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REVIEW

Food Microbiology Update

Emerging Foodborne Pathogens

R. G. BRYANT

Microbial Diseases Laboratory Section, Laboratory Services Branch, State of California Dept. of Health Services, 2151 Berkeley Way, Berkeley, CA 94704

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Abstract

A review of three "emerging" foodborne pathogen groups is presented, including *Campylobacter jejuni/coli, Yersinia enterocolitica,* and foodborne *Vibrio* sp.

Index Entries: Foodborne pathogens; foodborne *vibrio* infections; cholera; *Campylobacter jejuni; Campylobacter coli;* campylobacter in foods; *Yersinia enterocolitica;* foodborne yersinosis; cholera toxin.

Introduction

The phrase, "emerging foodborne pathogens," may be thought of as encompassing new disease agents that have just sprung onto the scene as well as more familiar organisms that may have recently emerged. This is certainly the case with the organisms I will discuss today. The *Campylobacter* and *Yersinia* organisms have been known under various names for some time, and yet their importance in foodborne disease has only been recently recognized. Foodborne cholera and other vibrio infections are not actually new to the continental United States, but may possibly be revisiting after a long absence.

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Foodborne *Campylobacter* Enteritis

Since the early 1970s when investigators first began to isolate Campylobacter routinely from human stool specimens, it has become apparent that the *Campylobacter jejuni/coli* organisms, previously known as the *C. fetus* ss *jejuni* group, are very important agents of bacterial enteritis. Campylobacter enteritis is characterized by abdominal pain, diarrhea, blood or mucus in the stool, and fever after an incubation period of from 2 to 10 d. Vomiting and dehydration have not been reported as common. The disease may last for 10 d to 2 wk and is usually self-limiting. Severe illness has been reported and death has occurred in certain compromised patients. Treatment with erythromycin is effective. The finding that food may be a source of infection has caused investigators to be more aware of these organisms, and as a result, some important outbreaks have been uncovered.

One of the first recorded isolations of Campylobacter related to human disease may have been foodborne, since the 1946 outbreak investigated by Levy was tentatively linked to the consumption of raw milk (*1*).

In 1977, a *Campylobacter* sp. was isolated from one of five people ill with enteritis out of 29 that attended a wedding breakfast in England (*2*). Serological evidence of infection was demonstrated in three other patients. Raw chicken that had contaminated food preparation surfaces was strongly suspected as the source.

In May of 1978, up to 2000 people were affected by gastroenteritis in Bennington, Vermont (*3*). A strong association with drinking water from the town supply was noted. Campylobacter organisms were cultured from five of nine patients.

In other outbreaks, the source of the Campylobacter organisms has varied from infected dogs to poorly cooked chicken (*4–6*). Many recent reports of Campylobacter enteritis have been noted from the USA, Europe, and Canada that are associated with the consumption of raw milk (*7–9*). Investigators now believe that raw milk will prove to be a major factor in the control of foodborne Campylobacter infections. In addition to the wild and domestic animal reservoirs of infection, houseflies have recently been shown to be vectors in the transmission of these organisms from animals to humans or foods (*10*).

Several microbiological surveys have been done to determine the incidence of Campylobacter in market foods. In England, the reported incidence in market poultry varies from a high of 92% in turkeys, to lower values near 25% for chilled processed chicken. In the United States, the reported incidence in chicken varies from a high of around 68% to a low of about 20% (*11*). These findings are, of course, very dependent on the microbiological methods used in each study. The findings are not surprising, however, when one realizes that the contamination rate for poultry is virtually 100% at the processing plant level. The poultry processing methods commonly used are not effective in producing a Campylobacter-free product.

Other food products also appear to be a source of Campylobacter. Turnbull and Rose in England analyzed the results from 31 laboratories that tested 4933 red meat samples from retail outlets during 1979 for the presence of Campylobacter (*12*). Of these samples, 3576 were also tested for Salmonella. The isolation rates for both

Salmonella and Campylobacter were low—about 1% were positive for Campylobacter and 2.5% were positive for Salmonella. Stern reported in 1981 on the recovery rate of Campylobacter from eviscerated pork, lamb, and beef carcasses (*13*). Campylobacter was isolated from 2% of beef, 24% of sheep, and 38% of swine carcasses. Syedhem and coworkers in Sweden reported isolation of Campylobacter from all of nine samples of minced beef and pork (*14*). Studies were done on the survival of the Campylobacter organisms in the samples under various conditions. The bacteria survived for 1 wk at 4°C and for 3 months at −20°C. None of the strains isolated were able to survive heating at 60°C for longer than 15 min. This suggests that proper heat processing and cooking of these foods would be sufficient for the destruction of Campylobacter. Very little, if any, work has been done to determine the incidence of Campylobacter in heat-processed foods. This may be caused by the lack of highly sensitive methods for the recovery of injured Campylobacter organisms.

The infective dose of Campylobacter ingested in food is suspected to be low, especially in debilitated individuals. A recent report in the *British Medical Journal* by D. A. Robinson confirms this for contaminated milk (*15*). The author ingested 180 mL of pasteurized milk containing 500 *Campylobacter jejuni* organisms. In 2 d, his stool specimens began to show large numbers of the same serotype of *Campylobacter jejuni*. He developed abdominal pain and diarrhea on the fourth day that lasted 3 d. Complement-fixing antibody, which was not present before the experiment, was detected at a titer of 1:16 after 10 d.

Methods

Reports in the literature are just now appearing that describe sensitive as well as selective procedures for the isolation of Campylobacter species from foods. In order to prepare for the laboratory investigation of foodborne Campylobacter outbreaks, the California State Department of Health Services laboratories have developed methods to isolate Campylobacter organisms from solid and semisolid foods and are currently working on recommended methods for fluid milk. Work with seeded food samples at the Microbial Diseases Laboratory of the California State Department of Health Services has resulted in a procedure that can be used to investigate possible foodborne outbreaks. The method is based on the selective filtration of centrifuged food suspensions, similar to the filtration techniques originally used to examine feces specimens (*16*). The relatively large pore size of the filter (0.65–0.8 μM) removes suspended food particles and most bacteria larger than 0.2–0.4 μM in diameter. The Campylobacter organisms are small enough to pass through the filter and be plated out with the filtrate onto selective media for isolation. The food suspensions are usually made with the aid of a stomacher or simply shaken in a flask containing glass beads. The centrifuging step is performed at low speed so as to throw down only the larger food particles. Centrifuge speeds that result in about 1000g of relative centrifugal force are used for a short period not to exceed 5 min. A portion of this light suspension can then be plated directly onto Campylobacter Selective Medium. The remaining portion can then be passed

through an 0.8 μ*M* filter with the use of a syringe and a swinnex filter holder. The filtered portion can then be plated out. The plating media used can be any one of several, including those formulated by Skirrow (*17*), Blaser (*18*), and Butzler (*19*). If a selective agar is not available, the filtrate can still be plated on a nonselective medium such as lysed sheep blood agar. This is practical because the filtration will often provide adequate selectivity. The plates are incubated in a reduced oxygen atmosphere at 42°C and examined at the end of 24, 48, and 72 h. A gas-replacement chamber is recommended for incubation that provides an atmosphere of 6% oxygen, 7–10% carbon dioxide, and the balance nitrogen. Campylobacter colonies growing up on the plates will give different colony morphologies depending on the type of plate used. On Skirrow's medium, the colonies will be flat, slightly spreading, and have a pinkish tinge. The reverse of the colonies will be red on this medium. Colonies may be screened by the oxidase test, using the Kovac's method. This will eliminate Proteus types. A wet mount for motility will often show the typical darting motion characteristic of the species. After examination of a gram-stained smear for typical "S" shaped or slightly curved, tiny gram-negative rods, a presumptive report can be issued of "*Campylobacter* sp."

In order to detect lower numbers of Campylobacter organisms, it is necessary to enrich food samples in a selective or nonselective liquid medium prior to plating on Campylobacter selective media. Various types of broth media have been used, usually with the addition of one or more antibiotics. Some examples include:

1. Brucella broth with vancomycin (15 μg/mL), trimethoprim (10 μ/mL), and cephalothin (10 μg/mL). Ferrous sulfate (0.2%), sodium metabisulfite (0.025%), and sodium pyruvate (0.05%) is also added.
2. Semisolid motility test medium (BBL) with 7% lysed horse blood and three antibiotics: Vancomycin (10 μg/mL), polymyxin B (5 IU mL) and trimethoprim (5 μg/mL).
3. Thioglycollate broth with vancomycin (10 μg/mL), trimethoprim (5 μg/mL), polymyxin B (2.5 IU/mL), and Cephalothin (1.0 μg/mL).

Strains of Campylobacter vary considerably in their minimal nutritional requirements. Since the actual composition of the various base media varies from lot-to-lot, many investigators have experienced difficulty in demonstrating consistent results with enrichment media formulations. The most promising results, however, will probably come from work with such selective enrichment broths. A recent paper in the *Journal of Applied and Environmental Microbiology* describes a method that employs Brucella Broth supplemented with lysed horse blood, sodium succinate, and cysteine (*20*). The medium is made selective by the addition of vancomycin, trimethoprim, polymyxin B, and cycloheximide. Foods are enriched for only 16–18 h in a shaker waterbath. The method claims detection levels down to one cell in 10 g of food.

Methods to recover injured Campylobacter organisms in foods have not yet been fully developed. The use of a nonselective enrichment broth for various incubation times up to 24 h may be one basic method. Alkaline peptone water has been used by Tanner and Bullin as an enrichment broth for Campylobacter (*21*).

Confirmation of Campylobacter and Identification of Species

Pickings from isolation plates can be made to brucella broth for further biochemical and growth tests. The Campylobacter organisms do not utilize carbohydrates as a source of energy and are relatively inert in routine biochemical tests. A series of characteristics can be demonstrated, however, that will allow for identification of isolates. The typical reactions are shown in Table 1. Note that all the species shown are oxidase- and catalase-positive, and reduced nitrate. *Campylobacter jejuni* and *C. coli* grow in the presence of 1% glycine, but not in 1.5 or 3.5% sodium chloride. Hydrogen sulfide detectable by a lead acetate strip is produced. A few recently developed tests have been proposed to separate *Campylobacter jejuni* and *C. coli*. These include: the ability to grow at 30.5°C, sensitivity to 4% triphenyltetrazolium chloride, and sodium hippurate hydrolysis. These tests are currently being evaluated by a number of labs. So far, mixed results have been obtained. The most promising test appears to be the hippurate hydrolysis. Some difficulty in reading the simple tube tests that detect the glycine end product, has been experienced. Newer tests that detect the benzoic acid end product of the hippurate hydrolysis may work out better. *Campylobacter jejuni* is positive and *C. coli* strains are negative.

Raw Milk and Campylobacter

Since California is one of the states that still allows the sale of raw milk, food microbiologists must recognize the potential role of this product as a source of Campylobacter infection. This will depend, of course, on the sensitivity of the methods used to detect Campylobacter organisms in raw milk.

One such method has been proposed by Chong E. Park and Z. K. Stankiewicz (*22*). It involves centrifugation of the milk sample at high speed in a refrigerated centrifuge, suspension of the pellet in brucella broth prior to streaking on Skirrow's plating medium, and enrichment in supplemented brucella broth incubated under constant flow of a special gas mixture. Samples containing larger numbers of Campylobacter will produce typical colonies on the direct plates. The lower numbers of organisms will come through the enrichment procedure. After 2–3 d of incubation at 42°C, 5 mL of the enrichment culture is passed through an 0.65 μM membrane filter, and the filtrate is plated in Skirrow's medium. The plates are incubated at 42°C in a static gas mixture. The authors claim sensitivity of two Campylobacter cells per 10 mL of milk containing 100–10,000 contaminants.

The Microbial Diseases Laboratory has done some work with the hope of simplifying the methods that have appeared in the literature. So far, work with antibiotic supplemented brucella broth has allowed the isolation of 90 Campylobacter cells/L of milk containing 25 million contaminants/mL. Although some simplification has been achieved, much more work needs to be done to produce a workable method that could be performed in any Public Health Laboratory. If such a method were available, Public Health would have a powerful tool to investigate milk-borne Campylobacter outbreaks.

TABLE 1
Typical Reactions of Campylobacter

	C. coli	*C. jejuni*	*C. fetus* ss *fetus*
Oxidase	+	+	+
Catalase	+	+	+
Nitrate	+	+	+
H_2S (TSI)	−	−	−
H_2S (Strip)	+(−)	+(−)	+(−)
Growth: 1% glycine	+	+	+
Growth: 1.5% NaCl	−	−	?
Growth: 3.5% NaCl	−	−	−
Growth: 25C	−	−	+
Growth: 30.5C	+	−	+
Growth: 35C	++	+	+
Growth: 42C	+++	+++	−
Naladixic acid	S[a]	S	R
TTC	R	S	S
Na hippurate	−	+	−

[a]The NARTC (naladixic acid resistant thermophilic Campylobacter) of Smibert are sensitive to TTc, grow in 1.5% NaCl, and are hippurate negative.

Foodborne Yersiniosis

The eighth edition of Bergey's Manual of Determinative Bacteriology classifies the gram-negative rod-shaped bacterium *Yersinia enterocolitica* as a member of the family *Enterobacteriaceae*. This organism is now recognized as an important cause of human disease. The major clinical symptoms of *Yersinia enterocolitica* infections include: acute gastroenteritis, mesenteric lymphadenitis, and terminal ileitis (*23*). In many cases, a syndrome that mimics appendicitis is found (*24*). Other forms of the disease include septicemia, urinary tract infections, arthritis, and meningitis. The incubation period for foodborne outbreaks has not been determined with high accuracy, but most likely is in the range of 2–5 d. The foods most commonly suspected are raw or lightly-cooked protein foods of animal origin. A heat-stable enterotoxin may be produced by some strains that is similar to the heat stable enterotoxin of enterotoxigenic *E. coli* strains (*25*).

Production of enterotoxin does not appear to be the main factor for pathogenicity, however. A plasmid-mediated virulence factor has been found that finds expression as V and W antigens. These antigens are nearly identical to the V and W antigens of other pathogenic Yersinia such as *Y. pestis* and *Y. pseudotuberculosis* (*26–28*). The V and W antigens are produced at 35–36°C and in the presence of 2.5 mmol of Ca^{2+} ion. A simple screening test for virulence has been developed wherein virulent cells autoagglutinate in tissue culture medium when incubated at 36°C (*29*). Some virulence associated plasmids can be detected

by the mouse lethality test and some workers have begun to develop direct assays for the plasmids as screening tests for clinical isolates (*30*). Another test for virulence is provided by the inhibition of such strains by magnesium oxylate medium (*28*). Recently, 100 strains of *Y. enterocolitica* were examined at CDC (*31*). These strains had been submitted from 21 states from 1970 through 1980. Several virulence tests were used in the study: two strains caused conjunctivitis in guinea pigs, seven were lethal for mice, 54 invaded HEP2 cells, 18 produced heat-stable enterotoxin, nine were Calcium dependent, 20 autoagglutinated, and 34 had distinctive colonies at 37°C. All of this shows the marked differences between strains, and that no one virulence test is satisfactory for all strains. Unfortunately, the expression of virulence may be lost when these organisms are repeatedly subcultured in the laboratory. For this reason, many workers prefer to separate virulent from avirulent strains by biotyping schema. In addition to biotyping, serotyping is done for epidemiological studies. The serotypes that have been most often associated with human disease are 0:3 and 0:9 in Europe; 0:3 in Japan and Canada; and 0:5 and 0:8 in the United States. Recently, the number of reported 0:3 isolates has been increasing, at least in California and New York (*32*).

A wide variety of environments may harbor *Y. enterocolitica*. The ogranisms have been isolated from the feces of many animals, and swine may be an important reservoir of infection for man. Food and water may also serve as a source of infection. Raw or inadequately cooked foods, especially meats, eggs, and milk, are most suspect. The organism can grow at refrigerator temperatures, but is inactivated by cooking, as is the case with other enteric pathogens. Growth can occur in milk at 22 or 4°C, but preformed heat-stable enterotoxin has not been found in milk cultures (*33*). The actual incidence of *Y. enterocolitica* in foods is not of most concern to food microbiologists. If one examines enough samples of selected foods, especially if enrichment methods are used, these organisms will be found. Actual surveys of raw milk, raw or rare meats, fresh vegetables, raw shellfish, and nonchlorinated drinking water have been quite successful in isolating *Y. enterocolitica*. The real problem involves the much less frequent occurrence of the truly virulent strains in foods that may cause outbreaks of intestinal disease. One of the greatest difficulties with this work involves the detection of virulent strains in the presence of a multitude of nonpathogenic environmental strains.

Well-documented cases of foodborne Yersiniosis have been fairly few and far between. The foodborne route of infection is suspected in many instances, but never proven because of the failure to isolate the organism in the suspected foods. A well-publicized exception to the rule occurred in 1976 and affected the school students in an upper New York state community (*34*). Over 150 persons became ill, including 36 children who were admitted to various hospitals in the area. Sixteen of these children had appendectomies performed. Chocolate milk served at the school cafeterias was found to be contaminated with serotype 0:8 *Y. enterocolitica*, the same type that was isolated from the ill students. An investigation revealed the product was prepared by a small dairy that added chocolate syrup to pasteurized milk using a manual mixing procedure.

Another large outbreak occurred at a coed summer camp in Sullivan County, New York, in July of 1981 (*35*). Out of 455 campers, 239 became ill with gastro-

enteritis. Five of seven hospitalized patients had appendectomies performed. *Y. enterocolitica*, serogroup 0:8, was isolated from 37 out of 69 persons examined and from foods served at the camp. Dissolved powdered milk and turkey chow mein were involved.

Methods

The method currently used in many laboratories to detect *Y. enterocolitica* in foods is based on the methods given in the 1976 edition of the APHA *Compendium of Methods for the Microbiological Examination of Foods*. This method depends on the cold enrichment of food samples (*36*). *Yersinia enterocolitica* grows at 4°C, while many other enteric bacteria tend to die off at this temperature. This method has been preferred by most workers in the United States because of the difficulty in recovery of the predominantly 0:8 serotypes by other types of enrichment procedures. The two selective enrichment media used are Selenite F broth and magnesium chloride–malachite green–carbenacillin medium. Not all strains of *Y. enterocolitica* will grow well on any one of the combinations given in the method.

When the incubation of each set of plates is complete, they are examined for the presence of typical Yersinia-like colonies. In the case of MacConkey and Salmonella-Shigella agars, the Yersinia will show up as small, colorless, nonlactose fermenting colonies. Cellobiose arginine lysine agar gives a deep pink colony because of the fermentation of cellobiose, without decarboxylation of arginine or lysine. Yersinia pectin agar is a differential agar only and has no inhibitory substances. It has the ability to detect pectinase producing bacteria by their formation of depressions in the agar as the medium is digested. Other organisms, such as *Klebsiella oxytoca, Aeromonas hydrophila*, and some vibrios will also show pectinolytic activity. Cefsulodin-Irgasan-Novobiocin agar (CIN) gives colonies with sharp edges and deep red centers. There is also a clear zone surrounding the colonies on this medium (*37*). A recent report in the *Journal of Clinical Microbiology*, showed that the CIN medium gave the best recovery of both mixed and pure cultures of all of the media tested (*38*).

Various other enrichment type procedures have been used that involve different enrichment broth media. Examples of these broths include: sorbitol bile broth, hemoglobin-oxylate-bile-sorbitol broth, veal infusion broth, and brain heart infusion broth. The methods given in the current *Bacteriological Analytical Manual* of the Federal Food and Drug Administration, makes use of sorbitol bile broth for animal products and hemoglobin oxylate-bile-sorbitol broth for vegetable products (*39*). These broth cultures are streaked out directly on MacConkey agar initially, and after 10, 14, and 18 d of cold enrichment.

A recent modification aimed at increased selectivity for isolation of *Y. enterocolitica* has been described by Aulisio and coworkers (*40*). The method makes use of a potassium hydroxide treatment for liquid enriched food samples. Just prior to streaking out the enrichment culture, a loopful (0.04 mL) of culture is mixed with 0.1 mL of a 0.5% KOH solution (made in 0.5% NaCl) for a few seconds. A loopful of the mixture is then streaked onto plating media. The original

authors used MacConkey plates for isolation. This method is claimed to increase the yield of Yersinia isolations fourfold over the conventional cold enrichment technique. The incubation time of the enrichment culture can be reduced in many cases with this method, as reported by the authors. Enrichment incubation for 2 d at 26°C was found to be sufficient for low levels of *Y. enterocolitica* and 7–10 d at 4°C was recommended for high levels of non-Yersinia contamination. A similar method, using phosphate-buffered saline enrichment for 1–2 d at 25°C has been developed by Doyle et al. for meat samples (*41*). This method also involves preplating treatment with KOH.

Recent papers have dealt with the injury of Yersinia cells when subjected to freezing, sublethal radiation, and heating. The use of nonselective enrichment broths at both 4 and 26°C may be most productive for Yersinia isolation in these cases.

Confirmation and Identification of Yersinia Species

Once the suspicious colonies have been selected, at least two and preferably five, of each type are picked to TSI agar, LIA, and motility-indole-ornithine medium. These media are incubated at 25°C. Pickings that give typical reactions—that is, an acid slant and butt, without gas or H2S on the TSI, acid in the butt of the LIA tube, positive motility, and evidence of ornithine decarboxylase—are worked up further. Conventional biochemical tests can be used or test kits such as the API or Pathotec.

Table 2 shows the pertinent biochemical characteristics used in the identification of *Y. enterocolitica* and *Y. pseudotuberculosis*. *Y. enterocolitica* shows a difference in its motility at 35°C and 25°C, being positive at 25°C, but not at 35°C. The same is true of the VP reaction. The reactions on lysine, arginine, and ornithine show a positive only for ornithine. About 50% of the *Y. enterocolitica* strains are indole-positive. Looking at the sugar reactions, we should note that lactose fermentation may be late. In comparing the reactions of the two species, we can see that *Y. enterocolitica* is sucrose and cellobiose negative, VP negative, and is negative on the lysine, arginine, and ornithine media. Several biotypes are possible within the species designation of *Y. enterocolitica*, as shown in Table 3. This is the Wauter's Biotype Schema. The typical strains are types 1 through 4, which are all sucrose, trehalose, ornithine, and β-galactosidase positive. Type 5 is rather inactive biochemically. The type 5 strains appear to be pathogenic in animals only. Most of the pathogenic serotype 0:3 isolates are indole negative, and belong to biotype 2. Biotype 1 organisms are generally considered environmental isolates.

Three groups of organisms that previously had been included in the *Y. enterocolitica* designation are now split off as separate proposed species. DNA homology studies support this separation. Table 4 shows the biochemical differentiation. The three species are *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia*. Besides the differences shown in the table, these strains can also be separated by their bacteriophage pattern using a battery of 24 phages as reported by Baker and Farmer (*42*). *Y. frederiksenii* and *Y. intermedia* have not been associated with in-

TABLE 2
Biochemical Tests for Yersinia

Test	Results[a]	
	Y. enterocolitica	*Y. pseudotuberculosis*
TSI	A/A (K/A)	A/A (K/A)
Oxidase	Negative	Negative
Christensen's urea	Positive	Positive
Glucose	A (gas var.)	A (no gas)
Lactose	Negative (late A)	Negative
Sucrose	A	Negative
Cellobiose	A	Negative
Melibiose	Negative (late A)	A
Raffinose	Negative (late A	Negative
Rhamnose	Negative (late A)	A
Motility, 25C	Positive	Positive
35C	Negative	Negative
VP, 25C	Positive	Negative
35C	Negative	Negative
Lysine decarboxylase	Negative	Negative
Arginine dihydrolase	Negative	Negative
Ornithine decarboxylase	Positive	Negative
Indole	Variable (~50% positive)	Negative
ONPG	Positive	Positive

[a]A = acid; K = alkaline.

TABLE 3
Wauter's Biotype Schema

Tests	Biotypes				
	1	2	3	4	5
Lipase	+	−	−	−	−
Indole[a]	+	+	−	−	−
Lactose (OF)	+	+	+	−	−
Xylose, 48 h	+	+	+	−	−
Sucrose	+	+	+	+	V
Voges-Proskauer	+	+	V	+	+
Nitrate	+	+	+	+	−
Trehalose	+	+	+	+	−
Ornithine decarboxylase	+	+	+	+	−
β-galactosidase	+	+	+	+	−

[a]Incubated at 29°C; all other tests incubated at 25°C.

TABLE 4
Differentiation of Yersinia Species

Species	Tests				
	Sucrose	L-Rhamnose	Raffinose	Melibiose	VP
Y. enterocolitica	+ (V)	−	−	−	+ (V)
Y. kristensenii	−	−	−	−	−
Y. frederiksenii	+	+	−	−	+
Y. intermedia	+	+	+	+	+

testinal disease, being isolated mainly from wound and skin infections. *Y. kristensenii* is an environmental type and has not been implicated in human disease.

Further testing on isolates that prove to be one of the *Y. enterocolitica* biotypes can be done at a reference laboratory. The isolates can be screened for potential virulence by the autoagglutination procedure. If necessary, isolates can also be tested in one of the assays for enterotoxigenicity, usually at a reference center.

Foodborne Vibrio Infections

The Vibrio organisms are gram-negative, nonsporing rods that may or may not exhibit a single rigid curve. All are motile by means of a single polar flagellum, and all are oxidase positive. Two Vibrio species have been universally recognized as important human pathogens that can infect man through contaminated food and water. They are *Vibrio cholerae* and *Vibrio parahaemolyticus*. Of course, the pathogenicity of these organisms has been known for quite some time; however, recent food outbreaks and other developments have generated a new wave of interest in these Vibrios that might qualify them as "emerging" pathogens.

Vibrio cholerae

Vibrio cholerae, serogroup 01, is the well-known cause of epidemic cholera and is currently divided into two biotypes—the classical and the El Tor. Each biotype is further divided serologically into the Ogawa, Inaba, and Hikojima serotypes. These organisms produce heat-labile enterotoxins. Typically, the disease produced by the classical 01 organisms results in a sudden onset of vomiting, without nausea or retching, and painless diarrhea after an incubation period of 1–5 d. The average incubation time is 2 d in most common source outbreaks. There is an enormous loss of fluids—up to one-half the victim's body weight in 24 h. Renal function is suppressed, leg cramps are common, with extreme fatigue and weakness progressing for the next 12 h or so. If untreated, many victims die within 24 h after

onset. Properly treated and managed, this disease is seldom fatal. The El Tor biotypes appear to give a lower morbidity and mortality rate. The infective dose is about 1000 organisms, but is greatly affected by the buffering effect of food in the stomach (*43*).

Organisms that appear to be very similar, both morphologically and biochemically, to *Vibrio cholerae*, but do not agglutinate in the group 01 antiserum are known as non-01 vibrios or nonagglutinable vibrios. These organisms are found in environmental waters and commonly contaminate marine shellfish. Sporadic outbreaks of mild to severe diarrhea have been attributed to these organisms. The most severe of these outbreaks approach the classical cholera outbreaks in symptomology. Unlike the classical strains, non-01 vibrios commonly cause extra-intestinal infections. The non-01 organisms may or may not produce cholera-like enterotoxins.

Recently, one of the more typical non-01 organisms has been shown by DNA relatedness and other studies to be a separate species, named *Vibrio mimicus*. These studies are sucrose-negative, appear to be pathogenic, and produce either a heat-labile or heat-stable enterotoxin (*44*). Infection with both *V. cholerae* 01 and the non-01 Vibrios can very often lead to the asymptomatic or carrier state, although this appears to be most common with the non-01 organisms.

Epidemic cholera had not been reported in the United States since 1911, when in 1973, a single case was reported from Port Lavaca, Texas. This case may have been linked to shellfish from the Gulf Coast waters (*45*). In 1978, 11 cases of cholera inolved the consumption of contaminated crabs (*46*). All of these cases implicated a toxigenic *V. cholerae* 01, biotype El Tor, serotype Inaba. Since 1978, there have been few serogroup 01 cases associated with seafood, but several cases of non-01 *Vibrio cholerae* infections—mostly associated with oysters from polluted shellfishing grounds.

There have been several surveys of US shellfishing areas and coastal waters for *V. cholerae* 01 and non-01 organisms. One survey of the East coast and Gulf coast areas examined 791 freshly harvested oysters collected during a year-long period starting in early June of 1979 (*47*). Some 111 samples (14%) were positive for *V. cholerae*, including both serogroup 01 and non-01 organisms. A higher percentage of positives were seen during the warmer months of the year. None of these isolates were found to produce cholera enterotoxin by the microslide gel diffusion or the Y-1 mouse adrenal cell tissue culture assay. When tested for pathogenicity using the infant rabbit, none of the 01 isolates were pathogenic, but two of the non-01 isolates were. Other studies and surveys have yielded isolates with a much higher ratio of pathogenic to nonpathogenic strains. The work of Colwell, Kaper, and others has established that both serogroup 01 and non-01 *vibrio cholerae* organisms, including toxigenic and pathogenic strains, are resident in Chesapeake Bay and in other coastal waters and that multiplication of the organisms takes place (*48*).

It is apparent that *V. cholerae* has established an endemic foci in certain US coastal waters. The exact relationship between its incidence, the effect of pollutants, and the influence of microbiological and biological flora remains to be worked out. A recent article in the *Journal of Applied and Environmental Microbiology* describes the attachment of both 01 and non-01 *Vibrio cholerae* to living ma-

rine copepods (*49*). This finding may be an important factor in the epidemiology of foodborne cholera. Proper handling, storage, and cooking of shellfish from the various coastal fishing grounds around the USA undoubtedly prevents much of the potential foodborne disease associated with *V. cholerae*. The organism can grow in seafoods containing up to 6% salt and at temperatures between 15 and 42°C. Optimum pH is between 7.6 and 8.6, but growth can occur from pH 6.0–9.0. Workers at the Federal Food and Drug Administration have suggested an in-the-can pasteurization process for canned oysters (*50*). Preliminary results indicate that somewhere between 15 and 30 min at 57.2°C would be effective.

Vibrio parahaemolyticus and Other Vibrios

Foodborne *V. parahaemolyticus* infection has been reported since the early 1950s in Japan and may cause over 50% of all bacterial foodborne illness in that country. It is the most important of the halophilic Vibrios as far as human disease is concerned. The first confirmed outbreak in the United States occurred in 1971. The disease has been associated almost exclusively with raw or lightly cooked fish, shellfish, or compound dishes with seafood ingredients. The symptoms include: watery diarrhea, abdominal cramps, nausea, vomiting, headache, and fever. Blood and mucus may be present in the stool in severe cases. The onset time ranges from 4 to 96 h, but is usually 6–24 h. *V. parahaemolyticus* can also cause non-enteric infections in humans—usually wound infection contracted while swimming in coastal waters. Another halophilic Vibrio that has received more attention recently is *V. vulnificus* (*51, 52*). Several cases of fatal sepsis have been reported after consumption of contaminated raw oysters or contact with seawater in the Chesapeake Bay region. Most of these infections have involved individuals with underlying liver disease. *V. vulnificus* can also cause wound infections similar to those caused by *V. parahaemolyticus*. *V. parahaemolyticus* has been demonstrated to be invasive in the adult rabbit and produces endotoxins similar to those of the Enterobacteriaceae. A heat-stable direct hemolysin for human red blood cells is produced by most strains isolated from stools of patients with the infection.

As with the cholera Vibrio, *V. parahaemolyticus* is found in coastal waters of the world and tends to be more concentrated in water with higher levels of organic matter. Sewage pollution plays a part and estuarine and brackish waters are particularly suited to these organisms. As one might expect, higher levels are present during the summer months, when most outbreaks occur. The number of *V. parahaemolyticus* organisms naturally occurring in seawater and seafoods is low—usually less than 100/g (*53–56*). Growth to very high levels can occur in a few hours if seafoods are not promptly chilled, frozen, or heat processed. Chilling has been reported as more lethal than freezing. The organisms may persist in frozen shellfish for prolonged periods. Growth can occur in seafoods at temperatures ranging from 5 to 44°C although the usual limits are from 10 to 40°C. Optimum growth occurs at 35–37°C and at pH 7.5–8.6 with 2–3% salt. During optimum growth, some strains can exhibit generation times as low as 8–9 min (*57*)! All of this means that processors, food service personnel, and consumers must take spe-

cial care when handling, storing, and preparing seafoods. It appears that high numbers of *V. parahaemolyticus* are needed to cause infection in humans—normally in the millions per gram. Most of the reported outbreaks in the United States have involved inadequate cooking or recontamination after cooking of contaminated seafoods.

Methods

Methods for the isolation of the pathogenic Vibrios from foods are similar, as one might expect. In the investigation of diarrheal disease involving seafoods from a public health point of view, one must be able to detect any of the pathogenic types. There have been several isolation methods proposed and used for foods. Most methods make use of an enrichment broth incubated at 35°C followed by streaking on gelatin agar plates and thiosulfate-citrate-bile salts-sucrose agar, abbreviated as TCBS. A new medium called trypticase soy-sucrose-triphenyltetrazolium agar has recently been described (*58*). It is so new that most workers have not had a chance to evaluate it. Suspect colonies are picked to TSI agar slants and other differential media for screening before going on to a battery of biochemical tests. The enrichment broths most commonly used for *V. cholerae* are alkaline peptone water and gelatin-phosphate-salt broth. For the halophilic Vibrios, alkaline peptone water with 3% salt, glucose-salt-teepol broth, and arabinose-ethyl violet broth have been used. Chill-stressed cells of *V. parahaemolyticus* have been successfully recovered by supplementing enrichment broths with magnesium and iron salts (*59*).

The procedure that is used at the Microbial Diseases Laboratory makes use of a combination of media. These methods are based on those given in the FDA bacteriological analytical manual, Chapters IX and XXIV (*60*). Initially, 1 : 10 sample homogenates are prepared using alkaline peptone water and 3% sodium chloride dilution water. TCBS agar plates are then streaked directly to detect large numbers of Vibrio organisms commonly found in outbreak situations. The alkaline peptone water homogenate is incubated at 35°C for 6–8 h and then streaked out on TCBS and gelatin agar plates in order to detect *V. cholerae* organisms. The 3% sodium chloride homogenate is used to seed tubes of alkaline peptone water and glucose-salt-teepol broth that are incubated overnight at 35°C. These tubes are later streaked onto TCBS agar plates for detection of lower numbers of the pathogenic halophiles.

On TCBS, *V. cholerae* will produce a large smooth yellow colony that is slightly flattened. The centers are opaque. Some of the related Vibrios will produce greenish colonies. *V. parahaemolyticus* and *V. vulnificus* will give smaller green to blue–green colonies with darker centers on TCBS agar. On gelatin agar, *V. cholerae* and related Vibrios will show up as semitransparent colonies surrounded by a cloudy zone. *V. parahaemolyticus* and other halophilic Vibrios will give a similar appearance, but the colonies may be smaller. Suspicious colonies can be picked to TSI, lysine iron agar, and motility medium. *V. parahaemolyticus* will typically give an alkaline slant with an acid butt and no H_2S or gas in TSI agar. The lysine iron agar slant will have an alkaline slant and butt with no evidence of

H2S. The organism will be motile. *V. cholerae* will produce an acid slant and butt on TSI with no gas or H2S. The lysine iron agar slant will show an alkaline slant and butt. Suspect isolates can be further screened by a gram stain and the oxidase tests. Those that are pure, gram-negative, straight or slightly-curved oxidase + rods, can be inoculated into a battery of biochemical tests as shown in Table 5. Here we see the minimal reactions needed for identification of *V. cholerae*, *V. parahaemolyticus*, and *V. mimicus*. The reactions are fairly similar for the three species shown, with the reactions of lysine, arginine, inositol, gelatin, and mannitol serving to screen out other, non-Vibrio organisms. *Aeromonas hydrophila* and *Plesiomonas shigelloides* are two such organisms that might be encountered.

V. parahaemolyticus can be distinguished from the other Vibrios by the halophism test where it grows in salt concentrations of from 1 to 8%, but not at 10% or in 1% tryptone medium without salt. *V. parahaemolyticus* is also VP-

TABLE 5
Vibrio Minimal Biochemicals[a]

Test	*Vibrio cholerae*	*Vibrio mimicus*	*Vibrio parahaemolyticus*	*Vibrio vulnificus*[b]
Oxidase	+	+	+	+
H_2S (TSI slant)	−	−	−	V
VP	V (65)	−	−	−
Indole	+	+	+	+
Glucose, ferm/gas	+/−	+/−	+/−	+/−
Gelatin	+	+	+	+
Mannitol	A	A	A	A
Inositol	−	−	−	NT
Sucrose	A	−	−	−
Lysine decarboxlylase	+	+	+	+
Arginine dihydrolase	−	−	−	−
Ornithine decarboxylase	+	+	+	V (66)
Growth in 1% Tryp. 0% NaCl	+	+	NG	NG
Growth in 1% Tryp. 1% NaCl	+	+	+	NT
Growth in 1% Tryp. 6% NaCl	V (60)	V (54)	++	+
Growth in 1% Tryp. 8% NaCl	NG (8)	NG (2)	+	NG
Growth in 1% Tryp. 10% NaCl	NG	NG	NG	NG
Growth at 42°C	+	+	++	NT

[a]V = variable reaction, A = acid reaction, NG = no growth, NT = not tested, ( ) = % positive.
[b]*V. vulnificus* also ferments lactose and salicin.

negative and does not ferment sucrose. Two other halophilic Vibrios that may be isolated from seafoods are *V. alginolyticus* and *V. anguillarum*. These organisms have not been associated with human disease. *V. alginolyticus* is VP positive, grows in 10% salt broth and ferments sucrose. *V. anguillarum* is lysine decarboxylase negative, and does not grow in 8–10% salt. *V. mimicus* is sucrose negative and VP negative but otherwise similar to the *V. cholerae* strains. Some of the reactions for *V. vulnificus* are shown. The main differential characteristics are the fermentation of lactose and salicin by *V. vulnificus*—these two sugars are not fermented by the other commonly isolated halophilic Vibrios. *V. vulnificus* will not grow in 8–10% salt broths. Further work may be done at this point, involving Kanagawa testing and serotyping of *V. parahaemolyticus* isolates or biotyping of *V. cholerae* isolates. This will most likely be done at a reference laboratory—usually an FDA laboratory or CDC. Tests for production of enterotoxins may also be undertaken.

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