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Occurrence of *Vibrio parahaemolyticus* in Dutch Mussels

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A study was carried out on the occurrence of *Vibrio parahaemolyticus* in Dutch mussels originating from the East Schelde Estuary. In a total of 79 10-g tissue samples, 3 (3.8%) were found to contain *V. parahaemolyticus*. In a second survey, 6 out of 23 bags of mussels (26%) contained one or more strains of *V. parahaemolyticus* in 5-g tissue samples. The many limitations of current methodology used in such surveys are stressed. Positive samples can be missed because viable cells may die during refrigerated transport. Surviving cells also may not be detected because they have been sublethally stressed. In addition, the unreliability of the identification criterion of no growth in 10% NaCl was demonstrated.

In the last 10 years some data have been published on the occurrence of *Vibrio parahaemolyticus* in North Sea water and in fish, mussels, and oysters from Dutch waters. Surveys by Nakanishi et al. (19) revealed that *V. parahaemolyticus* could not be isolated from 170 samples of fresh fish from the North Sea. In 1969 Kampelmacher et al. (13) isolated *V. parahaemolyticus* for the first time from fish from the North Sea. They isolated the species from one haddock amongst 407 samples of fish and shellfish. In a second survey Kampelmacher et al. (14) examined 288 samples of mussels, 80 samples of oysters, and 64 water samples of 200 ml each, all drawn from the East Schelde Estuary (The Netherlands). A total of 2.4% of the mussels, 0% of the oysters, and 4.7% of the water samples were found to contain *V. parahaemolyticus*. Barrow and Miller (3) isolated 18 strains of *V. parahaemolyticus* from market fish and shellfish in Britain. In 1972 Golten and Scheffers (10) collected 50-ml water samples at 67 sites along the Dutch coast, including the East Schelde Estuary; none of these was found to contain *V. parahaemolyticus*. In the North Sea region of the British coastal waters, Ayres and Barrow (1) examined samples of water, sediment, and shellfish for the distribution of *V. parahaemolyticus* during a 2-year period, from January 1975 to December 1976. They found 2.4% of the water samples, 10.2% of the sediments, and 10.7% of the shellfish positive for *V. parahaemolyticus*.

This report is concerned with the isolation of *V. parahaemolyticus* from mussels from the East Schelde Estuary. Hence, we present some more recent information concerning the incidence of this species in the region of the North Sea.

MATERIALS AND METHODS

Preliminary investigations. Since no fully reliable enrichment medium for *V. parahaemolyticus* was available, the medium Kampelmacher and co-workers (13, 14) used in their surveys of the East Schelde Estuary was compared with four other enrichment media in an attempt to find a better one. These media were: (i) freshly prepared meat broth with 5% NaCl, prepared according to Kampelmacher and co-workers (13, 14); (ii) modified Kristensen medium (16) (0.3 g of meat extract, 1.0 g of peptone, 7.0 g of NaCl, 0.3 g of Tensaryl 72 BS, 100 ml of distilled water, pH 8.6, with 0.2 g of soluble starch added after sterilization); (iii) same as (ii), but with 0.3 g of Tensaryl 80 B in place of Tensaryl 72 BS; (iv) buffered alkaline peptone-water, containing glucose as carbohydrate, 7.5% NaCl for inhibition of bacteria with relatively low salt tolerance, and sodium lauryl sulfate as an inhibitory agent (18) (1.0 g of peptone, 0.5 g of glucose, 0.66 g of $Na_2HPO_4 \cdot 2H_2O$, 7.5 g of NaCl, 0.01 g of sodium lauryl sulfate, 100 ml of distilled water, pH 8.6); (v) same as (iv), but glucose was replaced by soluble starch (final pH, 8.6).

Five pure cultures of *V. parahaemolyticus* were used, which had been made available by The Netherlands National Institute of Public Health. The five enrichment media were each inoculated with a loopful of a pure culture of *V. parahaemolyticus* grown in tryptone soya broth (Oxoid) for 18 h at 37°C. Enrichment was done in duplicate, one series being incubated for 24 h at 37°C and one being incubated for 24 h at 42°C (2). After enrichment, subcultures were made by streaking loopfuls onto thiosulfate-citrate-bile-salt-sucrose agar plates (Oxoid TCBS cholera medium) and incubating these for 18 to 24 h at 37°C.

Surveys. (i) First survey. In the period March 1974 to December 1975, a total of 79 samples of mussels were taken from the East Schelde Estuary. After landing, the samples were transported by train in a cooling box with melting ice. Bacteriological examination took place the day after each sampling. The shell of each mussel was scratched with a knife to

remove the epifauna and mud and subsequently disinfected with a cotton wool swab impregnated with 70% ethanol. After the shells had been opened aseptically, the pooled mussel tissue of each sample was homogenized in a sterile Waring blender by applying a total of 20,000 revolutions. For enrichment and isolation, the procedures recommended by Kampelmacher and co-workers (13, 14) were followed strictly. Enrichment was carried out by incubating 10 ml of mussel tissue in 100 ml of 5% salt meat broth at 37°C. After 18 to 24 h of incubation, isolation of *V. parahaemolyticus* was attempted by streaking a loopful onto TCBS agar (Oxoid) and incubating it for 18 to 24 h at 37°C. Typical green colonies surrounded by green halos were examined for identity with *V. parahaemolyticus* by carrying out the tests recorded in Table 1 (8). All the media used for identification contained 2% NaCl unless otherwise indicated.

(ii) Second survey. From September 1977 to March 1978, 23 2-kg bags of mussels originating from the East Schelde Estuary were bought in retail stores in Utrecht. Before bacteriological examination, each bag was subdivided into 10 parts. All 10 subsamples were examined for the presence of *V. parahaemolyticus*. The same procedure as in the first survey of cleaning, disinfecting, and shucking the mussels was then followed. Before homogenization, an equivalent weight volume of tryptone soya broth (Oxoid) containing 1% NaCl was added to each pooled subsample of mussels in a Waring blender. Enrichment was carried out directly and also after a resuscitation step wherein 10 ml of homogenate was transferred and incubated in 100 ml of tryptone soya broth containing 1% NaCl at room temperature for 2 h. Enrichment media used in this second survey were: (i) 5% NaCl meat broth (13, 14) for all samples and (ii) Hörie arabinose–ethyl violet broth (11) for samples examined from 14 December 1977 until the end of survey. For this purpose, 1 ml of the homogenate (0.5 g of mussel tissue) or 1 ml of the resuscitated mixture (0.5 g of mussel tissue) of each subsample was transferred into 100 ml of the enrichment medium. After 18 to 24 h of enrichment at 37°C, these cultures were subcultured by streaking loopfuls onto TCBS plates. After overnight incubation, isolation and identification of *V. parahaemolyticus* was attempted as described for the first survey.

TABLE 1. *Properties assumed to be of determinative value in the assessment of the identity of presumed isolates of V. parahaemolyticus*

Property	+ or −
Gram stain	−
Oxidase	+
Motility	+
Catalase	+
Indole	+
Glucose	+[a]
Sucrose	−
Lactose	−
Anaerobic attack on starch in presence of 5% NaCl	+
Citrate assimilation	+
Methyl red	+
Acetoin (Voges-Proskauer)	−
Lysine decarboxylase	+
Ornithine decarboxylase	+
Arginine dihydrolase	−
Growth in 1% tryptone–water with: 0% NaCl	−
8% NaCl	+
10% NaCl	D[b]
Growth at 42°C	+
Susceptibility to pteridine derivative O 129	+

[a] Fermentative, without gas.
[b] D, Dependent on mode of assessment (see below).

RESULTS AND DISCUSSION

Preliminary investigation. Enrichment in 5% NaCl meat broth (13, 14) incubated at 37°C gave the best results, all five strains being recovered on TCBS agar. When incubated at 42°C only three strains were recovered. The other four enrichment media gave less satisfactory results at both temperatures. This prompted us to select 5% NaCl meat broth as the enrichment medium for the surveys.

Surveys. (i) First survey. The results of the first survey are listed in Table 2. A strain of *V. parahaemolyticus* was isolated from only 3 (3.8%) of the 10-g samples of mussels. This correlates well with the figure of 2.4% of Kampelmacher et al. (14) for Dutch mussels in 1971, following the same isolation procedures. Isolation was successful in February, October, and December 1975. This is quite striking, since *V. parahaemolyticus* is a thermotrophic organism (12), whereas in these months the water temperature is relatively low. The average water temperatures in the East Schelde Estuary were 4.4°C in February 1975, 13.4°C in October, and 6.0°C in December. Kaneko and Colwell (15) established an annual cycle of *V. parahaemolyticus* in the Rhode River subestuary of Chesapeake Bay. *V. parahaemolyticus* survives in the sediment during the winter and is released into the

TABLE 2. *Survey 1: detection of V. parahaemolyticus in 10-g samples of mussel tissue in 1974–1975*

Sampling date	No. of samples		Mean water temp (°C) during month of sampling
	Total	Positive	
3/21/74	5	0	4.5
3/26/74	5	0	4.5
4/25/74	4	0	10.0
6/26/74	6	0	16.4
7/1/74	7	0	17.0
7/8/74	7	0	17.0
1/13/75	9	0	5.9
2/13/75	6	1	4.4
10/7/75	8	1	13.4
10/28/75	7	0	11.0
11/19/75	8	0	9.3
12/15/75	7	1	6.0

water column when the water temperature rises to between 14 and 19°C. After release, *V. parahaemolyticus* can associate with plankton, which is consumed by mussels. At this stage the bacteria can be detected in the mussels.

The fact that no *V. parahaemolyticus* could be isolated in the warm summer months, when the water temperature in the East Schelde Estuary rises to 20°C, might have been an artifact, viz., a finding due to *V. parahaemolyticus* not surviving the cold shock which would occur during transport to the laboratory, when samples are kept in a cooling box at low temperatures (0 to 6°C) for 18 to 24 h. This effect is likely to occur in summer, but very much less so under Dutch winter conditions. Experiments to support this theory showed a dramatic decrease in numbers of colony-forming units of *V. parahaemolyticus* obtained on selective media when exposed 1 or 2 days in mussel broth to 2°C (25). The same explanation for the falsely low recovery of *V. parahaemolyticus* in small samples shipped on ice for even short periods of time was subsequently given independently by Clark (7). These observations indicate that any surviving cells of *V. parahaemolyticus* are likely to be sublethally injured. Improved recovery, especially when initial numbers are low, is therefore likely if a resuscitation treatment (17) is applied before exposing the cells to inhibitory agents in enrichment or isolation media.

(ii) Second survey. In the second survey, resuscitation before enrichment was carried out in tryptone soya broth (Oxoid) containing 1% NaCl. As a comparison, the enrichment media were also inoculated directly. Besides the salt meat broth used in survey 1, the arabinose–ethyl violet broth of Horie et al. (11) was also used. This was because Beuchat (5, 6) found the latter broth most suitable for detecting uninjured, heat-stressed, chilled, and frozen *V. parahaemolyticus*.

Resuscitation before enrichment did indeed lead to a considerably higher estimate of the incidence of *V. parahaemolyticus* than that obtained by using enrichment only (Table 3). Of 23 2-kg bags of mussels, 6 (26%) were positive in one or more 0.5-g subsamples. In total, 3 subsamples were positive without a resuscitation step, whereas 11 subsamples were positive for *V. par-*

TABLE 3. *Survey 2: detection of V. parahaemolyticus in mussels in 1977 and 1978*[a]

Sampling date	No. of subsamples positive					Serovar[c]
	Total	Detection method[b]				
		K	R + K	H	R + H	
1977: 9/19/77	0	0	0	ND[d]	ND	
10/4/77	0	0	0	ND	ND	
10/11/77	0	0	0	ND	ND	
10/18/77	1	1	0	ND	ND	O3 K37
10/25/77	0	0	0	ND	ND	
11/8/77	0	0	0	ND	ND	
11/15/77	0	0	0	ND	ND	
11/23/77	0	0	0	ND	ND	
11/30/77	1	0	1	ND	ND	Not typable
12/7/77	0	0	0	ND	ND	
12/14/77	0	0	0	0	0	
12/20/77	0	0	0	0	0	
12/28/77	0	0	0	0	0	
1978: 2/7/78	1	0	0	0	1	Not typable
2/15/78	0	0	0	0	0	
2/21/78	0	0	0	0	0	
2/28/78	2	0	0	0	2	O4 K8
3/7/78	1	0	0	0	1	O4 K8
3/14/78	0	0	0	0	0	
3/21/78	0	0	0	0	0	
3/29/78	0	0	0	0	0	
4/4/78	4	1	2	1	4	O4 K8
4/18/78	0	0	0	0	0	

[a] At each date, one 2-kg bag of mussels was examined (10 0.5-g mussel tissue subsamples from each bag).
[b] K, 5% NaCl meat broth of Kampelmacher and co-workers (13, 14); H, arabinose–ethyl violet broth of Horie et al. (11); R, resuscitation before enrichment.
[c] All strains Kanagawa negative on Wagatsuma agar.
[d] ND, Not done.

ahaemolyticus with resuscitation before enrichment.

In 130 0.5-g subsamples of mussels examined by using the two enrichment media, 12 contained one or more viable cells of *V. parahaemolyticus*. These were found in 3 subsamples when 5% NaCl meat broth was used and in 9 subsamples when Horie broth was used. One 0.5-g subsample on 4 April 1978 was positive for *V. parahaemolyticus* by all four methods of detection. Another was positive, after resuscitation, in both enrichment media. In all other subsamples from which *V. parahaemolyticus* was isolated, only one procedure of detection was successful.

Identification. All suspect strains in the second survey examined for identity with *V. parahaemolyticus* by carrying out all tests recorded in Table 1 were sent for confirmation, serotyping, and the Kanagawa test to a reference laboratory of the Public Health Laboratory Service in England. The results are also recorded in Table 3.

During the survey it was often a problem to pick suspect green colonies on the primary isolation medium (TCBS agar) out of the mass of colonies of sucrose-positive bacteria, often identified as *V. alginolyticus*. Attempts were therefore made to suppress the growth of *V. alginolyticus* but not that of *V. parahaemolyticus* by the addition of various antimicrobial agents to enrichment media or TCBS agar. For this purpose pure cultures of strains of *V. parahaemolyticus* and *V. alginolyticus* were suspended in tubes containing peptone-saline. From these suspensions 0.1 ml was streaked on to TCBS agar plates containing, respectively, 0, 2, 3, 4, and 5 mg of oxytetracycline or chlortetracycline per 100 ml added at 47°C to the tempered TCBS agar.

Numbers of cells of *V. parahaemolyticus* or *V. alginolyticus* recovered after overnight incubation at 37°C on TCBS agar did not vary greatly. The colony sizes decreased with increasing concentrations of antimicrobial agents. There was no evidence that *V. alginolyticus* was suppressed more than *V. parahaemolyticus*.

In the enrichment media of Horie, the vibriostatic agent pteridine phosphate was applied in concentrations of 0, 0.1, 0.2, and 0.3 g/100 ml in an attempt to suppress growth of *V. alginolyticus* in favor of *V. parahaemolyticus*. These attempts were also fruitless. At 0.3 g of pteridine phosphate per 100 ml in Horie broth, most strains of *V. parahaemolyticus* and *V. alginolyticus* inoculated in relatively low numbers of viable cells (<10/ml) failed to grow.

The classical criterion in the identification of *V. parahaemolyticus* is that no growth shall occur in media containing 10% NaCl. This seems disputable. Thompson et al. (24) had to reject most of their typical sucrose-negative isolates because these grew in such media. Sakazaki (21) found 0.6% of his strains capable of growing in tryptone broth with 10% NaCl. Sakai et al. (20) tested 100 strains of *V. parahaemolyticus*; 51 of these were tolerant to 10% NaCl. One of the criteria Sutton (23) used in identifying *V. parahaemolyticus* was slight growth in 10% NaCl.

To settle this point, 25 strains of *V. parahaemolyticus* were tested for this criterion. Pure cultures were inoculated in tryptone-water and tryptone soya broth (Oxoid), both containing 10% (wt/vol) NaCl and incubated at 30 and 37°C. In tryptone-water, 16 strains of *V. parahaemolyticus* grew within 3 days of incubation at 30°C, and 7 grew within 3 days when incubated at 37°C. In tryptone soya broth, 8 strains grew within 2 days of incubation at 30°C, 9 grew within 2 days at 37°C, 11 other strains grew within 3 days of incubation at 30°C, and 2 others grew within 3 days at 37°C. Hence, NaCl tolerance seems to depend on various factors, such as incubation temperature, duration of incubation, and nutrient composition of the medium.

Health significance of results. The occurrence of the established low levels of *V. parahaemolyticus* in Dutch mussels does not seem to pose a pressing public health problem. In Holland, mussels are eaten cooked, fried, or marinated. Under these conditions of processing, *V. parahaemolyticus* is very unlikely to survive (4, 9).

Recontamination of mussels after processing followed by storing at lukewarm temperature can clearly always lead to considerable populations of *V. parahaemolyticus*. Sanyal and Sen (22) demonstrated that in human volunteers 10^5 to 10^7 colony-forming units of *V. parahaemolyticus* are required to trigger gastroenteritis. *V. parahaemolyticus* is a thermotrophic species that does not grow at all at temperatures below the range 9.5 to 10.5°C (12) and still grows very slowly at slightly higher temperatures. In a natural environment the minimum temperature for growth for *V. parahaemolyticus* is 10°C (15). Only under special conditions (alkaline pH, 3% NaCl) does it seem possible that growth occurs at a temperature of 5°C (4).

Therefore, the risk of this bacterium causing food-borne enteritis in The Netherlands, with its temperate climate and where, in addition, the species occurs only sporadically, seems rather remote. Nevertheless, good food preparation practices are still required to avoid occasional failures to control *V. parahaemolyticus* and other thermotrophic enteric pathogens.

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