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Nature of the Kanagawa Phenomenon of *Vibrio parahaemolyticus*

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In a study of the Kanagawa phenomenon of *Vibrio parahaemolyticus*, both Kanagawa-positive and -negative strains were found to produce hemolytic factors that could not be differentiated on Wagatsuma blood agar. The presence of fermentable carbohydrates in media containing high concentrations of NaCl promoted the growth of *V. parahaemolyticus* and resulted in a marked decrease in medium pH and increased hemolysin production. The Kanagawa hemolysis of test strains differed according to the carbohydrates added. Clearly defined Kanagawa hemolysis was observed in blood agars of high salt content, but the distinction was lost in media containing 3% NaCl. From the results of this study, the Kanagawa hemolysis was interpreted as an expression of quantitative difference in hemolysin production, a conclusion that is clearly demonstrated on special blood agar of high salt content.

A well-defined, clear hemolysis produced by *Vibrio parahaemolyticus* on specially prepared media has been considered closely related to its enteropathogenicity and has been termed "the Kanagawa phenomenon" by Japanese investigators studying *V. parahaemolyticus* (4). Several reports in Japan have indicated that strains of *V. parahaemolyticus* isolated from stools of food poisoning patients are Kanagawa positive (K+), whereas almost all strains of marine origin are Kanagawa negative (K−) (3, 4, 6). A heat-stable, direct hemolysin was recently described as responsible for Kanagawa hemolysis (KH) (12), and purification of the hemolysin has been reported (7, 8). However, Twedt et al. (11) stated in their detailed study that KH resembled hemolysis on ordinary human blood agar. Reports on food poisoning cases associated with K− strains are accumulating (5, 9), and experimental results on the relationship between KH and enteropathogenicity are contradictory, as reviewed by Twedt and Brown (10). We reported previously that KH appeared to be an expression of quantitative rather than qualitative difference in hemolysin production (2). This report describes the effect of carbohydrates and other factors on KH.

MATERIALS AND METHODS

Cultures studied. Test strains of *V. parahaemolyticus* (Table 1) were supplied by R. Sakazaki, National Institute of Health, Japan; H. Zen-Yoji, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan; and Y. Miyamoto, Kanagawa Prefectural Public Health Laboratory, Yokohama, Japan. These included K− strains from stools of diarrheal patients.

Culture media. Special blood agar used for the observation of KH was Wagatsuma blood agar (WBA), which contained 5% washed human erythrocytes in modified Wagatsuma agar (Eiken, Japan). To test various effects on KH, Wagatsuma agar (without carbohydrates) composed of 0.5% yeast extract, 1% peptone, 7% NaCl, 0.0001% crystal violet, and 1.5% agar (pH 7.5) and Wagatsuma broth without agar were prepared according to the formula for modified Wagatsuma agar, and necessary components were incorporated.

Unless otherwise noted, 3% NaCl was always incorporated in the other media used.

Determination of hemolytic activity. KH was determined according to the description of Miyamoto et al. (4). Briefly, cultures of test strains in nutrient broth were streaked linearly on WBA plates, and the result was read after incubation for 24 h at 37 C. Well-defined, clear hemolysis around the bacterial growth was recorded as positive, no hemolysis was negative, and a very narrow zone of hemolysis was doubtful. The observation of hemolysis was sometimes extended to 48 h. To test the activity of hemolytic factors diffused into agar underneath and around the bacterial growth, WBA and nutrient agar (NA) plates were inoculated with K+ and K− strains and incubated for 24 h. After removing the bacterial lawn, the hemolytic zone of WBA and the zone of bacterial growth of NA were cut and extracted by repeated freeze-thawing. The extracts, sterilized through membrane filters (0.45 μm; Millipore Corp.), were added to antibiotic assay cylinders placed on WBA and NA-blood plates, and the hemolytic activity was determined by hemolysis of media around the cylinders after 25 h at 37 C.

TABLE 1. *Origin and designation of Vibrio parahaemolyticus*

Strain no.	Original designation	Kanagawa hemolysis	Source	Donor
1	5507	+	Fish	Y. Miyamoto
3	8741	+	Fish implicated in food poisoning	Y. Miyamoto
5	9065	+	Fish implicated in food poisoning	Y. Miyamoto
8	9379	+	Patient's stool	Y. Miyamoto
10	9382	+	Patient's stool	Y. Miyamoto
12	9384	+	Patient's stool	Y. Miyamoto
23	T-3011	+	Patient's stool	H. Zen-Yoji
26	T-3031	+	Patient's stool	H. Zen-Yoji
51	9166	-	Patient's stool	Y. Miyamoto
54	9369	-	Patient's stool	Y. Miyamoto
56	8848	-	Fish	Y. Miyamoto
57	9331	-	Fish	Y. Miyamoto
61	70-3062	-	Fish	R. Sakazaki
62	70-3079	-	Fish	R. Sakazaki
66	T-3095-1	-	Patient's stool	H. Zen-Yoji
68	T-3232-1	-	Patient's stool	H. Zen-Yoji

Determination of growth and pH. The growth of test strains in fluid media was determined turbidometrically at 540 nm, using a Bausch & Lomb Spectronic 20 spectrophotometer. The pH was measured with a Beckman Expandomatic SS-2 pH meter. For measuring the pH of solid media, 1 g of media extracted in 9 ml of distilled water was used.

RESULTS

Typical hemolysis of WBA plates caused by extracts of WBA cultured with K+ and K- strains is shown in Fig. 1. Two kinds of hemolytic zones, a clearly defined KH-like zone around the cylinder and a hazy zone of hemolysis outside the clear zone, were produced by all extracts of WBA without qualitative differences between K+ and K- cultures. Different extracts of cultured media produced different sizes of hemolytic zones, with K+ cultures tending to produce slightly larger zones than K- cultures. Hemolysis underneath the bacterial growth of some K- strains at 24 h had a colored, maplike appearance with a mixture of clear and greenish-red zones, but zones produced by K+ strains were clear.

Figure 2 shows the hemolysis by cultured NA extracts on WBA plates. Again, no qualitative differences was noted in hemolytic activities between extracts of media from K+ and K- strains, and slightly larger zones were produced by extracts of K+ cultures than by those of K- cultures. The hemolytic patterns were essentially the same as with WBA extracts, but diameters of zones produced by NA extracts were smaller than those of WBA extracts. When these extracts were tested on ordinary blood agar plates, a narrow and hazy zone of hemolysis without well-defined margin was produced, and no marked difference was noted between extracts of WBA and NA from both K+ and K- cultures.

Hemolysis by K+ and K- strains was ob-

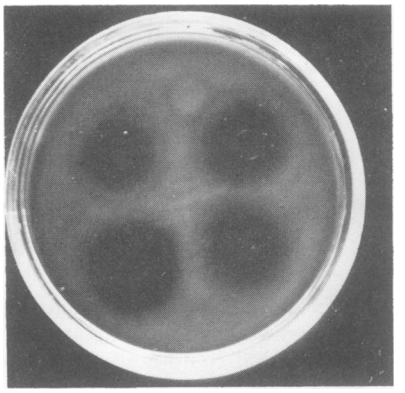

FIG. 1. *Hemolysis of Wagatsuma blood agar by extracts of Wagatsuma blood agar cultured with Kanagawa-positive (K+) and -negative (K-) strains of Vibrio parahaemolyticus. Upper left, strain no. 8 (K+); lower left, no. 12 (K+); upper right, no. 51 (K-); and lower right, no. 68 (K-). The dark zone around the cylinder is clear hemolysis with distinct margin; the outer zone is hazy hemolysis without distinct margin.*

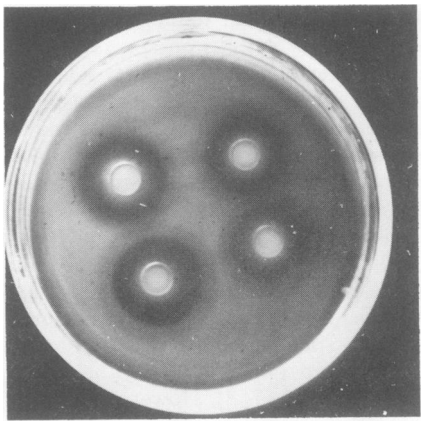

FIG. 2. *Hemolysis of Wagatsuma blood agar by extracts of nutrient agar (3% NaCl) cultured with Kanagawa-positive (K+) and -negative (K-) strains of Vibrio parahaemolyticus. Upper left, strain no. 8 (K+); lower left, no. 12 (K+); upper left, strain no. 8 (K+); lower left, no. 12 (K+); upper right, no. 51 (K-); and lower right, no. 68 (K-). The dark zone around the cylinder is clear hemolysis with distinct margin; the outer zone is hazy hemolysis without distinct margin. The white zone at center indicates precipitate produced by extracts of nutrient agar culture.*

TABLE 2. *Effects of carbohydrates on Kanagawa hemolysis of Vibrio parahaemolyticus*[a]

Strain no.	Kanagawa hemolysis on media containing[b]:									
	No carbohydrates	Lactose	Sucrose	Dextrose[c]	Mannitol	Mannose	Maltose	Galactose	Trehalose	Arabinose
1	−	−	−	+	+	+	+	−	−	±
3	−	−	−	+	+	+	+	−	+	−
5	−	−	−	+	+	+	+	−	+	−
8	−	−	−	+	+	+	+	−	+	−
10	−	−	−	+	+	+	+	±	+	−
12	−	−	−	+	+	+	+	−	+	+
23	−	−	−	+	+	±	±	−	−	−
26	−	−	−	+	+	+	+	±	+	±
51	−	−	−	+	−	±	±	−	+	−
54	−	−	−	+	−	−	−	−	+	−
56	−	−	−	+	−	−	+	−	+	−
57	−	−	−	+	−	±	+	+	+	−
61	−	−	−	+	−	+	−	−	+	−
62	−	−	−	+	−	+	−	−	−	−
66	−	−	−	+	−	±	+	−	+	+
68	−	−	−	+	−	−	+	+	−	+

[a] Symbols: +, positive; −, negative; ±, doubtful.
[b] Wagatsuma blood agar was incorporated with selected carbohydrates at 0.5% concentration.
[c] Bacterial growth was frequently surrounded by brownish turbidity.

served on WBA containing selected carbohydrates at 0.5% concentration to determine the effects of carbohydrates on KH (Table 2). No hemolysis was noted with either K+ and K− strains after 24 h at 37 C in media without carbohydrates or with nonfermentable sucrose and lactose. The KH was produced in the presence of fermentable carbohydrates, with results differing according to the strains and carbohydrates added. The inclusion of maltose and trehalose in media converted more than half of the K− strains to K+, and most K+ strains became K− in the presence of galactose and arabinose. Some K− strains were positive in mannose-containing media. The dextrose-containing medium generally produced a large KH zone, but the bacterial growth was frequently surrounded by a brownish turbid zone.

Next studied was the effect of NaCl concentrations on KH (Table 3). When mannitol was added to the media, K+ strains produced KH after 24 h in Trypticase soy blood agar and nutrient blood agar containing 7% NaCl, but K− strains did not. The inclusion of dextrose in the media containing 7% NaCl produced large zones of KH by both K+ and K− strains, but the brownish turbid zone was frequently noted around the bacterial growth. When NaCl was reduced to 3% in these media, all K+ and a majority of K− strains showed hazy and narrow zones of hemolysis without distinct margin that were not considered to be KH. Furthermore, in media containing dextrose and 3% NaCl, most strains showed a tendency toward swarming growth. In other studies we noted that hemolysis appeared Kanagawa-like when NaCl concentration was 5% or more.

Three strains were selected for study of the relationship between KH and change of pH in media: no. 5, which is K+ on WBA with mannitol and mannose; no. 54, K− with mannitol and mannose; and no. 61, K− with mannitol and K+ with mannose. Strains were inoculated on WBA containing mannitol and/or mannose, and the KH and pH of the media were observed during incubation at 37 C (Table 4). The K+ strains began to show KH after 15 h in the presence of mannitol, whereas K− strains were KH negative up to 24 h. After 48 h of incubation, hemolysis for all test strains would not be considered KH, since the margin was not well defined. The pH of mannitol-containing media decreased gradually during the first 18 h of incubation; the decrease was more marked in the medium grown with the K+ strain than with K− strains, and it was followed by a pH increase at 24 and 48 h. The inclusion of mannose in the media produced results similar to those of the mannitol-containing media by strains no. 54 and lightly inoculated no. 5. When no. 5 was heavily inoculated, KH was noted at 15 h with a medium pH of 5.5; the pH decreased to 5.4 at 18 h accompanied by brownish turbidity around the bacterial growth. The

TABLE 3. *Effect of NaCl concentrations on Kanagawa hemolysis of Vibrio parahaemolyticus*

Strain no.	Kanagawa hemolysis[a]							
	TSA[b] + 7% NaCl		TSA + 3% NaCl		NA[c] + 7% NaCl		NA + 3% NaCl	
	Mannitol	Dextrose	Mannitol	Dextrose	Mannitol	Dextrose	Mannitol	Dextrose
1	+	+	(+)[d]	(+)	±	+	(+)	(+)
3	+	+	(+)	(+)	+	+	(+)	(+)
5	+	+	(+)	(+)	±	+	(+)	(+)
8	+	+	(+)	(+)	+	+	(+)	(+)
10	+	+	(+)	(+)	+	+	(+)	(+)
12	+	+	(+)	(+)	+	+	(+)	(+)
23	+	+	(+)	(+)	+	+	(+)	(+)
26	+	+	(+)	(+)	+	+	(±)	(+)
51	−	+	(+)	(+)	−	+	(±)	(+)
54	−	+	(±)	(+)	−	+	(±)	(±)
56	−	+	(+)	(+)	−	±	(+)	(+)
57	−	+	(+)	(+)	−	+	(±)	(+)
61	−	+	(−)	(+)	−	+	(+)	(+)
62	−	+	(+)	(+)	−	+	(+)	(+)
66	−	+	(+)	(+)	−	+	(+)	(+)
68	−	+	(+)	(+)	−	+	(±)	(+)

[a] Symbols are as in Table 2.
[b] Trypticase soy agar with 5% erythrocytes.
[c] Nutrient agar with 5% erythrocytes.
[d] Ordinary hemolysis (beta) without clear margin.

TABLE 4. *Change in pH and Kanagawa hemolysis of Vibrio parahaemolyticus on Wagatsuma blood agar*

Carbohydrate added	Strain no.[a]	pH of medium and appearance of hemolysis during incubation for:						
		0 h	6 h	9 h	15 h	18 h	24 h	48 h
Mannitol	5	8.2	7.7	7.2	6.0	5.7	5.8	8.7
		−[b]	−	−	±	+	+	(+)[c]
Mannitol	54	8.2	7.9	7.6	7.0	7.0	7.4	8.7
		−	−	−	−	−	−	(+)
Mannitol	61	8.2	7.7	6.9	7.4	7.4	7.8	8.8
		−	−	−	−	←	−	(+)
Mannose	5[d]	8.2	6.8	6.4	5.5	5.4	5.4	5.4
		−	−	−	+	B[e]	B	B
Mannose	5[f]	8.2	7.5	7.0	6.2	6.0	6.9	8.8
		−	−	−	−	±	+	(+)
Mannose	54	8.2	7.9	7.7	6.3	6.8	7.9	8.7
		−	−	−	−	−	−	(+)
Mannose	61	8.2	7.3	6.6	5.8	6.3	6.9	8.6
		−	−	−	+	+	+	(+)

[a] No. 5 is K+ in Wagatsuma blood agar with mannitol and mannose, no. 54 is K− with mannitol and mannose, and no. 61 is K− with mannitol and K+ with mannose.
[b] Kanagawa hemolysis.
[c] Ordinary hemolysis (beta) without clear margin.
[d] Heavily inoculated.
[e] Chocolate-like brownish turbidity in medium.
[f] Lightly inoculated.

pH level and color of media remained the same during the subsequent incubation. When mannose-containing media were inoculated with no. 61, KH began after 15 h when the pH decreased to 5.8. Test strains were inoculated in WBA of different pH values to determine the effect of pH on the growth of solid media. WBA became brownish and no or scanty growth was noted when pH was 5.4 or below.

The growth of test strains and change of pH in Wagatsuma broth with or without mannitol and mannose were observed by inoculating 200 ml of test media with 0.1 ml of nutrient broth cultures (Fig. 3–5). A slight decrease in pH and gradual increase in optical density as a result of growth were noted during incubation for 9 to 24 h in the absence of fermentable carbohydrates. In the presence of mannitol and mannose, strains no. 5 and no. 61 propagated rapidly from 9 to 15 h of incubation, accompanied by marked decreases in pH of the media. However, the initial growth of no. 54 was slow compared with the other strains, and the decrease in the pH of the media was also less marked. In a comparison of strains no. 5 and no. 61, the latter showed somewhat more marked growth and lowered pH than the former in media tested.

The growth rates of K+ and K− strains were compared in fluid media (Table 5). Test strains showed poorer growth in Wagatsuma broth containing mannitol than in nutrient broth, and the increase of NaCl concentration to 8% resulted in a decrease of colony counts. The addition of erythrocytes to Wagatsuma broth with mannitol did not improve the growth.

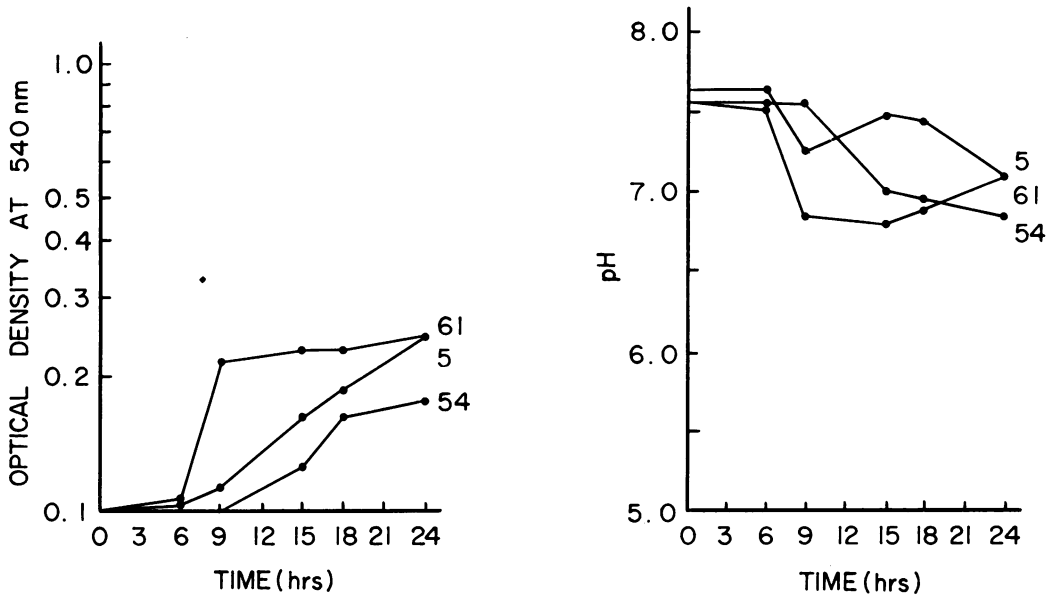

FIG. 3. *Growth and pH change of Vibrio parahaemolyticus strains in Wagatsuma broth containing no carbohydrate source. Numeral indicates strain number.*

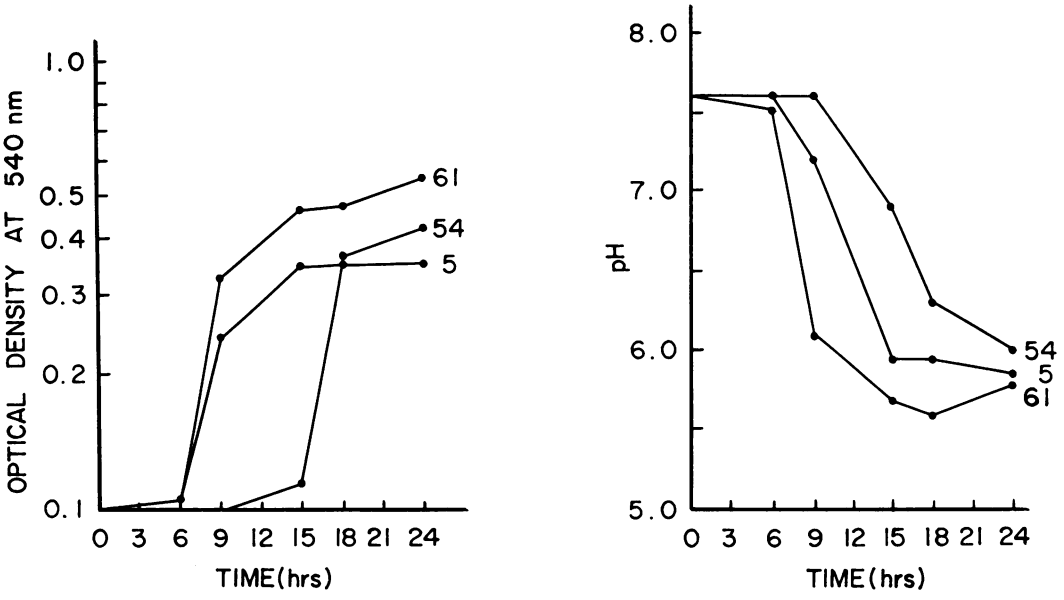

FIG. 4. *Growth and pH change of Vibrio parahaemolyticus strains in Wagatsuma broth containing mannitol. Numeral indicates strain number.*

DISCUSSION

Contrary to reports by Japanese investigators that KH could be produced only in special blood agar (3, 4), the results of this study showed that factors causing KH can be produced in special blood agar and even in NA. Several investigators (7, 8, 12, 13) reported the purification of a hemolysin from young culture supernatants of K+ strains but not from those of K− strains. We noted that K+ strains initiated KH on WBA after 15 to 18 h of incubation, and that the hemolytic factors produced in NA by K− strains were less active than those produced by K+ strains. These results indicate that fluid media without fer-

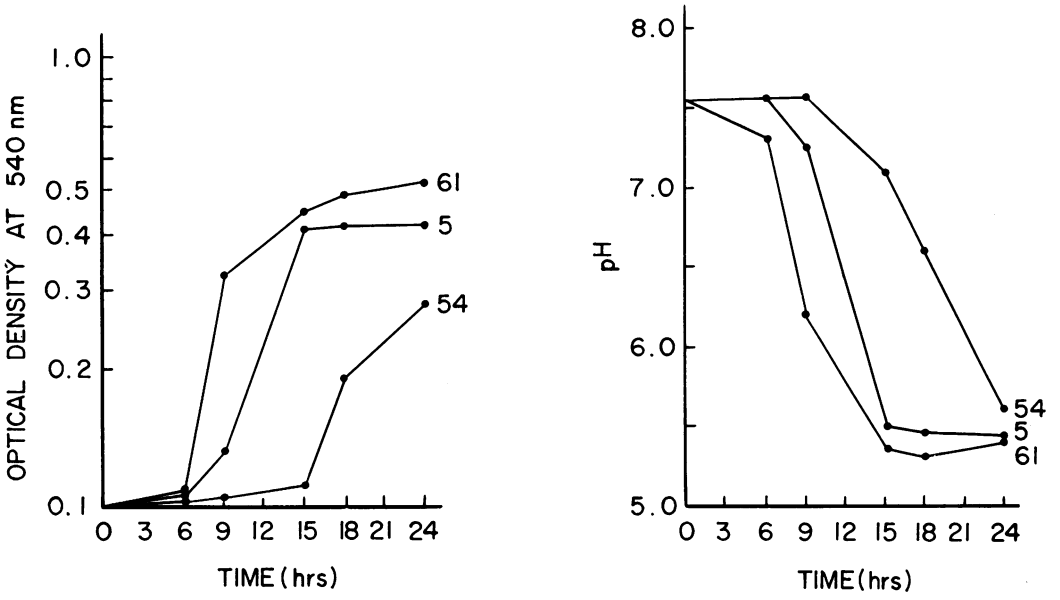

FIG. 5. *Growth and pH change of Vibrio parahaemolyticus strains in Wagatsuma broth containing mannose. Numeral indicates strain number.*

TABLE 5. *Growth of Vibrio parahaemolyticus in fluid media*

Medium	Strain no.	Number of viable cells per 0.1 ml after observation period of:				
		0 h	1 h	3 h	6 h	24
Nutrient broth (3% NaCl)	1	565	725	1,940	780,000	35,700,000
	51	175	370	1,870	1,010,000	30,800,000
Wagatsuma broth[a] (7% NaCl)	1	320	255	585	13,400	5,900,000
	51	203	226	621	14,500	3,600,000
Wagatsuma broth (8% NaCl)	1	181	277	305	1,950	1,950,000
	51	316	331	287	1,380	1,100,000
Wagatsuma broth (7% NaCl, 5% erythrocytes	1	543	485	436	12,400	3,200,000
	51	562	377	635	20,200	4,620,000

[a] Contains 0.5% mannitol.

mentable carbohydrates may not be good media with which to produce hemolytic factors, especially by weakly hemolytic strains.

The presence of fermentable carbohydrates was important for KH on WBA, with results varying according to the carbohydrates used. Our results also suggest that the growth of *V. parahaemolyticus* is responsible, at least in part, for KH. WBA is a poor medium, probably because of the high salt content, and the breakdown products of carbohydrates may help to promote growth. The acidic condition of the media resulting from the breakdown of carbohydrates may also favor the growth of some strains, as suggested by Barrow and Miller (1). It is possible that the breakdown products of carbohydrates and low pH may influence the production of KH factors, since KH was observed in media with selected carbohydrates at about pH 6.0 or below; but this possibility was not carefully investigated in the present study. The differing abilities of various strains to produce KH factor were suggested by Chun et al. (2), and the suggestion is supported in this study by the more marked growth of K− strain no. 61 as opposed to K+ strain no. 5 in fluid media containing mannitol.

The finding that WBA with mannose and dextrose sometimes showed brownish turbidity around the bacterial growth of some strains accompanied by a pH of 5.4 indicates that the presence of some carbohydrates in solid media supports fermentation to a degree that yields the brownish denaturation of blood-containing media.

As suggested by Miyamoto et al. (4), KH was clearly demonstrated on WBA and other blood media containing 7% NaCl but not in media of low NaCl concentration; high NaCl concentration appeared to influence the diffusibility of KH factor.

Our findings indicate that the addition of fermentable carbohydrates to poor media containing 7% NaCl results in the promotion of growth and increased hemolysin production by breakdown products, and KH factor produced

by strains of differing hemolytic abilities could diffuse rapidly in media of high salt content. Thus, the quantitative difference of KH factor would be demonstrated easily and rapidly. Kato et al. (3) used a medium with 2.5% NaCl and no carbohydrates, but our result with this medium showed no clear difference in hemolysis between K+ and K− strains (unpublished data). Twedt et al. (11) reported that the patterns of KH resembled the hemolysis seen in ordinary human blood agar, and the use of special blood agar would be a method of clearly differentiating strongly hemolytic strains from weakly hemolytic ones.

The relationship between KH and the enteropathogenicity of *V. parahaemolyticus* was evaluated on an empirical basis (4). Different KH patterns were produced by the addition of different carbohydrates, and no reasonable elucidation has been presented for the relationship between KH produced by various strains in media containing mannitol and their pathogenicity.

Various hemolysins or enzymes of different molecular weights would be involved in KH, a rather crude method of hemolysis testing, and our further study will be directed to the differential determination of hemolytic factors involving KH in extracts of cultures in solid and fluid media containing different fermentable carbohydrates.

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