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Immunological Detection of the Kanagawa Phenomenon of *Vibrio parahaemolyticus* on Modified Selective Media

TAKESHI HONDA,* CHATCHAI SORNCHAI,† YOSHIFUMI TAKEDA, AND TOSHIO MIWATANI

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan

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Selective media for *Vibrio parahaemolyticus*, BTB-Teepol agar and modified arabinose-ammonium sulfate-cholate agar, were modified for use in immunological detection of the thermostable direct hemolysin produced by this organism. The modified BTB-Teepol agar and modified arabinose-ammonium sulfate-cholate agar were both found to be useful for the modified Elek test and immunohalo test with antiserum (or immunoglobulin G) against the thermostable direct hemolysin. With these modified media it is possible to isolate *V. parahaemolyticus* and identify the Kanagawa phenomenon on a single plate and thus save time in obtaining results.

Some strains of *Vibrio parahaemolyticus* produce thermostable direct hemolysin (5, 9, 11, 12), which has been found to be closely related with the pathogenicity of *V. parahaemolyticus* in humans (4, 8, 10). This hemolysin is usually detected on a special blood agar medium (Wagatsuma medium), and the hemolytic action of this organism in Wagatsuma medium has been designated as the Kanagawa phenomenon (7, 8). Recently we reported (2) a simple, reliable immunological method for detecting the Kanagawa phenomenon of *V. parahaemolyticus*.

This paper reports immunological methods for detection of the Kanagawa phenomenon on modified conventional selective media for *V. parahaemolyticus*. These methods are useful for both isolation of *V. parahaemolyticus* and detection of the Kanagawa phenomenon on a single plate and thus save time in obtaining results.

All bacterial strains used were from the Laboratory for Culture Collections, Research Institute for Microbial Diseases, Osaka University. Anti-thermostable direct hemolysin antiserum was prepared as described previously (2).

The immunological tests used were the modified Elek test and immunohalo test essentially as described previously (2, 3). In brief, the modified Elek test was carried out by adding antihemolysin serum to a well cut in agar medium near a colony(ies) grown on the medium, and the immunohalo test was carried out by inoculating bacteria onto agar medium containing antihemolysin antiserum or antihemolysin immunoglobulin G.

† Present address: Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 7, Thailand.

Three different media are widely used as selective media for the isolation of *V. parahaemolyticus*: bromthymol blue (BTB)-Teepol (Shell Oil Co.) agar (7), thiosulfate-citrate-bile salts-sucrose agar (7), and MAAC (modified arabinose-ammonium sulfate-cholate) agar (6). Experiments were carried out with BTB-Teepol agar and MAAC agar because they were found to be better for hemolysin production by *V. parahaemolyticus* than thiosulfate-citrate-bile salts-sucrose agar.

Kanagawa phenomenon-positive strains of *V. parahaemolyticus* did not produce precipitin lines in the modified Elek test on the original BTB-Teepol agar, or an immunohalo around colonies grown on antiserum-containing BTB-Teepol agar. As this suggested that *V. parahaemolyticus* grown on the original BTB-Teepol agar did not produce sufficient thermostable direct hemolysin for reactions, we tried to modify the BTB-Teepol agar to obtain better production of the hemolysin. Biken agar no. 1 is the best medium for hemolysin production on agar medium found so far (2). The composition of this modified BTB-Teepol agar, which is similar to that of Biken agar no. 1, is shown in Table 1. The most critical component of the modified BTB-Teepol agar is glucose, because it affects hemolysin production by *V. parahaemolyticus* and also the color change of colonies as a result of glucose fermentation. In the modified Elek test, addition of less than 0.2% glucose at pH 7.5 gave no precipitin line, or a faint line, with a green colony, whereas addition of 0.5% glucose or more gave a clear precipitin line with a yellow colony. Therefore, we added 0.4% glucose to the medium, and this gave a clear precipitin line

TABLE 1. Composition of modified BTB-Teepol agar[a]

Component	Original BTB-Teepol agar (per liter)	Modified BTB-Teepol agar (per liter)
Beef extract	5 g	5 g
Peptone (Difco)	10 g	30 g
NaCl	30 g	40 g
$Na_2HPO_4 \cdot 12H_2O$		30 g
Saccharose	20 g	20 g
Glucose		4 g
Teepol	2 ml	2 ml
Agar	15 g	
Noble agar (Difco)		15 g
Bromothymol blue	0.08 g	0.08 g
Bromocresol purple		0.01 g
Antihemolysin[b]		50 ml
pH	7.8	8.0

[a] We used 10 ml of autoclaved medium per petri dish (90 by 15 mm).

[b] For the immunohalo test, antihemolysin antiserum with a titer of 1:32 was mixed with autoclaved medium after it had cooled to 50°C.

or immunohalo around typical green colonies of *V. parahaemolyticus*. We tested six Kanagawa phenomenon-positive and six negative strains of *V. parahaemolyticus* by the modified Elek test and immunohalo test on modified BTB-Teepol agar. All strains gave typical green colonies, and the results of Kanagawa phenomenon were consistent.

Modified BTB-Teepol agar was tested for its selectivity for *V. parahaemolyticus* with the various bacterial strains shown in Table 2. Results showed that the modified BTB-Teepol agar was almost as selective as the original BTB-Teepol agar.

MAAC agar was first developed for isolation of *V. parahaemolyticus* from materials other than patients' feces, such as fish and shellfish. These materials usually contain large numbers of *Vibrio alginolyticus*, which interfere with the isolation of *V. parahaemolyticus* on BTB-Teepol or thiosulfate-citrate-bile salts-sucrose agar (6).

MAAC agar was tested for hemolysin production by *V. parahaemolyticus*. With minor modi-

TABLE 2. Selectivity of modified BTB-Teepol agar

Strain	Original BTB-Teepol agar		Modified BTB-Teepol agar	
	Growth (colony size)	Color of colonies[a]	Growth (colony size)	Color of colonies[a]
Escherichia coli				
8-697-1	Tiny	—	None	—
8-686-1	Tiny	—	None	—
8-679-1	Small	Y	Tiny	—
8-700-1	Tiny	G	Tiny	—
Aeromonas hydrophila				
No. 5	Tiny	—	None	—
Klebsiella pneumoniae				
RIMD 1102001	Tiny	—	None	—
Enterobacter aerogenes				
RIMD 0502001	Small	Y	Tiny	Y
Proteus vulgaris				
RIMD 1643001	None	—	Tiny	Y
RIMD 1643006	None	—	None	—
RIMD 1643005	None	—	None	—
Vibrio parahaemolyticus[b]	Normal	G	Normal	G
Vibrio alginolyticus				
RIMD 2201008	Normal	Y	Normal	Y
Vibrio cholerae				
569B	Small	Y	Small	Y
ME 12938	Tiny	Y	Tiny	Y

[a] Y, Yellow; G, green; —, no visible color change.

[b] Six Kanagawa phenomenon-positive strains (RIMD 2210260, 2210385, 2210388, 2210390, 2210391, and 2210394) and six negative strains (RIMD 2210046, 2210048, 2210049, 2210050, 2210051, and 2210052) behaved in the same way.

TABLE 3. Composition of modified MAAC agar[a]

Component	Original MAAC (g/liter)	Modified MAAC (g/liter)
Arabinose	5	5
Polypeptone	1	
Peptone (Difco)		1
Ammonium sulfate	1	1
NaCl	40	40
Sodium thiosulfate	10	10
Sodium cholate	1	
Sodium deoxycholate		0.2
Bromothymol blue	0.04	0.04
Thymol blue	0.04	0.04
Agar	15	
Noble agar (Difco)		15
Antihemolysin[b]		50 (ml)
pH	8.6	8.6

[a] We used 10 ml of autoclaved medium per petri dish (90 by 15 mm).

[b] Immunoglobulin purified by column chromatography of protein A-Sepharose CL-4B and adjusted to a titer of 1:32 (2) was added to cooled autoclaved medium (at 50°C) for the immunohalo test.

fications (Table 3), it was found to permit sufficient hemolysin production by *V. parahaemolyticus* to give a precipitin line by the modified Elek test. However, addition of antihemolysin antiserum (5% final concentration) for the immunohalo test resulted in the formation of green colonies of *V. parahaemolyticus*, which made it impossible to distinguish *V. parahaemolyticus* from green colonies of *V. alginolyticus*. The formation of green colonies of *V. parahaemolyticus* on the modified MAAC agar with 5% antihemolysin antiserum was found to be due to some component(s) in the serum other than antibody against the hemolysin. Therefore, immunoglobulin G against the hemolysin was obtained by affinity column chromatography on a *Staphylococcus aureus* protein A-Sepharose CL-4B column (1). Addition of this immunoglobulin G to the modified MAAC medium gave an immunohalo around typical yellow colonies of Kanagawa phenomenon-positive strains of *V. parahaemolyticus*.

Thus it is concluded that the modified BTB-Teepol agar and the modified MAAC agar can be used for both isolation of *V. parahaemolyticus* and identification of the Kanagawa phenomenon of colonies grown on the agar plates.

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