

A survey on the occurrence of *Vibrio parahaemolyticus* on fish and shellfish, marketed in The Netherlands

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SUMMARY

A survey was carried out on the occurrence of *Vibrio parahaemolyticus* on fish and shellfish, as sold in The Netherlands.

The optimal mode of detection of this bacterium appeared to be: (i) enrichment of swabs taken from the surface and the gills in freshly prepared meat broth with 5% NaCl; (ii) streaking onto Teepol bromothymol blue agar (BTB) and taurocholate bromothymol blue sucrose agar (TCBS); (iii) confirmation of suspect colonies by testing for mode of growth in butts/slants of a Kligler type glucose sucrose iron thiosulphate agar, formation of indole in 2% NaCl 2% trypticase water, anaerobic utilization of starch in the presence of 5% NaCl and oxidase reaction according to Kovacs (1956).

A total of 407 samples, stemming from 17 types of fish and shellfish, taken at three fish shops, was examined by this technique. Only one specimen, i.e. a haddock, was found to contain *V. parahaemolyticus*. This contamination rate of approximately 0.3% correlates well with data found earlier for fish landed in Northern Germany.

INTRODUCTION

Epidemiological investigations carried out in Japan have demonstrated that the halotrophic *Vibrio* species *V. parahaemolyticus* can cause outbreaks of infectious enteritis when absorbed with food in great numbers (Sakazaki, Iwanami & Fukumi, 1963; Zen Yoji *et al.* 1965; Sakazaki *et al.* 1968). Such vibrios have their main habitat in estuarine waters, fish and shellfish. Outside Japan, these bacteria have been encountered on fish and shellfish landed in the United States (Ward, 1968; Krantz, Colwell & Lovelace, 1969) and in Northern Germany (Nakanishi, Leistner, Hechelmann & Baumgart, 1968).

This evidence prompted a search for *V. parahaemolyticus* in fish and shellfish marketed in The Netherlands. Before starting the survey proper, systematic attempts were made to develop a method of detection of these vibrios in such products, which would combine the convenience required for survey work with reasonable sensitivity.

DEVELOPMENT OF A METHOD OF DETECTION

As *V. parahaemolyticus* is a non-psychrotrophic mesophilic organism (Temmyo, 1966), it may be assumed that on fresh, chilled fish and shellfish only relatively low numbers of vibrios will occur. This requires that a search for these bacteria in fish, etc. is made by a suitable enrichment technique, followed by subculturing for isolation on an appropriate solid medium.

The value of various combinations of media for this purpose was assessed.

*Methods**Strains*

Eight pure cultures of *V. parahaemolyticus* were used. These had been made available by Dr R. Sakazaki, National Institute of Health, Tokyo, Japan.

In addition pure cultures of various other food bacteria, stemming from the Institutes' collections, were used.

Enrichment media

The following media were used: (i) freshly prepared meat broth with 5% NaCl; (ii) the Japanese selective enrichment medium, containing 2% NaCl and 60 µg./ml. colistin methane sulphonate (Sakazaki *et al.* 1963); (iii) the same as (ii), but with 10 µg./ml. of the allied antibiotic polymyxin B sulphate instead of colistin, since we had found earlier that this adequately suppresses many Gram-negative rod-shaped bacteria (Mossel, 1959); (iv) the same as (iii), but with, in addition, 5 µg./ml. tylosin added, to suppress particularly bacilli and catalase positive cocci (Greenberg & Silliker, 1962*a, b*); (v) the enrichment medium, currently used for the detection of *V. cholerae* in stools, containing peptone, 5 g.; NaCl, 5 g.; glucose, 5 g.; teepol (Shell Netherlands), 1 ml.; methyl violet, 2 mg.; water, 1 l.; pH = 8.5 (Sakazaki, 1965). In order to attain maximal inhibition of the *Pseudomonas-Acinetobacter* association that predominates on chilled fish and shellfish, enrichment cultures were tentatively also made under anaerobic conditions, attained by covering the broth surface with sterile beef tallow.

Isolation media

In the initial work the two original Japanese isolation media were used. These are: (i) BTB-agar (Akiyama *et al.* 1963), containing meat extract, 3 g.; peptone, 10 g.; sucrose, 15 g.; NaCl, 20 g.; teepol (Shell Netherlands), 1 ml.; bromothymol blue, 80 mg.; agar, 15 g.; water, 1 l.; final pH = 7.8; (ii) TCBS-agar (Kobayashi, Enomoto, Sakazaki & Kuwabara, 1963), of the following composition: yeast extract powder, 5 g.; meat extract paste, 5 g.; peptone, 10 g.; NaCl, 10 g.; Na₂S₂O₃ · 5H₂O, 10 g.; dried ox bile, 5 g.; sodium taurocholate, 3 g.; sodium citrate, 10 g.; ferric citrate, 1 g.; sucrose, 15 g.; bromothymol blue, 40 mg.; thymol blue, 40 mg.; agar, 15 g.; water 1 l.; final pH = 9.2.

In tentative studies with mixtures of pure cultures of *V. parahaemolyticus* and some other types of bacteria of common occurrence on fish the selectivity of these

media was found rather disappointing. Hence we attempted to modify them so as to inhibit particularly Gram-positive organisms, *Pseudomonadaceae* and some *Enterobacteriaceae*. This was tried by the addition to BTB-agar of 5% NaCl, 5 µg./ml. tylosin and 2 µg./ml. polymyxin B sulphate as inhibitors. After such an agar, called PTSS (Polymyxin Tylosin Salt Sucrose), had been found sufficiently selective, its productivity was tested, using the eight pure cultures of *V. parahaemolyticus*, referred to earlier. Although this appeared to be rather low, viz. 10^{-3} – 10^{-6} of colonies growing on blood agar developing on this agar medium, we yet decided to assess its value in comparison with the other two media.

Identification procedures

The cardinal taxonomic properties of *V. parahaemolyticus* are the following: catalase+, oxidase+, glucose attacked by a fermentative pathway, sucrose–, H₂S from cysteine and thiosulphate–, indole+, nitrate+, urea–, gelatin+, arabinose+, growth in the presence of 10% Na–Cl (Sakazaki *et al.* 1968). In addition Baross & Liston (1968) observed that *V. parahaemolyticus* utilizes starch under anaerobic conditions in the presence of 5% NaCl. We have investigated whether this criterion could be used as a rapid screening test for the confirmation of suspect colonies obtained on BTB-and/or TCBS-agar.

Various fresh isolates of the genera *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Aeromonas*, *Klebsiella* and *Staphylococcus* were stabbed to the bottom of tubes, containing a 10 cm. butt of freshly steamed agar of the following composition: trypticase, 10 g.; yeast extract, 3 g.; NaCl, 50 g.; soluble starch, 10 g.; bromothymol blue, 80 mg.; agar, 15 g.; water 1 l.; pH = 7.2. After 24–40 hr. incubation at 37°C. growth and change of the colour of the indicator to yellow in the lowest part of the tube were checked. We had established earlier that the latter phenomenon indicates very reliably whether an isolate is capable of anaerobic dissimilation of carbohydrates (Mossel & Martin, 1961). The results of these tests were that, although some of the tested organisms, e.g. staphylococci and some *Enterobacteriaceae*, would tolerate the NaCl concentration used, none of these would attack starch; whereas the generally amylase positive aeromonadaceae (Schubert, 1967) would not grow in the presence of 5% NaCl. Attempts to inhibit the former bacteria by increasing the NaCl content to the required level of 8% failed, because at this reduced a_w -value the anaerobic attack on starch by *V. parahaemolyticus* became less consistent.

In the survey therefore a freshly steamed deep tube of 5% NaCl starch agar was used in the identification, together with a Kligler type sucrose glucose thiosulphate iron agar in butt/slant form, a tube of 2% trypticase 2% NaCl broth for testing indole formation and the oxidase reaction by the method of Kovacs (1956).

Results

Enrichment media

Amongst the five enrichment media described under Methods, the alkaline peptone teepol methyl violet medium lacked sufficient selectivity. The three other

Table 1. A comparison of the results with various techniques for the detection of *V. parahemolyticus* added to fish swabs

Strains of <i>V. parahemolyticus</i> used	Approx. numbers of cells initially present per 10 ml. enrichment fluid	Enrichment in polymyxin 2% NaCl meat broth														
		Aerobic incubation 20 hr. at 37°C.					Anaerobic incubation 20 hr. at 37°C.									
		BTB	TCBS	PTSS	BTB	TCBS	PTSS	BTB	TCBS	PTSS	BTB	TCBS	PTSS			
<i>V</i> ₁	ca. 400 40	++†	+	+	+	+	++	+	+	+	++	++	++	++	++	++
<i>V</i> ₂	ca. 660 66	++	+	+	+	+	++	+	+	+	++	++	++	++	++	++
<i>V</i> ₄	ca. 360 36	++	+	+	+	+	+	+	+	+	++	++	++	++	++	++
<i>V</i> ₅	ca. 480 48	++	+	+	+	+	+	+	+	+	++	++	++	++	++	++
<i>V</i> ₆	ca. 300 30	++	+	+	+	+	+	+	+	+	++	++	++	++	++	++
<i>V</i> ₈	ca. 310 31	++	+	+	+	+	+	+	+	+	++	++	++	++	++	++
Number of missed isolations in the case of lowest inoculum		0	2	3	2	1	5	0	0	0	0	0	0	0	0	0
Growth index†		26	19	12	19	15	4	28	29	18	27	28	18	18	18	18
Control: non-inoculated enrichment fluid with fish swabs		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

† Growth scale used for colonies of *V. parahemolyticus*.
 + Scarce growth (< 30 col.).
 ++ Growth (30-300 col.).
 +++ Luxuriant growth (> 300 col.).
 - No growth

BTB Bromothymol blue teepol sucrose salt agar (pH 7.8).
 TCBS Taurocholate citrate bromothymol blue sucrose salt iron agar (pH 9.2).
 PTSS Polymyxin tylosin sucrose salt agar.

selective media used for enrichment gave excellent results with pure cultures of *V. parahaemolyticus*, but when these same strains were added to fish swabs at about 100 organisms per swab all these three media showed a lower recovery rate than the antibiotic-free 5% NaCl meat broth, which was therefore chosen for use in the survey.

Isolation media

The relative values of the three solid media for isolating *V. parahaemolyticus* from two different enrichment media were determined by the following procedure.

Tubes of the enrichment media were inoculated with two different concentrations of the *Vibrio* suspensions giving about 400 and 40 organisms per 10 ml. of medium respectively. Swabs which had been infected with normal fish flora by rubbing them against fresh Dutch sole were added to these inoculated tubes, which were thereupon incubated aerobically and anaerobically for 20 hr. at 37°C. Each tube was then subcultured on plates of the three different isolation media, which were incubated for 24–48 hr. at 37°C.

The results shown in Table 1, particularly the numbers of missed isolations shown in line 13 of the table, demonstrate clearly that PTSS-agar is too inhibitory for this purpose. Although there were no missed positives when PTSS-agar was used in conjunction with 5% NaCl meat broth, the number of colonies on the plates was always low, with a 'growth index' of 18 against 27–29 for the other two media. It should be stressed, however, that this does not detract from the value of the PTSS medium for other purposes, such as replication of master plates of food dilutions for tentative grouping of bacteria encountered in foods (Mossel, 1969).

Anaerobic incubation of polymyxin broth gave a low recovery compared with aerobic, but with 5% NaCl broth the two methods were equally good.

Sensitivity of the method finally adopted

As a result of these preliminary tests the best method for the isolation of *V. parahaemolyticus* from fish appeared to be by enrichment in 5% NaCl meat broth for 20 hr. at 37°C. followed by plating on BTB-agar or TCBS-agar, or both, with incubation for 20 hr. at 37°C.

To assess the sensitivity of this method, inocula of about 100 cells of each of the *Vibrio* strains studied were added to the enrichment medium, simultaneously with 10^4 – 10^5 viable cells of a mixed inoculum of *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Arizona* sp., *Escherichia coli* and *Enterobacter aerogenes*. By the use of the selected procedure the *Vibrio* strains could invariably be isolated. Under more severe conditions, i.e. in the presence of large numbers of the *Pseudomonas*–*Acinetobacter* association of fish, the procedure appeared to be less productive: when artificially inoculated with 1–10 cells of *V. parahaemolyticus* these organisms were successfully recovered from 13 out of 26 swab cultures; when inoculated with 10 – 10^2 cells 40 out of 48 of such cultures were positive.

THE SURVEY PROPER

Principles

Any *V. parahaemolyticus* present on fish may be expected to occur on the surface, because these bacteria stem primarily from estuarine waters. Therefore it seemed justified to sample fish by a non-destructive surface technique. Plugs of cotton wool, previously found to preserve *V. parahaemolyticus* adequately (Mossel, Kampelmacher & van Noorle Jansen, 1968), were used for this purpose.

The entire body surfaces of the fish and shellfish were streaked intensively. In fish particularly the gills were included in this swabbing. The swabs were immediately transferred to tubes of 5% NaCl meat broth. Depending on the size of the fish, two to three swabs were used per object.

In this survey a check on the functioning of the procedure was built in. For this purpose two swabs, artificially inoculated with 10–100 *V. parahaemolyticus*, were tested in parallel with the fish samples. *V. parahaemolyticus* was recovered from those controls in all instances, except one, which was carried out along with a series of tests done on an extremely warm day, with an ambient temperature over 30°C.

Material, results and discussion

In the period June–September 1968 and 1969 a total of 407 samples were examined. The samples originated from the North Sea and were taken in three different fish shops in the City of Utrecht. The types of fish and shellfish examined in this survey and their numbers are summarized in Table 2.

From one sample only, viz. a haddock, *V. parahaemolyticus* was isolated. The

Table 2. *Review of types of fish and shellfish samples examined for V. parahaemolyticus*

Type	Number of samples examined
Bass	9
Cod	62
Codling	2
Garfish	6
Green cod	3
Gurnard	20
Haddock	49
Herring	2
Mackerel	28
Mullet	15
Plaice	42
Sole	42
Turbot	19
Whiting	24
Lobster	26
Shrimp	33
Cuttle fish	25
Total	407

contamination rate was therefore 0.25%. Nakanishi, Leistner & Baumgart (1967) and Nakanishi *et al.* (1968) had earlier found no positives in fish landed from the North Sea, and 3–31%, dependent on the month in which sampling was carried out, in fish landed from the Baltic Sea. Our results lie between these limits.

It appears from this survey that *V. parahaemolyticus* is not frequently encountered on fisheries products as sold in The Netherlands. Most of the commodities studied in this survey are stored in chilled, unsalted form. In addition, they are always eaten after cooking or frying. These two circumstances respectively, do not favour the growth of *V. parahaemolyticus* and will eliminate any organism of this type that might develop. Together with the low degree of contamination found in fish and shellfish, these circumstances make it rather unlikely that *V. parahaemolyticus* plays an important role in gastro-enteritis in The Netherlands.

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