Research Note

Misidentification of *Vibrio cholerae* O155 Isolated from Imported Shrimp as O Serogroup O139 due to Cross-Agglutination with Commercial O139 Antisera

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ABSTRACT

Fish and shellfish products imported into Denmark are routinely analyzed for pathogenic *Vibrio* spp., particularly *V. cholerae*, if products originate from subtropical or tropical areas. A *V. cholerae* strain that agglutinated commercial O139 antiserum but not the O1, Inaba, or Ogawa antisera was isolated from imported raw frozen shrimp. The toxigenicity of the strain was analyzed, and the results of a polymerase chain reaction showed that the *V. cholerae* strain did not contain the virulence genes *ctx*, *tcpA*, and *zot*, which are normally found in *V. cholerae* O1 and O139. The strain was resistant to colistin and spectinomycin. The high susceptibility of the strain to antimicrobial agents was confirmed by the lack of an SXT element, a self-transmissible, chromosomal genetic element that is normally present in O139 strains and encodes resistance to sulfonamides, trimethoprim, and streptomycin. The strain contained two plasmids, in contrast to other O139 strains, which normally do not contain plasmids. The characteristics of the strain led to further agglutination testing with other antisera that are not commercially available, and the strain was found to agglutinate O155 antisera in repeated testing. Manufacturers of O139 antisera should be aware of the closely related O antigens of the O139, O22, and O155 serogroups and should be aware that their commercial diagnostic O139 antisera must be absorbed to remove cross-reacting agglutinins of O22 and O155 strains.

Fish and shellfish products are subjected to mandatory inspection, including microbiological analyses for bacterial pathogens like *Vibrio parahaemolyticus* and *Vibrio cholerae*, by importing countries. In particular, products originating from subtropical and tropical areas are routinely analyzed for *V. cholerae* belonging to the O1 and the O139 serogroups, the only two serotypes causing the disease cholera as defined by the World Health Organization (21). Microbiology standards for the analysis of *V. cholerae* usually include enrichment in an alkaline broth medium followed by subculturing onto thiosulfate–citrate–bile salt–sucrose agar; biochemical testing of suspected isolates; and, finally, serogrouping of *V. cholerae* isolates by using commercial O1 and O139 antisera (2). If a product contains *V. cholerae* O1 or O139, the product is withheld and either rejected or, in rare cases, processed further until the product is deemed safe for human consumption.

It is well recognized that *V. cholerae* is part of the normal bacterial flora found in aquatic environments (3, 11). However, the prevalence of O1 and O139 serogroups in such environments appears to be lower than that of other serogroups of *V. cholerae* (5, 11). With rare exceptions, all *V. cholerae* O1 and O139 strains contain a virulence gene cassette including the cholera toxin gene (*ctx*), which enables the strains to cause cholera. In contrast, so-called *V. cholerae* non-O1, non-O139 strains rarely contain such genes, including *ctx* (10). Authorities will take action and condemn products if *V. cholerae* strains isolated from these products are found to agglutinate with *V. cholerae* O1 or O139 antisera, whereas different actions seem to be taken if non-O1 and non-O139 strains are isolated.

The number of different O serogroups of *V. cholerae* continues to increase, and currently there are more than 190 recognized O serogroups (16–18). Several somatic (O) antigenic relationships among serogroups of *V. cholerae* have been described by Shimada et al. (18). Strains assigned to serogroups O22 and O155 have been shown to possess antigen factors in common with *V. cholerae* O139 (17). Thus, for practical use, any diagnostic antisera prepared against *V. cholerae* O139 must be absorbed with the reference strains representing serogroups O22 and O155 to remove cross-reacting agglutinins of the O22 and O155 strains (17).

In the present case report, we describe the isolation of a *V. cholerae* strain from imported raw frozen shrimp that agglutinated commercial O139 antisera but in subsequent analyses was found to be nontoxicogenic and to belong to the O155 serogroup.
MATERIALS AND METHODS

Sample and bacteriological analysis. Fish and shellfish products imported into Denmark are routinely analyzed for pathogenic *Vibrio* spp., particularly *V. cholerae*, if products originate from subtropical or tropical areas (4). In September 2000, a consignment of raw frozen shrimp originating from Thailand was received at the Division of Microbiology, Region Ringsted, Ministry of Food, Agriculture and Fisheries, for microbiology analyses. The sample was analyzed for pathogenic *Vibrio* spp. by a standard procedure including enrichment of 25 g of samples in 225 ml of alkaline peptone water (2% NaCl, pH 8.6 ± 0.2) followed by incubation at 37°C for 6 h and again for 18 to 24 h before subculturing of the surface pellicle onto thiosulfate–citrate–bile salt–sucrose agar (Merck, Darmstadt, Germany). Following overnight incubation at 37°C, sucrose-positive colonies (yellow colonies) were restreaked onto blood agar (Oxoid, Basingstoke, UK) to assure purity, and then characterization was carried out with the commercial API 20E assay according to the manufacturer’s instructions (bioMérieux, France). Profiles were analyzed with API 20E software (version 3.1, bioMérieux). Suspected *V. cholerae* isolates were sent to the Royal Veterinary and Agricultural University for verification by standard biochemical testing (14).

Serology. Sucrose-positive yellow colonies typical of *V. cholerae* were tested by agglutination tests employing commercial polyvalent O1, monospecific Ogawa and Inaba antisera, and so-called Bengal O139 antisera. All antisera were obtained from Denka Seiken Co., Ltd., Tokyo, Japan. Isolates were also sent to the reference laboratory at the National Institute of Health, Tokyo, Japan, for agglutination testing according to the scheme established by Shimada et al. (18).

Antimicrobial susceptibility testing and isolation of plasmid DNA. The strain’s susceptibility to 15 antimicrobial agents was tested by disk diffusion on Mueller Hinton agar as recommended by the manufacturer (Oxoid) and according to the guidelines of the National Committee for Clinical Laboratory Standards (12). The strain was incubated aerobically in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) for 18 to 24 h at 37°C. After the transfer of 1.5 ml of brain heart infusion broth culture into an Eppendorf tube, plasmid preparation was carried out by the method of Kado and Liu (9), modified by incubation of the cells at an elevated pH (12.75) for 30 min at 65°C during the lysis step. Electrophoresis and visualization of plasmids were essentially carried out as previously described (13).

PCR analysis for the detection of virulence-associated genes and the SXT element. Two of the most important and commonly found virulence factors of *V. cholerae* O1 and O139 are cholera toxin, a potent enterotoxin, and the toxin-coregulated pilus antigen, an essential intestinal colonization factor (6). Furthermore, an essential gene called *zot* has also been reported to encode a biological activity producing zonula occludens toxin (1, 19). Accordingly, we analyzed the isolated *V. cholerae* strain and adequate controls for the presence of *ctxA*, *tcpA*, and *zot* by polymerase chain reaction (PCR) as previously described (7, 15).

Waldor et al. (20) identified in *V. cholerae* O139 a ~62-kb self-transmissible, chromosomally integrating genetic element, which they termed the SXT element. The SXT element was found to contain genes encoding for resistance to sulfonamides, trimethoprim, and streptomycin (20). We attempted to determine whether our strain contained the SXT element by using PCR primers int1-F (GCT GGA TAG GTT AAG GGC GG) and int1-B (CTC TAT GGG CAC TGT CCA CAT TG) (F = forward; B = backward), which produce a 592-bp internal fragment of the integrase of the SXT element (8). *V. cholerae* O139 strain MO10 was used as a positive control (20).

RESULTS AND DISCUSSION

At the Division of Microbiology, Ringsted, typical *V. cholerae*-like colonies were seen on thiosulfate–citrate–bile salt–sucrose agar as yellow, flat colonies 2 to 3 mm in diameter. Further morphological and biochemical characterization, including API 20E profile 5347124, confirmed the identification of these colonies as *V. cholerae* colonies. A bacterial specimen sent to the Royal Veterinary and Agricultural University confirmed these findings by demonstrating typical biochemical reactions of *V. cholerae*, including the production of lysine and ornithine decarboxylases but not arginine dihydrolase, no acid production from cellobiose and salicin, growth in media containing 0 and 6% NaCl, and the utilization of o-nitrophenyl-β-d-galactopyranoside. The strain was sensitive to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine phosphate).

Repeated antiserum agglutination confirmed that the isolate agglutinated O139 antiserum but not the O1, Inaba, or Ogawa antiserum. The verification that the isolate was *V. cholerae* O139 led the Division of Microbiology to withhold the consignment of the frozen shrimp. Because the isolation of *V. cholerae* O139 from commercial seafood products had, to our knowledge, not been reported previously, we wanted to test the toxigenicity of the strain. To our surprise, the results of the PCR for *ctx*, *tcpA*, and *zot* showed that the *V. cholerae* strain did not contain any of these virulence genes.

The strain was susceptible to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, neomycin, streptomycin, sulfonamides, tetracycline, trimethoprim, and the vibriostatic agent O/129, whereas resistance was shown only to colistin and spectinomycin. This resistance pattern is different from the antimicrobial susceptibility patterns normally found for O139, which, with few exceptions, is resistant to sulfonamides, trimethoprim, and streptomycin (20). The high susceptibility of the strain to antimicrobial agents was confirmed by the absence of the SXT element, as shown by PCR. In contrast to other O139 strains studied, which normally lack plasmids, repeated plasmid analysis of our O139 strain showed that it contained two plasmids of 5.2 and 3.8 kb.

The characteristics of the *V. cholerae* O139 strain, particularly the absence of virulence genes, a finding that is extremely rare for O139, led us to send a strain specimen to the National Institute of Health, Japan, for confirmation of the O139 serogroup. Repeated agglutination testing with O139 antiserum prepared against the *V. cholerae* O139 reference strain (MO45, ATCC 51394) revealed that the strain did not agglutinate O139 antiserum. Further repeated agglutination testing of the strain with other antiserum showed that the strain agglutinated O155 antisera.

In 1994, Shimada et al. (17) found that a strain isolated from a patient with diarrhea in Thailand and a *V. cholerae* O22 reference strain possessed somatic (O) antigen factors
in common with *V. cholerae* O139. Because the strain isolated in Thailand could not be placed in any of the 154 established O serogroups of *V. cholerae*, it was assigned to a new serogroup, O155 (17). O antigens of strains belonging to the O22 and O155 serogroups were shown to be closely related to that of *V. cholerae* O139 in an a, b-a, c type of relationship, but they were not completely identical to those of serogroup O139 (17).

According to the manufacturer’s (Denka Seiken) written information enclosed with the vial containing the O139 antiserum, the antiserum was prepared by hyperimmunizing rabbits with a so-called Bengal type O139 strain. Furthermore, nonspecific agglutinins were said to have been removed, although the manufacturer did not specify which agglutinins had been removed or how they were removed. Thus, our results strongly suggest that the antiserum used was not sufficiently absorbed with the O22 and O155 reference strains to remove agglutinins. It is therefore urgent that Denka Seiken and other manufacturers of O139 antiserum be aware of the closely related O antigens of the O139, O22, and O155 serogroups and that their commercial diagnostic O139 antiserum be absorbed to remove cross-reacting agglutinins of O22 and O155 strains.

In conclusion, our case study shows how the use of a nonspecific commercial antiserum caused an initial misidentification of a *V. cholerae* O155 strain as a O139 serogroup strain.

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**REFERENCES**


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