1961) and PE-20 (Philippines, 1961), though at low rate in this group and with a low titer. These two S_1-positive

strains which had been excluded from test-strains in the study of Horikawa on account of their colonial roughness and instability in saline were qualified for test-strains this time and represented not only R-agglutination due to T.7 and H.3 sera, but also showed the complete sensitivity in the dilution of the complement 1 : 40. And contrariwise, 7 out of 10 S_1-negative strains showed the maximum complement resistance as indicated with the sensitivity pattern 4/4/4/4. In fine, it can be said that the same comment on the results of CV can be applied to this case too.

In 6 qualified strains of NAG group, no agglutination was observed as to S_1 and R (H.3) sera. Only one strain, Heiberg I 1774, reacted in the minimum degree with R (T. 7). This reaction, however, was considered to have been an accidental occurrence in view of the fact that the end titer of T.7 was lower than that of H.3. Viewed from an angle of the complement sensitivity test which resulted in an intense growth-inhibition due to the complement up to 5 out of 6 strains, it is strange that the NAG strains did not react with both the S_1 and R sera. After all, there may be nothing to do but consider that the mentioned relation among S_1, R agglutinations and the complement sensitivity is limited to *V. cholerae*.

Results on newly isolated ET strains : There was a prevalence of cholera amounting to 1,390 patients (122 deaths) caused by the non-hemolytic ET of Ogawa type from August 26 to November 4, 1969 in South Korea.[2)3)7)9)] In February 1970, the present author had a chance to get 320 epidemic strains isolated there and to use for this study together with the other 20 fresh isolates collected afterwards from Philippines, Hong Kong, and other countries. In March 1970, all the Korean strains were examined for their colonial features and their spontaneous agglutinability in saline unheated or heated, from which 20 strains were screened out owing to the weak agglutinability under heating, and the remaining 300 strains were tested for S_1-agglutination. What is to be noted here is that all the Korean strains were of the typical smooth form, and the colonial morphology of them on agar plate still remained unchanged even after one week or so.

In the first time agglutination using S_1-serum, 23 strains carrying S_1 in a titer 1: 80 or more were found among them (left-most row in Table 5). From the viewpoint of the freshness of the strains and complete smoothness of their colonies, it was entirely an unexpected result, and to the author's regret, he was obliged to omit the other items of test. The second time agglutination with S_1-serum of these strains were performed again at the beginning of August in this year, together with the two kinds of R-agglutination and the test for complement sensitivity including 10 S_1-negative strains collected at random from the remainder as controls.

What is noteworthy in data of Korean strains shown in the other part of Table 5 is that first the S_1-positive strains decreased by 14 from 23 and secondly there was little change in the complement sensitivity of the 14 strains possessing S_1 or of the 11 strains possessing S_1 and R. As for the second finding, it may be said that these S_1-positive strains have been somewhat sensitive to the complement

Table 5. Results of newly isolated ET strains

Strain and source		S_1 agglutination March	S_1 agglutination August	R agglutination T.7	R agglutination H.3	Sensitivity to complement
43 out of 300 ET Ogawa strains isolated in Korea, 1969	No. 72	160	320(160)	80	320	3/4/4/4
	306−1	80	160(320)	40	80	1/3/4/4
	2−3	320	160(160)	−	80	1/3/4/4
	73	80	160(160)	−	160	3/4/4/4
	158	160	160(160)	80	320	1/3/4/4
	226	80	160(160)	80	320	1/4/4/4
	236	80	160(160)	−	160	2/3/4/4
	146	160	80(80)	−	−	1/3/4/4
	188	80	80(80)	40	160	2/3/4/4
	208	80	80(80)	−	−	4/4/4/4
	214	160	80(80)	80	320	3/3/4/4
	225	80	80(80)	40	160	1/3/4/4
	381	80	80(80)	80	160	2/4/4/4
	309	80	80(−)	−	−	0/3/4/4
	139	160	−(−)	−	−	3/4/4/4
	145	160	−(−)	−	−	3/3/4/4
	164	160	−(−)	−	−	3/4/4/4
	177	160	−(−)	−	−	4/4/4/4
	304	160	−(−)	−	−	3/4/4/4
	204	80	−(−)	−	−	4/4/4/4
	221	80	−(−)	−	−	4/4/4/4
	235	80	−(−)	−	−	3/3/4/4
	251	80	−(−)	−	−	4/4/4/4
	10 strains (random sampling)	All negative				3/4/4/4 : 3 3/3/3/3 : 4 2/4/4/4 : 1 2/3/3/4 : 1 2/3/4/4 : 1
	11 str. ET Ogawa Philippines, 1969	All negative				4/4/4/4 : 10 3/4/4/4 : 1
	2 str. ET Ogawa Africa, 1970	All negative		All		4/4/4/4
	6 str. ET Inaba Hong Kong, 1969 1 str. ET Inaba Jordan, 1970	All negative		All		4/4/4/4

Only S_1 agglutination was carried out twice, in March and August, 1970.
The other tests were performed in August, 1970.

from the viewpoint of the results of the first tube (1 : 40), but the difference between the main and control tests is little worth consideration in comparison with that observed in the cases of laboratory strains (Table 2, 3, and 4). In short, it seemed likely that in the case of Korean strains the serological factor S_1, the factor R as well, are discriminated from the complement sensitivity.

As the samples of fresh isolates other than the Korean strains, 13 Ogawa type and 7 Inaba type strains of ET were used in addition. The results indicating that they were all lacking either in the reactivity to S_1 and R sera or in the complement sensitivity are also shown in this table.

Discussoin

By additional use of anti-R-sera, it is seen definitely clear that the strains carrying the antigen S_1 are in a rough state broadly speaking. As it is generally accepted that the smooth-to-rough variation is a gradual or step-like change, it is natural that there can exist the strains exhibiting both the serological R (used as a synonym of S_1) reaction and the complement sensitivity and the other strains exhibiting serological R reaction only, besides typical rough strains characterized by roughness or granularity of colonies, instability in saline, loss of proper antigens, etc.

In the case of the author's studies, atypical rough or semi-rough strains of the former state could be detected only in the stock laboratory strains. The appearance of this type variant seems to be related to storage life or age of the strains, rather than to the difference of biotypes CV and ET or of serotypes Inaba and Ogawa. In fact, CV strains tested were mainly stock cultures of several decades, a half of which were S_1-positive and sensitive to the complement, while all the ET strains, among which only 2 of 12 strains were determined as R of this type, were those isolated in Southeast Asia in 1961 and 1962.

Other non-colonial semi-rough strains detectable only by the serological R-reaction were found at the rate of 14 to 300 epidemic strains isolated in South Korea, 1969. It is hardly possible to apply extensively the mentioned "age relation" concerning the laboratory strains to the Korean strains, and some other inducements should be taken into consideration in this case. The fact that the epidemic in Korea was one of the large-scale incidents experienced in recent years and mighty countermeasures were provided for it, appears to be a key to the question, and the present author is inclined to explain the appearance of the variants by the following reason, in conformity with the opinion established mainly by Japanese workers in the epidemic of cholera in Shanghai and other cities in the Continent in 1942 (Akisada[1]; Higashi[5]; Joya[8]; Yasuda and Shinagawa[10]) : the serological variation in Korean strains occurred in patients and carriers at the end of the epidemic, probably under the influence of enforced vaccination or sufficient immunity acquired naturally. In addition it is instructive that variation can take place in patients treated with antibacterial drugs

also, especially at the end of the disease, as well as in carriers (Felsenfeld[4]).

From the observations of serological R-reaction, complement sensitivity, instability in saline, and colonial morphology, the present author supposed that they were all the manifestation of "rough" in a broad sense and appeared in due order in the course of variation of smooth-to-rough. As the serological R-reaction among them is considered to be the sharpest measure for demonstration of roughness in an early stage of the variation, the author thinks that it must be applied more widely to experiments and practice on cholera vibrio, e. g. antigen analysis, preparation of diagnostic factor serum, animal inoculation, preparation of prophylactic vaccine, etc. It has already been described in the paper of Horikawa[6] that the existence of S_1 or R antigenic factor in cholera vibrio sometimes will hinder the progress of experiments and practical works.

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玉置の抗原因子 S1 についての研究続報，エルトール型コレラ菌におけるその出現と本因子の本態

堤　　恒　雄

長崎大学医学部細菌学教室（主任　青木義勇教授）

摘　　要

抗原因子 S_1 はエルトールコレラ菌の教室保存菌株でも検出された．ただその検出率はクラシック型コレラ菌に比して低率であった．保存菌全株についてのその出現率は菌株の保存期間すなわち「古さ」に関連し，上記のコレラ菌の区分やその血清型とは無関係であった．S_1 保有菌は抗R血清によって同時に凝集されまた補体の殺菌作用に感受性であった．本菌は通常の基準によるR型変異菌ではないが，S→R変異の前駆的段階にあるものと称し得よう．本因子はエルトール菌新鮮分離株の一部においても立証されたが，補体感受性は期待に反して認め得なかった．この場合には上記の「古さ」の関係は適用できない．血清学的R型変異が流行菌株の場合このように早期にも起る可能性を文献上から考察した．