



SELECTIVE MEDIUM FOR ISOLATION OF VIBRIO PARAHAEMOLYTICUS

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SUMMARY

An elective-selective medium containing carbenicillin and a percentage of NaCl has been set up for the research of Vibrio parahaemolyticus in water, sea sediments and foods, with special regard to bivalve molluscs. This medium was tested and showed to be particularly selective with regard to the saprophytic microbial flora.

The suitability of the medium was confirmed by comparative tests carried out on well known substrata, commonly used for research of Vibrio parahaemolyticus.

INTRODUCTION

Vibrio parahaemolyticus, as reported by WHO,¹ is being considered more and more important as an agent of food-borne infections associated with sea foods.

In 1951 Fujino et al.² showed that V. parahaemolyticus was responsible for several food-borne outbreaks in Japan, associated with the high consumption of fish and raw molluscs.

The presence of V. parahaemolyticus was subsequently detected in other parts of Asia,^{3,4,5} in Australia,⁶ in North America,^{7,8} in Great Britain⁹ and in the Baltic Sea.¹⁰ A high rate of cases was also reported in the Mediterranean region and in Italy.^{11,12}

The areas where V. parahaemolyticus has been reported are increasing in number and, consequently, the interest in finding more suitable detection methods, both in the marine environment and in sea foods.

Vibrio parahaemolyticus cannot be easily cultivated in routine media. It is very sensitive to chemical substances and to variations in the physical environment. In addition, it is sensitive to any competitive action by the saprophytic microbial flora. Because of this, some years ago a semi-synthetic medium of composition similar to that of a mussel homogenate was set up in order to reproduce certain environmental conditions in which marine Vibrio spp. can live naturally.¹³

In the field the medium showed good selectivity but little inhibiting effect with regard to the rich saprophytic bacterial flora of fish and of sea products in general.

The aim was to make this cultivation medium more selective, both by exploiting the marine characteristics of V. parahaemolyticus and by utilizing an effective selecting agent.

To date, a series of surveys have been carried out based on the following factors:

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- (1) the selection of suitable concentrations of NaCl to inhibit the saprophytic microflora without interfering with the development of V. parahaemolyticus;
- (2) the selection of an active antimicrobial substance to add in sufficient quantity in relation to the schizomycetes which can be detected more frequently in the marine environment and in fish products, but which is also practically devoid of inhibiting action towards V. parahaemolyticus under the physical and chemical conditions of the culture medium.

The proposed modifications were carried out following a series of preliminary experiments on cultures with and without marine vibrios and saprophyte microbes;

- (3) checking the possibility of improvement of the medium by comparing it to other more widely used enrichment media;
- (4) research of V. parahaemolyticus in bivalve molluscs (Mitilus galloprovincialis), as well as in water and marine sediments, by means of the proposed medium.

MATERIALS

Bacterial strains

- <u>Vibrio parahaemolyticus</u> , negative Kanagawa	ISS VP1
- <u>Vibrio parahaemolyticus</u> , positive Kanagawa	ISS VP3
- <u>Vibrio parahaemolyticus</u> , positive Kanagawa	ISS VP5
- <u>Vibrio alginolyticus</u>	ISS VA1
- <u>Vibrio cholerae</u>	ISS V1002
- <u>Proteus mirabilis</u>	ISS PR3
- <u>Pseudomonas fluorescens</u>	ISS PS4
- <u>Micrococcus varians</u>	ISS M126
- <u>Staphylococcus aureus</u>	ATCC 12610
- <u>Escherichia coli</u>	ATCC 12015
- <u>Salmonella typhimurium</u>	ATCC 7823
- <u>Streptococcus faecalis</u>	ATCC 349

Several bacterial strains pertaining to the genera Pseudomonas, Aeromonas and Proteus, isolated from samples of molluscs, have been tested. These strains are not reported on in the tables because they were not identified.

Enrichment cultivation media

- Polimixine salt broth (PSB) - Difco
- Glucose salt teepol broth (GSTB) - Oxoid
- Phyton peptone sulfisoxazole broth (PPS)
- Phyton peptone carbenicillin broth (PPC) - (new medium)

Yeast extract	3 g
Peptone	10 g
NaCl	60 g
Phyton	5 g
H ₂ O	add to 1000 ml
pH 7.5	

The above media should be sterilized at 120°C for 20 minutes. Before use, carbenicillin should be added at a concentration of 0.1 mg/ml (to 1000 ml of the media - 10 ml of a solution of carbenicillin). After this, the medium lasts nine days at 4°C or 72 hours at room temperature.

Isolation cultivation medium

- Thiosulfate citrate bile saccharose agar - TCBS - Difco

Confirming cultivation ground

- Triple sugar agar - TSI - +2% NaCl

Confirming biochemical kits

- Pathotec for enterobacteria

Tested antimicrobial products

- Ampicillin - Farmitalia
- Carbenicillin - Duncan Farmaceutici S.p.A.
- Cloxacillin - Proter SpA
- Eritromicin - Proter SpA
- Flucloxacillin - Proter SpA

Apparatus

- Colorimeter "Spectronic 20" Baush and Lomb
- Coulter Counter Electronics LT Model ZF for standardizing broth cultivation

METHOD

All tests were carried out by injecting 10 ml of the cultivated medium under examination with 0.01 ml of a 24-hour broth culture of the above-mentioned microorganisms. The cultures were carefully standardized with the Coulter Counter, in order to attain initial concentrations at about 10⁴ germs/ml.

Tests were carried out on basic phyton and peptone broth (PP) medium, devoid of inhibitors, in the following sequence:

- (1) The following concentrations were tested: NaCl equal to 2%, 4% and 6%.
- (2) The antibiotics mentioned above were tested at various gradual concentrations, selected on the basis of their minimum inhibiting concentration (MIC) against various microorganisms. Tests were performed by using the PP medium with the addition of 6% of NaCl. The microbial growth was controlled by turbidimetry at 525 nm.
- (3) The medium proposed containing carbenicillin (PPC) was tested at the same time as the medium devoid of antibiotics (PP) and with the more widely used media for research on V. parahaemolyticus in foods, such as GSTB, PSB and PPS. Control was performed by turbidimetry, as mentioned above.
- (4) The validity of the above described medium was finally checked also by tests on bivalve mollusc samples, sea water and sediments previously polluted with V. parahaemolyticus. These were carried out at the same time on the different media (Table 3).

Each sample from cultivations in brackish water of lake Caprolace (Latina), immediately after sampling, was subdivided into six sub-samples; each of them was placed in purpose-made stabulation basins and consisted of one-tenth brackish water, 200 g of marine sediment and 20 average size molluscs.

With the exception of a sub-sample used for control, the others were injected with various gradual concentrations of V. parahaemolyticus (ranging from 10^4 germs/ml to 1 germ/ml, according to the Miles-Misra method).¹⁴

After 24 hours another experiment was carried out on the molluscs in brackish water and in the sediments, by means of feeding PPC and GSTB concentrations into the medium. Following 24-hour incubation at 37°C by both cultivations, isolation was carried out by multiple streaks on TCBS agar plates.

Eventually, every suspected colony was subjected to confirmatory biochemical tests.

RESULTS AND CONSIDERATIONS

(1) The results of the tests carried out on various concentrations of NaCl are reported in Table 1.

According to the analysis of these results, the concentration of 6% NaCl in the substratum does not inhibit V. parahaemolyticus, whereas it clearly inhibits organisms pertaining to groups of Micrococcus (t = 19.5), Pseudomonas (t = 20) and Proteus (t = 11.5).

(2) Following a series of preliminary tests carried out with various antibiotics - namely, eritromicin, cloxacillin, flucoxacillin, ampicillin and carbenicillin - it was found that the best results were attained with the combination of 1 µg/ml of carbenicillin and of 60 mg/ml of NaCl.

This combination was selected primarily on the grounds of testing different concentrations (0.1, 1 and 10 µg/ml of the carbenicillin in the medium) in the presence of 4%, 6% and 8% of NaCl.

Thus, a clear inhibition has taken place towards Proteus, Staphylococcus and Escherichia coli, both in pure cultures and in mixed cultures of these three microorganisms. However, it does not occur with V. parahaemolyticus (Table 2).

(3) The results attained by the tests carried out at the same time, as other well known media, also showed that the PPC develops a good elective action with regard to V. parahaemolyticus together with a strong selective action towards most of the tested germs. Furthermore, Proteus, Micrococcus and Staphylococcus are completely inhibited - contrary to what happens with other media (Table 3), including the GSTB.

These observations were confirmed by the statistical analysis carried out on the results.

The analysis of the bivalent variance according to Friedeman's grades¹⁵ showed - as seen in Table 4 - that there is a very wide difference among the various media.

In addition, variance analysis was carried out (Table 5) in order to show possible differences, not only in the media, but also in the response of the microorganisms at equal levels of culture conditions. It was shown that there were considerable differences (P 0.01) both between the media and among the tested strains.

As shown in Table 3, the group of the marine vibrios under experimental conditions recorded a good growth on all media used, whereas the other microorganisms underwent a strong inhibition in the PPC and showed a variable growth in the other tested substrata.

Finally, calculations were carried out on the mean values of the responses from the four media with the relative confidence intervals (Graph 1) and the analysis of the difference between the means (Table 5).

The results attained always confirmed that the behaviour of the PPC differs considerably from that of other media, whereas there are no special indications as to whether the latter differ at all between themselves.

(4) The tested samples, coming from scarcely and highly polluted sea waters, nearly always showed the presence of many strains such as Aeromonas, Pseudomonas, E. coli and Proteus.

Tests carried out in the stabulation basins showed that V. parahaemolyticus - even under slight continuous movement - tends to concentrate particularly in molluscs and in sediment. This observation ought to be supported by further research, in order to clarify the environmental relationships between molluscs and the marine environment.

In addition, in the above-mentioned samples it was possible to detect one V. parahaemolyticus cell/ml in our medium, whilst the GSTB provided positive results only for concentrations of about 10 cells/ml.

On the basis of what has been stated, it is possible to conclude that the combined action of NaCl and carbenicillin gives a medium already having high electivity⁴ and selectivity features, which make it particularly suitable for research on V. parahaemolyticus samples having a high saprophytic charge, such as molluscs, sediments and sea water.

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TABLE 1. GROWING OF BACTERIA IN BASE MEDIUM (PP) WITH THE ADDITION OF DIFFERENT CONCENTRATIONS OF NaCl

Average values of the readings expressed in transmittance percentage carried out in turbidimeter at 525 nm wavelength.

Tabular value of the Student $t = 6.86$ ($P = 0.001$).

Strains	2% NaCl T%	4% NaCl		6% NaCl	
		T%	Student t	T%	Student t
<u>V. parahaemolyticus</u> K ⁻	28	30	0.46	30	0.72
<u>V. parahaemolyticus</u> K ⁺	38	40			
<u>V. parahaemolyticus</u> K ⁺	30	31			
<u>V. alginolyticus</u>	35	36		36	
<u>V. cholerae</u>	38	40	0.7	60	8.3
<u>Pr. mirabilis</u>	36	48	5.55	58	11.48
<u>Ps. fluorescens</u>	60	100	20	100	20
<u>M. varians</u>	36	75	15.1	90	19.5
<u>St. aureus</u>	30	30	0	33	1.8
<u>E. coli</u>	30	45	6.3	69	14.1
<u>S. typhimurium</u>	34	37	1.96	58	12
<u>Str. faecalis</u>	28	50	6.20	50	6.20

TABLE 2. GROWING OF BACTERIA IN BASE MEDIUM PP + 6% NaCl (CONTROL)
WITH THE ADDITION OF GRADUAL CONCENTRATIONS OF CARBENICILLIN

Average values of the readings expressed in transmittance percentage carried out in turbidimeter at 525 nm wavelength.

Tabular value of the Student $t = 6.86$ ($P = 0.001$).

Strains	Control T%	Carbenicillin					
		0.1 $\mu\text{g/ml}$		1 $\mu\text{g/ml}$		10 $\mu\text{g/ml}$	
		T%	Student t	T%	Student t	T%	Student t
<u>V. parahaemolyticus</u> K ⁻	30	30		27		34	
<u>V. parahaemolyticus</u> K ⁺	42	42	0	41	0.26	41	0.24
<u>V. parahaemolyticus</u> K ⁺	33	34		34		34	
<u>V. alginolyticus</u>	36	35		35		35	
<u>V. cholerae</u>	60	60	0	84	12.53	86	19.7
<u>Pr. mirabilis</u>	58	60	0	100	72	100	72
<u>Ps. fluorescens</u>	100	100	0	100	0	100	0
<u>M. varians</u>	90	95	3.87	100	8.67	100	8.67
<u>St. aureus</u>	33	50	8.5	88	47.6	96	63
<u>E. coli</u>	69	70	0.43	83	7.3	100	26.8
<u>S. typhimurium</u>	58	63	2.33	80	11.48	95	19.3
<u>Str. faecalis</u>	50	58	3.36	65	7.83	96	28.17

TABLE 3. GROWING OF BACTERIA IN DIFFERENT MEDIA OF TESTED CULTIVATION

Average values of the readings expressed in transmittance percentage carried out in turbidimeter at 525 nm wavelength.

Strains	Media			
	PPC	GSTB	PSB	PPS
<u>V. parahaemolyticus</u> K ⁻	30	18	42	35
<u>V. parahaemolyticus</u> K ⁺	50	19	32	37
<u>V. parahaemolyticus</u> K ⁺	47	21	30	33
<u>V. alginolyticus</u>	24	15	43	25
<u>V. cholerae</u>	85	37	44	50
<u>Pr. mirabilis</u>	100	11	31	35
<u>Ps. fluorescens</u>	100	100	100	100
<u>M. varians</u>	100	30	45	51
<u>St. aureus</u>	98	95	35	97
<u>E. coli</u>	80	12	40	55
<u>S. typhimurium</u>	81	12	40	40
<u>Str. faecalis</u>	70	100	48	40

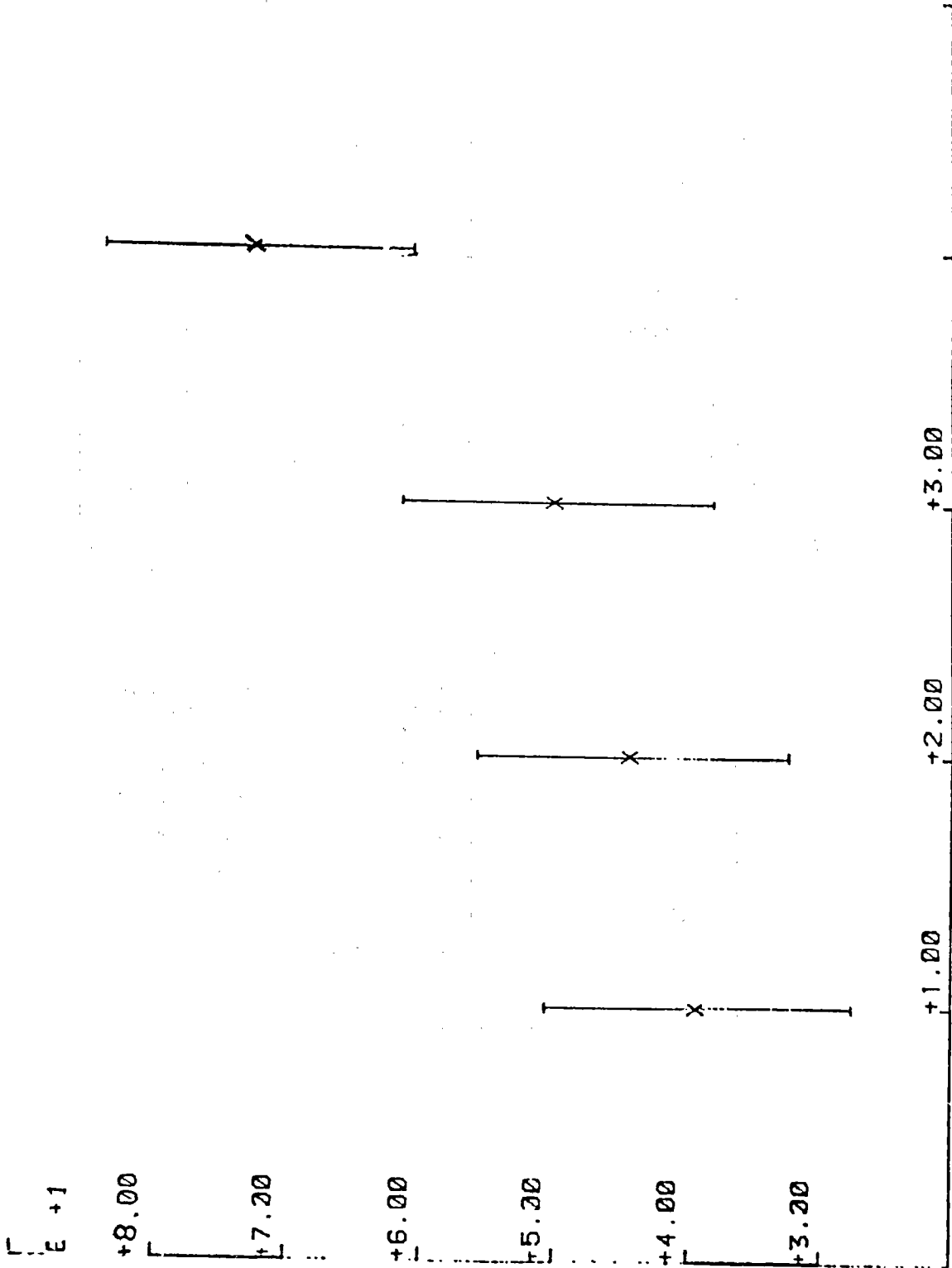
TABLE 4. TABLE OF GRADES

Strains	Media			
	PPC	GSTB	PSB	PPS
<u>V. parahaemolyticus</u> K ⁻	2	1	4	3
<u>V. parahaemolyticus</u> K ⁺	4	1	2	3
<u>V. parahaemolyticus</u> K ⁺	4	1	2	3
<u>V. alginolyticus</u>	2	1	4	3
<u>V. cholerae</u>	4	1	2	3
<u>Pr. mirabilis</u>	4	1	2	3
<u>Ps. fluorescens</u>	2.5	2.5	2.5	2.5
<u>M. varians</u>	4	1	2	3
<u>St. aureus</u>	4	2	1	3
<u>E. coli</u>	4	1	2	3
<u>S. typhimurium</u>	4	1	2.5	2.5
<u>Str. faecalis</u>	3	4	2	1

TABLE 5. COMPARISON BETWEEN MEDIA BASED ON THE DIFFERENCE BETWEEN THE ATTAINED AVERAGE TRANSMITTANCE VALUES

GSTB - PPC	Significant (P 0.00022)
PSB - PPC	Significant (P 0.00128)
PPS - PPC	Significant (P 0.0083)
GSTB - PSB	Not significant
GSTB - PPS	Not significant
PSB - PPS	Not significant

GRAPH 1. AVERAGE VALUES OF THE RESPONSES ATTAINED FROM THE FOUR TYPES OF MEDIUM WITH RELATIVE CONFIDENCE INTERVALS



REFERENCES

1. Zones côtières à usage récréatif et parcs à coquillages - Surveillance et qualité (Med VII), OMS ICP/RCE 206 (9), Copenhagen, 1979
2. Fujino, T. et al. (1953) On the bacteriological examination of Shirasu food poisoning, Med. J. Osaka Univ., 4, 229
3. Chatterjee, B. D., Neogy, K. N. & Gorbach, S. L. (1970) Study of Vibrio parahaemolyticus from cases of diarrhoea in Calcutta, Indian J. Med. Res., 58, 234
4. Velirimovic, B. (1972) The geographical distribution of the human disease due to V. parahaemolyticus in South-East Asia and Pacific, Zentbl. Bakt. Hyg., 227, 91
5. Smith, M. R. & Haga, K. (1971) Preliminary findings on the detection and occurrence of V. parahaemolyticus among the US military in the Far East, Bacteriol. Proc., 80
6. Battey, Y. M. (1970) Gastroenteritis in Australia caused by a marine vibrio, Med. J. Aust., 1, 480
7. Ward, B. O. (1968) Isolations of organisms related to V. parahaemolyticus from American estuarine sediments, Appl. Microbiol., 16, 543
8. Thompson, W. K. & Thacker, C. L. (1974) V. parahaemolyticus in Canadian shellfish. International Symposium on V. parahaemolyticus, Tokyo, 105
9. Barrow, G. J. & Miller, D. C. (1972) Vibrio parahaemolyticus: a potential pathogen from marine sources in Britain, Lancet, i, 485
10. Giammanco, G. et al. (1973) Isolamento di vibroni alofili riferibili a Vibrio parahaemolyticus e Vibrio alginolyticus da pesci e da campioni di acqua del Mediterraneo, Boll. Ist. Sieroter. Milano, 52, 1
11. Gianelli, F. et al. (1970) Isolamento di batteri correlati a Vibrio parahaemolyticus delle acque del mare Adriatico, Igiene Moderna, 68, 264
12. Liston, J. & Baross, J. (1973) Distribution of Vibrio parahaemolyticus in the natural environment, J. Milk Food Techn., 36, 113
13. De Felip, G. et al. (1974) Messa a punto di un terreno di arricchimento per il Vibrio parahaemolyticus ed il Vibrio alginolyticus, Annali Sclavo, 16, 641-654
14. Miles, A. A. & Misra, F. F. (1938) A direct count method, J. Hygiene Cambridge, 38, 732
15. Siegel, S. (1956) Non parametric statistics, McGraw Hill

