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NOTES AND SHORT COMMUNICATIONS

Isolation of Pandemic *Vibrio parahaemolyticus* from UK Water and Shellfish Produce

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Abstract *Vibrio parahaemolyticus* is a Gram-negative, halophilic bacterium found commonly in temperate and warm estuarine waters worldwide. *V. parahaemolyticus* is considered an emerging bacterial pathogen in Europe and has been responsible for several recent seafood-associated outbreaks. During ad hoc testing of raw shellfish produce in May 2012, pandemic group (O3:K6) *V. parahaemolyticus* was isolated from Pacific oysters (*Crassostrea gigas*), harvested in Southern England. Follow-on testing of water and shellfish, encompassing a small number geographically diverse sites, also retrieved pandemic group isolates. These strains are amongst the most northerly pandemic strains described to date and represent the first instance of pandemic *V. parahaemolyticus* isolated in the UK, highlighting the expanding geographical distribution of these foodborne pathogens in the environment.

Introduction

Globally, *Vibrio parahaemolyticus* is the leading cause of bacterial gastroenteritis associated with the consumption of seafood produce. Clinical characteristics of *V. parahaemolyticus* infections include abdominal cramps, diarrhoea, nausea, headaches, fever and chills [6]. The presence of *V. parahaemolyticus* in the marine environment is closely related to water temperature, with strains readily isolated when environmental temperatures exceed 15 °C [1, 8]. Until 1996, *V. parahaemolyticus* infections were typically sporadic, with few largescale foodborne outbreaks. In 1996, a sudden increase in *V. parahaemolyticus* infections emerged in Calcutta, India, with a distinctive serotype (O3:K6). This serotype, subsequently termed the 'pandemic group', rapidly disseminated around the globe and has been responsible for large foodborne outbreaks across Asia, Africa and America [13]. Recent reports suggest that the number of *V. parahaemolyticus* infections appear to be increasing in Europe [1]; however, regionally, little data exists on the role of pandemic *V. parahaemolyticus* strains as a cause of disease. A small number of recent O3:K6 strains responsible for foodborne infections have been reported in Spain [10], Italy [16, 17] and France [18]. Toxigenic *V. parahaemolyticus* is considered relatively rare in the natural environment, with typically less than 5 % of strains believed to be able to initiate disease in humans. Few studies have attempted to analyse the prevalence of toxigenic strains of *V. parahaemolyticus*, although reports in Southern Europe have indicated the presence of haemolysin genes exceeding 5 % in water and shellfish samples Serracca et al. [20]. The vast majority of pathogenic strains produce just two recognized virulence factors during pathogenesis. Of these, the thermostable direct haemolysin (TDH) [2, 14], responsible for the Kanagawa haemolysis, and the TDH-related haemolysin (TRH) [7] are considered the most predictive overall indicators of potential virulence [1] (Fig. 1).

Materials and Methods

During May–October 2012, we tested shellfish samples (Pacific oyster, *C. gigas*) from two commercial shellfish harvesting areas (sites 1 and 2), as well as 10 l marine water samples from a separate site, in the South West UK (site 3). The samples were obtained monthly from site 1, once only for site 2 (July 2012) and monthly (July–October 2012) for site 3. Shellfish samples were transported to the laboratory

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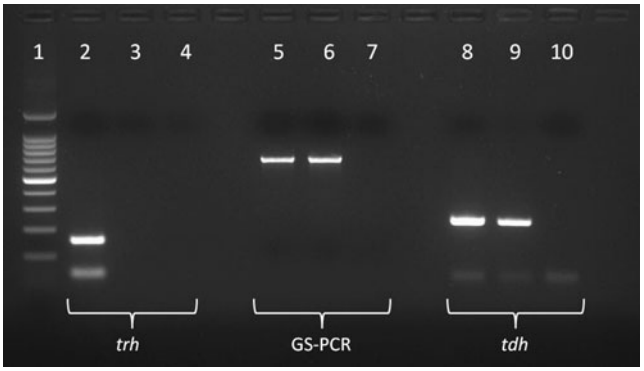

Figure 1 PCR analysis of a representative recovered *V. parahaemolyticus* strain, using assays specific for *trh*, *tdh* and group-specific pandemic PCR. *Lane 1*, 100 bp DNA ladder (Promega); *lane 2*, positive control DNA (*trh*+ isolate); *lane 3*, recovered strain; *lane 4*, negative control (water). *Lane 5*, positive control DNA (library pandemic strain); *lane 6*, recovered strain; *lane 7*, negative control (water). *Lane 8*, positive control DNA (*tdh*+ strain); *lane 9*, recovered strain; *lane 10*, negative control (water)

on ice and processed immediately upon receipt. Shellfish were washed and opened aseptically, and the meat and intravalvular fluid of ten individual animals were pooled together. From the sample, 25 g was removed and stomached (model 400 Seward stomacher; Seward, Ltd.) for 3 min. Sterile alkaline saline peptone water (ASPW) (75 ml) was added making a 1:3 dilution, and the mixture was stomached for 3 min. A further 150 ml of sterile ASPW was added and then incubated at 41.5 ±1 °C for 6 h. This enrichment broth was then subcultured onto selective media, including thiosulfate citrate bile sucrose (TCBS) agar (Oxoid Ltd.), chromID *Vibrio* agar and VID agar (Biomerieux, Marcy l'Etoile, France). Samples were incubated for 24 h at 37 °C. Following incubation, colonies were selected on the basis of distinctive morphology and colour, and further enriched on Marine Agar plates (Difco) for 24 h at 30 °C. Presumptive *Vibrio* strains were then identified to species level biochemically using API20E (Biomerieux).

As part of this short ad hoc microbiological testing, presumptive *Vibrio* species were isolated following alkaline APW broth enrichment, originally from site 1, in May 2012. Based on colony morphology on selective agar (marine agar, TCBS and VID agar) and tolerance to 8 % NaCl, *V. parahaemolyticus* was suspected. Presumptive *V. parahaemolyticus* strains were also isolated in July (site 2) and August (sites 1, 2 and 3). Microbiological analysis was subsequently performed on the isolated bacterial strains using a range of culture-based and molecular testing approaches. A preliminary identification of *V. parahaemolyticus* was made after biochemical analysis using API20E (Biomerieux, Marcy l'Etoile, France), according to manufacturers recommendations. For DNA extraction, a loopful of colonies was chosen and resuspended in 300-μl sterile distilled water and boiled for 10 min to lyse cells. Presumptive *V. parahaemolyticus* strains isolated from marine agar plates were subsequently analysed by polymerase chain reaction (PCR), using two species-specific assays (*tlh* and *toxR*) that target *V. parahaemolyticus* [2, 9, 15]. In addition, the presence of the virulence genes *tdh* and *trh* were determined by multiplex PCR according to the procedure described by Bej et al. [2]. The amplicons were analysed in a 2.0 % agarose gel. Following PCR for *toxR*, *tlh*, *tdh* and *trh*, all strains were subsequently tested using primers that target the group-specific sequence variation in the *toxRS* gene, as previously described [12]. Following boiling, the lysate was centrifuged briefly, and the supernatant containing DNA was used directly during PCR. As a further confirmatory step, strains were submitted to a separate laboratory (School of Biosciences, University of Exeter) for additional PCR testing using *toxR* and group-specific primer sets, essentially as described above.

Serological analysis of all strains was determined by agglutination using commercially available *V. parahaemolyticus* antisera (Denka Seiken Ltd., Tokyo). All isolates were grown on TSA agar with 3 % NaCl and 0.1 % Teepol. For K-type determination, a bacterial suspension was made in 3 % NaCl solution, whilst O-type determination was performed using bacterial suspension in 3 % NaCl supplemented with 5 % glycerol. Strains were boiled for 1 h and centrifuged for 5 m prior to the addition of 10 μl of polyvalent sera and 10 μl of boiled cell sample on glass slides (76×26 mm). The slide was tilted back and forth for 1 min until agglutination was observed. Reactions were performed using a range of monovalent O antigens (0–11) and K antigens (1–61) according to the manufacturer's antigenic scheme.

Table 1 Representative subset of *V. parahaemolyticus* strains isolated in Southern UK during the summer of 2012

Site of isolation	Matrix and month of isolation	Molecular and serotype characteristics
Commercial shellfish site 1	*C. gigas*, May 2012	Tdh+, Trh−, ToxR+, GS+, 03:K6
Commercial shellfish site 1	*C. gigas*, May 2012	Tdh+, Trh−, ToxR+, GS+, 03:K6
Commercial shellfish site 1	*C. gigas*, May 2012	Tdh+, Trh−, ToxR+, GS+, 03:K6
Commercial shellfish site 1	*C. gigas*, May 2012	Tdh+, Trh−, ToxR+, GS+, 03:K6
Commercial shellfish site 1	*C. gigas*, May 2012	Tdh+, Trh−, ToxR+, GS+, 03:K6
Marine site 1	Marine water, September 2012	Tdh+, Trh−, ToxR+, GS+, 03:K6
Commercial shellfish site 2	*C. gigas*, September	Tdh+, Trh−, ToxR+, GS+, 03:K6

Results and Discussion

During ad hoc sampling of shellfish and water samples, we isolated pandemic (O3:K6) *V. parahaemolyticus* from several sites in Southern England during the summer of 2012. All *V. parahaemolyticus* strains demonstrated typical molecular and biochemical characteristics associated with 'pandemic group' status strains, namely the possession of thermostable direct haemolysin (*tdh*+) coupled to the absence of the *tdh*-related haemolysin gene (*trh*). In addition, all tested strains demonstrated the serotype O3:K6, indicative of pandemic strains reported worldwide [13]. These strains represent, to our knowledge, the first report of pandemic O3:K6 *V. parahaemolyticus* isolated in the United Kingdom. These findings are significant for a number of reasons. Firstly, although other studies have demonstrated the presence of pandemic strains in marine water samples in Europe, as well as clinical cases, most instances to date have been reported in Southern Europe [10, 16–18]. With the exception of a single clinical case of pandemic *V. parahaemolyticus* in Norway in 2002, and believed to be domestically acquired [4], we believe that these bacterial strains represent the most northerly identified pandemic strains isolated directly from the environment. Secondly, these strains were recovered during a relatively mild summer in the UK in terms of sea surface temperatures. The UK mean temperature for summer was 13.9 °C, which is 0.4 °C below average. Other than 2011, the summer of 2012 was the coolest summer since 1998 [11]. For instance, for sites 1 and 2 (shellfish harvesting areas), temperatures did not exceed 20 °C during the entire summer period. Of interest, the environmental temperatures were typically less than 15 °C at site 1 when the pandemic strains were initially isolated in May 2012. Given that numerous *V. parahaemolyticus* outbreaks in Europe have been linked to anomalously warm weather episodes, the isolation of pandemic strains during a cold summer in Northern latitudes is therefore striking. Previous studies have demonstrated an inverse relationship between environmental temperature and the recovery of *tdh*+ strains [3, 19]. It will be of interest to determine if the *tdh*+ strains isolated as part of this work are adapted to survive at unusually low temperatures. Finally, the isolation of pandemic strains from a range of different geographical sites in Southern England during a relatively short sampling programme, including shellfish and water samples (May–September 2012, Table 1), is suggestive that these potentially pathogenic strains may be ubiquitous in the marine environment. Recent studies analysing the presence of *V. parahaemolyticus* in UK shellfish produce did not report the isolation of pandemic strains [21]; however, a lack of long-term and systematic surveillance data precludes us from establishing whether these strains have emerged recently or not in the UK waters.

We must stress that the relative numbers of recovered pandemic strains from raw shellfish produce were low, and these bacteria were isolated after an initial enrichment step. In addition, the shellfish produce that was selected for testing had not undergone purification—a legislative requirement for the majority of bivalve shellfish produced in Southern England—thus, the number of pandemic isolates in purified shellfish produce may be substantially lower than those likely to initiate infection based on current risk assessments [5]. Irrespective, the isolation of pandemic group strains from temperate environmental sources is striking and highlights the potential for temperature abuse of raw shellfish produce as a potential risk factor, as implicated in past outbreaks caused by pandemic strains in Europe [10]. Preliminary molecular analyses including PFGE on a subset of the strains presented here indicates that these bacteria may represent novel isolates when compared to other pandemic *V. parahaemolyticus* strains in Europe. It will be of interest to compare these strains alongside a geographically and clinically diverse group of pandemic strains, encompassing both European and non-European *V. parahaemolyticus* isolates. Current work utilising a range of typing approaches will provide further insights into the evolutionary and phylogenetic relatedness of these strains. Future work should include a quantitative surveillance analysis of the seasonal and geographical distributions of these pathogens in unpurified bivalve shellfish in the UK and potentially elsewhere in Northwest Europe.

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