Characterization of Virulence Profiles and Antimicrobial Resistance Patterns of Clinical and Environmental *Vibrio parahaemolyticus* Isolated in Canada

by

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Abstract

Vibrio parahaemolyticus is a naturally occurring marine organism with a worldwide distribution in estuaries and coastal environments. Consequently, *V. parahaemolyticus* bio-accumulates in the gut of filter-feeding molluscan shellfish (e.g. oysters) and is a common cause of seafood associated gastroenteritis. Recent data show increased rates of *V. parahaemolyticus* infection in Canada, particularly British Columbia (BCCDC, 2015; PHAC, 2014). To further improve our understanding of the types of *V. parahaemolyticus* strains that exist in the Canadian (primarily British Columbia) environment and facilitate assessment of risk to public health, virulence profiles and antimicrobial resistance (AMR) patterns were determined for 600 isolates collected between 1998 and 2011.

Clinical isolates (n=64) and environmental strains (n=536) cultured from oysters collected from Canadian growing areas were analyzed in this study. A multiplex PCR assay was used to confirm all isolates as *V. parahaemolyticus* and characterize virulence gene profiles (*tdh* and *trh*). AMR phenotypes were determined by a disc diffusion assay using a panel of 20 clinically relevant antibiotics. Further sub-typing using multi-locus sequence typing (MLST) was conducted on a subset (clinical, n=31; environmental, n=135) of isolates.

Results showed 53% of clinical and 8% of environmental isolates possess at least one of the virulence markers. Overall levels of AMR were low, with 7% of isolates exhibiting pansusceptibility and 0.5% displaying resistance to 4 antibiotics. Ampicillin resistance was widespread (91%) with resistance to cephalothin (29%), streptomycin (4.3%), sulfisoxazole (2.7%), piperacillin (2.3%), oxolinic acid (0.5%), ceftiofur (0.4%), gentamicin (0.2%), and kanamycin (0.2%) also seen. MLST analysis showed the *V.*

parahaemolyticus population structure is genetically diverse with 36 novel sequence types (STs) identified, all recovered from environmental isolates. In comparison, a smaller number of STs appear to be responsible for illnesses in British Columbia.

Generally, findings from this research provide evidence that differences in virulence, AMR, and STs exist among clinically and environmentally derived *V. parahaemolyticus*. Future investigations using more discriminatory analyses, such as whole genome sequencing, may elucidate other factors and/or predictors of *V. parahaemolyticus* virulence as well as mechanisms mediating antibiotic resistance; this may facilitate studies attempting to understand the interplay between virulence and AMR.

Preface

A component of Chapter 2, sub-section 2.2.2 (multiplex PCR testing of *V. parahaemolyticus*) is based on work primarily conducted by Canadian Food Inspection Agency Burnaby Laboratory staff. The author, Jennifer Liu, verified the results, performed testing on a small subset of isolates, and completed the analysis and interpretation of the results. Additionally, as part of Chapter 2, sub-section 2.2.3 (multi-locus sequencing typing of *V. parahaemolyticus*), Canadian Food Inspection Agency Burnaby Laboratory staff completed PCR reactions for 26 isolates. The author, Jennifer Liu, completed testing on the remaining isolates as well as conducted data analysis and interpretation for all isolates.

The images displaying geographic area within Canada seen in Figures 2.1, 2.2, and 3.5 were produced by Fisheries and Oceans Canada for the purpose of providing Canadians with access to information about the programs and services offered by the Government of Canada. Permission to reproduce the images for this thesis (i.e. non-commercial reproduction) has been granted by Fisheries and Oceans Canada.

The rest of the research in this thesis was completed solely by the author, Jennifer Liu, under the guidance of Dr. Kevin J. Allen and Dr. Swapan K. Banerjee.

This work is original and has not been previously published.

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List of Abbreviations

AMP	Ampicillin
AMR	Antimicrobial resistance
APW	Alkaline peptone water
ATCC	American Type Culture Collection
BC	British Columbia
BCCDC	British Columbia Centre for Disease Control
CC	Clonal complex
CEF	Cephalothin
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CTX	Cefotaxime
DLV	Double locus variant
ENR	Enrofloxacin
FAO	Food and Agriculture Organization of the United Nations
GEN	Gentamicin
goeBURST	Global optimal eBURST
KAN	Kanamycin
KP	Kanagawa phenomenon
MDR	Multi-drug resistant
MLST	Multi-locus sequence typing

MPN	Most probable number
NAL	Nalidixic acid
NOR	Norfloxacin
OA	Oxolinic acid
PCR	Polymerase chain reaction
PHAC	Public Health Agency of Canada
PIP	Piperacillin
PMB	Polymyxin B
PNW	Pacific Northwest
STR	Streptomycin
T	Oxytetracycline
T3SS	Type III secretion system
T6SS	Type VI secretion system
TCBS	Thiosulphate citrate bile salts sucrose
TDH	Thermostable direct hemolysin
TIO	Ceftiofur
TRH	*tdh*-related hemolysin
TSA	Tryptic soy agar
TSA-NaCl	Tryptic soy agar supplemented with 1.5% sodium chloride
TSB	Tryptic soy broth
TSB-NaCl	Tryptic soy broth supplemented with 1.5% sodium chloride
SLV	Single locus variant
ST	Sequence type
SUL	Sulfisoxazole
SXT	Trimethoprim/sulfamethoxazole

TET	Tetracycline
US	United States of America
WGS	Whole genome sequencing
WHO	World Health Organization

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Chapter 1 – Introduction

Since its discovery over 65 years ago, *Vibrio parahaemolyticus* has been implicated as the causative agent in multiple foodborne illnesses around the world and recent data show increased rates of infection in North America (Newton et al., 2012; Crim et al., 2014; BCCDC, 2015; PHAC, 2014). In Canada, the incidence rate of vibriosis increased from 0.14 per 100, 000 cases in 2009 to 0.23 per 100, 000 cases in 2014 with the majority of infections attributed to *V. parahaemolyticus* (PHAC, 2014). Similar increasing trends have been observed in British Columbia (BC) with the highest rate (1.9 per 100, 000) reported in 2015 when an outbreak of *V. parahaemolyticus* associated with the consumption of raw BC oysters occurred (BCCDC, 2015).

V. parahaemolyticus is a natural inhabitant of marine waters and consequently bio-accumulates in the gut of filter-feeding molluscan shellfish. Accordingly, human infections commonly present as acute gastroenteritis characterized by diarrhea, vomiting, and abdominal cramps through the consumption of raw, undercooked, or contaminated seafood (Butt et al., 2004; Drake et al., 2007; Nair et al., 2007). Not all strains of *V. parahaemolyticus* are considered to be truly pathogenic and two hemolysins, a thermostable direct hemolysin (TDH) and a *tdh*-related hemolysin (TRH), are well recognized virulence factors (FAO, 2011).

While antibiotics have revolutionized the treatment of infectious diseases, unfortunately, their use and misuse have resulted in the development and spread of antibiotic resistance (WHO, 2014). Antimicrobial resistance (AMR) is a growing global concern and a food safety problem. In particular, antibiotic use in food animals (for treatment, disease prevention, or growth promotion) allows for

resistant bacteria and resistance genes to spread from food animals to humans through the food chain (Aarestrup and Wegener, 1999; WHO, 2014). Furthermore, antimicrobial agents are released into marine environments from aquaculture activities as well as agricultural runoff providing opportunities for marine organisms, such as *V. parahaemolyticus*, to acquire resistance mechanisms and subsequently concentrate in filter-feeding shellfish.

Limited surveillance studies investigating AMR profiles and trends in *V. parahaemolyticus* have been conducted in North America. Furthermore, while information on the prevalence of *V. parahaemolyticus* possessing *tdh* and/or *trh* genes are more extensive, few publications on isolates originating from Canada exist. Accordingly, the present work is focussed on characterizing *V. parahaemolyticus* found in Canada (predominantly BC) with an emphasis on examining clinically and environmentally derived strains to determine virulence profiles and AMR patterns to facilitate assessment of risk to public health and contribute towards the Government of Canada's federal framework for action with regards to antimicrobial resistance and use in Canada (Huston et al., 2016).

1.1 The *Vibrio* Genus and *V. parahaemolyticus*

Ubiquitously found in the marine environment, members of the *Vibrio* genus are straight or curved Gram-negative, non-sporeforming rods that are 0.5 µm to 0.8 µm in width and 1.4 µm to 2.6 µm in length (Drake et al., 2007). However, when they are grown in the laboratory, they frequently revert to straight rod morphology (Drake et al., 2007). A single polar flagellum enables these bacteria to be highly motile in liquid media while lateral flagella allow for migration across semi-solid surfaces by swarming (Yeung and Boor, 2004; McCarter, 1999). Most species produce oxidase and catalase and ferment glucose without producing gas (Butt et al., 2004; Drake et al., 2007).

The *Vibrio* genus is comprised of over 100 species with nearly a dozen identified as pathogenic to humans including: *V*. *cholerae*, *V*. *mimicus*, *V*. *fluvialis*, *V*. *parahaemolyticus*, *V*. *alginolyticus*, *V*. *cincinnatiensis*, *V*. *vulnificus*, *V*. *furnissii*, *V*. *metshnikovii*, and *V*. *carchariae* (Drake et al., 2007; Baker-Austin et al., 2017; Lovell, 2017; Thompson et al., 2003; Smith et al., 1991). While all these bacteria have been reported to cause foodborne disease, *V*. *parahaemolyticus*, *V*. *vulnificus*, and *V*. *cholerae* are the most common and therefore considered the most significant agents (Drake et al., 2007; Newton et al., 2012; Thomas et al., 2013). Since *V. parahaemolyticus* was first discovered by Tsunesaburo Fujino in 1950 as the causative agent of a large outbreak associated with the consumption of semi-dried sardines in Japan (Shinoda, 2011), it has been recognized as a cause of gastrointestinal illness worldwide and accounts for the majority of foodborne-related vibriosis. For example, in the United States (US), the estimated annual number of episodes of foodborne acquired illnesses caused by *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* are 34 664, 96, and 84 respectively (Scallan et al., 2011). In Canada, the estimated annual number of domestically acquired foodborne illnesses caused by *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* are 1798, 1, and 0 respectively (Thomas et al., 2013).

V. parahaemolyticus is a mesophilic and mildly halophilic bacterium. Accordingly, warmer temperatures and moderate salinity levels promote its growth and survival. Optimal growth temperatures are between 35°C and 39°C and optimal sodium chloride concentrations are between 1% and 3% (Yeung and Boor, 2004). Under ideal conditions, the generation time of *V. parahaemolyticus* is less than 20 minutes. In fact, doubling times as quick as 5 minutes have been reported (Yeung and Boor, 2004). In contrast, under extreme conditions (such as starvation and temperature stress) that do not support optimal physiological functions, *V. parahaemolyticus* has been reported to enter a viable but non-culturable state (Jiang and Chai, 1996; Mizunoe et al., 2000).

1.2 Ecology and Occurrence

V. parahaemolyticus is considered part of the autochthonous microflora of estuarine and coastal ecosystems and there is no correlation between the presence of this microorganism and faecal contamination of the environment (FAO, 2011; Kaneko and Colwell, 1977). Accordingly, *V. parahaemolyticus* has been isolated from seawater, sediment, plankton, and various fish and shellfish species (FAO, 2011; Johnson et al., 2009; Venkateswaran et al., 1990).

1.2.1 Prevalence in Marine Environments

The presence of particulates, zooplankton, and other sources of chitin influence the prevalence of *V. parahaemolyticus* (Kaneko and Colwell, 1975a; Venkateswaran et al., 1990). For example, *V. parahaemolyticus* was observed to adsorb onto chitin particles and copepods with pH and salinity significantly affecting the efficiency of adsorption; an acidic pH as opposed to an alkaline pH was more favourable and adsorption decreased with increased salinity (Kaneko and Colwell, 1975a). Furthermore, an ecological study conducted in Chesapeake Bay, Maryland found the incidence of *V. parahaemolyticus* to be correlated with water temperature and reported that temperatures between 14°C and 19°C were critical for the appearance of *V. parahaemolyticus* in the water column (Kaneko and Colwell, 1973). Similarly, an early study by Kelly and Stroh (1988) found that *V. parahaemolyticus* was detectable in Pacific Northwest waters only during the summer months when water temperatures were greater than 17°C and salinities were below 13 ppt. Likewise, Cox and Gomez-Chiarri (2012) detected *V. parahaemolyticus* in water and oysters collected from Rhode Island when water temperatures were greater than 18°C.

A survey of nine states, representing Pacific, Gulf, and Atlantic coastal waters in the US, was conducted to compare seasonal levels of *V. parahaemolyticus* and the data showed strong correlations between water temperature and *V. parahaemolyticus* levels (DePaola et al., 1990). An average density of 4 cells/100 mL in seawater was observed when the water temperature was below 16°C; however, the levels of *V. parahaemolyticus* could increase to 100 cells/100 mL when water temperatures increased to 25°C (DePaola et al., 1990; Kaneko and Colwell, 1973). The Gulf Coast had the warmest mean temperature (22°C) and highest mean *V. parahaemolyticus* levels of 44 CFU/100 mL, while the Pacific Coast water was the coldest at 15°C and was associated with lower levels of *V. parahaemolyticus* (2 CFU/100 mL). More recent studies have attempted to develop models to predict the presence of *V. parahaemolyticus*. For example, Urquhart et al. (2016) analyzed data collected over a 7 year period from American oysters harvested from two sites in the Great Bay Estuary of New Hampshire. The authors concluded that inclusion of chlorophyll *a* concentration, to an empirical model based on temperature and salinity, further improved the accuracy for estimating the likelihood of *V. parahaemolyticus* presence in the Great Bay Estuary (Urquhart et al., 2016). Johnson et al. (2012) conducted a similar study to determine relationships between environmental parameters and *V. parahaemolyticus* populations in water, oysters, and sediment. The investigators analyzed samples over a 21 month period from three geographically distinct sites (Pacific Northwest, northern Gulf of Mexico, and Chesapeake Bay) and reaffirmed sea surface water temperature as an effective predictor of annual variation in *V. parahaemolyticus* abundance (Johnson et al., 2012).

It has also been reported that *V. parahaemolyticus* "over-winters" in sediment and is undetectable in the water column during the winter months (Kaneko and Colwell, 1973). From late spring to early summer, when water temperatures rose to 14°C, *V. parahaemolyticus* surviving in the sediment were released from bottom communities into the water column, attaching to zooplankton that were rich in

chitin and used as a food source to support *V. parahaemolyticus* proliferation as the temperature rose (Kaneko and Colwell, 1973). More recently, researchers demonstrated that Saharan dust nutrients, deposited in tropical marine waters experimentally and through natural events, can promote rapid blooms of *Vibrio* and suggested that dust-associated iron is an important driver in *Vibrio* population dynamics (Westrich et al., 2016). Through *in situ* field studies, Saharan dust atmospherically transported to the Caribbean and sub-tropical Atlantic coincided with high levels of dissolved iron, and was followed by up to a 30-fold increase in culturable *Vibrio* over background levels within 24 hours (Westrich et al., 2016).

1.2.2 Prevalence in Shellfish

V. parahaemolyticus has been detected and isolated from a wide variety of fish and shellfish, including codfish, sardine, mackerel, flounder, clams, octopus, shrimp, crab, lobster, crawfish, scallops, and oysters (Su and Liu, 2007; Odeyemi, 2016). As *V. parahaemolyticus* is naturally present in shellfish growing and harvesting areas (Deepanjali et al., 2005; DePaola et al., 1990; Kaysner et al., 1990; Kelly and Stroh, 1988; Kaneko and Colwell, 1975b), it consequently bio-accumulates and concentrates in filter-feeding molluscan shellfish, such as oysters, clams, and mussels, making these bivalve molluscs vehicles for foodborne disease agents. A recent meta-analysis identified oysters as the most common food associated with *V. parahaemolyticus* infections, with a prevalence rate of 63% (Odeyemi, 2016). Moreover, higher densities of the bacterium are found in oyster digestive tissues compared to muscle tissue (Cabello et al., 2005; Wang et al., 2010). In addition, oyster-to-oyster variability in *V. parahaemolyticus* levels has been described. Kaufman et al. (2003) tested 20 oysters and found that approximately 90% had *V. parahaemolyticus* levels between 200 to 2000 CFU per gram; however, a single “hot” oyster, containing levels nearly 10-fold higher (20 000 CFU per gram) than oysters simultaneously harvested within a 1 m^2 proximity, was observed. Similar results were obtained by Klein

and Lovell (2017) who tested 110 individual oysters and found two "hot" oysters that had *V. parahaemolyticus* levels ~20 times higher than the average (Klein and Lovell, 2017). Kaufman et al. (2003) further noted while standard sampling protocols specify pooling 10 to 12 animals for analysis are more than adequate for estimating the population mean, the detection of a "hot" oyster would not likely be detected in most instances and further hypothesized these "hot" oysters, which occur at low frequency, may explain the sporadic nature of *V. parahaemolyticus* infections (Kaufman et al., 2003).

Seasonal variations in the prevalence and levels of *V. parahaemolyticus* have been observed. During summer months, shellfish often have *V. parahaemolyticus* levels 200 times greater, on average, than those in the water (DePaola et al., 2000; DePaola et al., 1990). Moreover, it has been noted that in temperate waters, *V. parahaemolyticus* is often detected in oysters harvested in warmer months; however, in tropical waters, detection can occur throughout the year (Deepanjali et al., 2005). While temperature and salinity play important and inter-related roles in influencing the prevalence and levels of *V. parahaemolyticus* in temperate waters (DePaola et al., 2003), salinity appears to be the major factor in tropical waters (Deepanjali et al., 2005).

Between June 1998 and July 1999, 370 lots of oyster shell-stock were sampled from restaurants, oyster bars, retail and wholesale seafood markets throughout the US; oysters were harvested from the Gulf (49%), Pacific (14%), Mid-Atlantic (18%), and North Atlantic (11%) of the US as well as Canada (8%) (Cook et al., 2002b). The investigators found densities of *V. parahaemolyticus* in oysters from all harvest regions were highest in the summer months and those originating from the Gulf Coast produced the highest densities and often exceeded 10 000 MPN/g (Cook et al., 2002b). Similar retail studies conducted in China (Chen et al., 2010), Brazil (Sobrinho Pde et al., 2011), and Poland (Lopatek et al., 2015) isolated *V. parahaemolyticus* from shellfish samples and also found seasonal trends. More

recently, Cruz et al. (2015) investigated the incidence of *V. parahaemolyticus* in New Zealand oysters and greenshell mussels obtained from commercial shellfish-growing areas between December 2009 and June 2012 and found similar seasonal distributions; *V. parahaemolyticus* levels increased when seawater temperatures were high and samples exceeded 1000 MPN/g only when the seawater temperature exceeded 19°C.

Even if harvested shellfish initially had low *V. parahaemolyticus* concentrations, studies have shown that *V. parahaemolyticus* populations in unrefrigerated oysters can rapidly multiply and increase 50-fold after 10 hours and 790-fold after 24 hours if the oysters were held at 26°C (Gooch et al., 2002). In growing areas with semidiurnal tides (two high tides and two low tides per day), oysters may be exposed to ambient air temperatures during the intertidal period providing favourable conditions for rapid proliferation of *V. parahaemolyticus* within the oysters. Moreover, in some Pacific Northwest estuaries, intertidal harvest is practiced extensively; after the tide recedes from the harvest area, the shellfish are collected and placed in large baskets that are left in the harvest area until the tide rises sufficiently for a vessel to retrieve the baskets and transport the oysters to a processing facility (Nordstrom et al., 2004; Jones et al., 2016). Jones et al. (2016) also demonstrated that when oysters are submerged with the incoming tide, the accumulated *V. parahaemolyticus* can be purged. As intertidal harvest potentially exposes oysters to suitable conditions for *V. parahaemolyticus* growth, especially on sunny summer days, recommendations to reduce the risk of illness from *V. parahaemolyticus* in these situations include (i) harvesting oysters at high tide, on falling tide, or after the incoming tide has covered the oysters for a period of time, (ii) protecting harvested oyster from the sun, (iii) placing oysters under temperature control immediately after harvest and during transport to processing facilities, and (iv) rapidly cooling oysters to 10°C or less as quickly as possible to minimize post-harvest *V. parahaemolyticus* growth (CFIA, 2012).

1.3 Epidemiology

Vibrio infections present in one of three major clinical manifestations: 60 – 80% of infections cause gastroenteritis, 34% cause wound infections, and 5% cause septicemia (Butt et al., 2004; PHAC, 2011). When *V. parahaemolyticus* is isolated from stool alone, it is characterized as causing gastroenteritis (Drake et al., 2007). Typical symptoms include diarrhea (sometimes bloody and watery), abdominal cramps, nausea, vomiting, headache, chills, and low-grade fever (Nair et al., 2007; PHAC, 2011). Most cases of infection are self-limiting, of moderate severity, and can be treated solely with oral rehydration (Yeung and Boor, 2004; Butt et al., 2004; Daniels et al., 2000). However, treatment with antibiotics may be necessary in some situations (Butt et al., 2004; Daniels et al., 2000). For immunocompromised patients or for individuals with a pre-existing medical condition such as liver disease or diabetes, a *V. parahaemolyticus* infection can be fatal (Daniels et al., 2000; Yeung and Boor, 2004). In the United States, 100 hospitalizations and 4 deaths attributed to foodborne illnesses caused by *V. parahaemolyticus* are estimated to occur annually (Scallan et al., 2011). In Canada, 17 hospitalizations and 0.6 deaths related to domestically acquired foodborne illnesses caused by *V. parahaemolyticus* is estimated to occur annually (Thomas et al., 2015).

Traditionally, *V. parahaemolyticus* has been primarily classified according to a serotyping scheme based on a combination of O and K antigens (Nair et al., 2007). More recently, with the advent of molecular-based tools, efforts to elucidate the genetic diversity of *V. parahaemolyticus* circulating in different parts of the world have been undertaken (Han et al., 2015; Gonzalez-Escalona et al., 2008; Urmersbach et al., 2014; Han et al., 2014). For example, multi-locus sequence typing (MLST) methods have been widely applied in epidemiological investigations and have been successfully used to examine genetic diversity

of global isolate collections as well as geographically restricted populations (Struelens and Brisse, 2013; Cooper and Feil, 2004; Maiden, 2006). In MLST, bacterial isolate characterization is based on amplifying and sequencing internal fragments of housekeeping genes (Maiden, 2006). For each housekeeping gene, unique sequences are assigned distinct allele numbers and combined into an allelic profile and assigned a sequence type (ST). As a result, each isolate of a species is therefore unambiguously characterized by a series of integers which correspond to the alleles for each housekeeping loci (Maiden, 2006). The relatedness of isolates can then be determined by comparing allelic profiles with clonal complexes (CC) defined as groups of closely related but not identical isolates likely originating from a recent common ancestor (Maiden, 2006).

González-Escalona et al. (2008) developed a MLST scheme for *V. parahaemolyticus* based on the internal fragment sequences of seven housekeeping genes with allele and ST definitions posted on a PubMLST site (https://pubmlst.org/vparahaemolyticus/); accordingly, this tool can be used as a fingerprinting technique for molecular epidemiological and population genetic studies. Additionally, Banerjee et al. (2014) observed that there is congruence between MLST and serotype for *V. parahaemolyticus*; for example, among the 100 clinical isolates tested all ST36 strains identified belonged to the O4 serogroup and all ST417 isolates belonged to the O1 serogroup. Application of the MLST scheme to 100 *V. parahaemolyticus* strains isolated from geographically diverse clinical (n=37) and environmental (n=63) sources, indicated that this pathogen is genetically diverse (Gonzalez-Escalona et al., 2008). A total of 62 unique STs were identified; ST3 was the most common (22%) and consisted of *V. parahaemolyticus* pandemic strains isolated in four continents (Gonzalez-Escalona et al., 2008). The 62 STs were separated by eBURST analysis into three CCs with separate clonal complexes observed for *V. parahaemolyticus* isolates originating from the Pacific (CC36) and Gulf coasts of the US (CC34), while a third clonal complex consisted of strains belonging to the pandemic clonal complex (CC3) with worldwide distribution

(Gonzalez-Escalona et al., 2008). Later, Han et al. (2014) completed a broader phylogenetic analysis of 490 clinical *V. parahaemolyticus* isolates collected between 1951 and 2014 from 17 coastal countries. A total of 161 STs were identified (with ST3 being the most frequent and the sole sequence type with an international distribution) and the eBURST algorithm resolved the 161 STs into 8 CCs, 11 doublets, and 94 singletons with CC3 being the most populated (Han et al., 2014). The researchers also observed a high degree of genotypic diversity within the *V. parahaemolyticus* population and concluded that multiple sequence types contribute to the human infection around the world (Han et al., 2014).

1.3.1 Incidence in Asia

In 1950, the first *V. parahaemolyticus* outbreak in Japan resulted in 272 illnesses and 20 deaths following the consumption of semi-dried sardines known as *shirasu* (Shinoda, 2011). Since then, *V. parahaemolyticus* has accounted for many food poisoning cases in various parts of Asia including Japan (Alam et al., 2002; Hara-Kudo et al., 2012; Kubota et al., 2011), Taiwan (Wong et al., 2000; Yu et al., 2013), Bangladesh (Bhuiyan et al., 2002), Hong Kong (Chan et al., 2002), and Indonesia (Lesmana et al., 2001). Alam et al. (2002) reported that 20 – 30% of food poisoning cases in Japan were attributed to *V. parahaemolyticus*. Similarly, *V. parahaemolyticus* has been reported as one of the leading causes of foodborne disease in China (Qi et al., 2016; Wang et al., 2007). Between 1994 and 2005, *V. parahaemolyticus* was the most frequently (36%) identified agent of 1082 bacterial foodborne disease events (Wang et al., 2007). A study examining the prevalence of *V. parahaemolyticus* in Pacific shell-stock oysters sold at wholesale or retail food markets in southern China revealed that of the 202 oysters tested, 89% had detectable levels of *V. parahaemolyticus*, with 55 samples having levels greater than 100 MPN/g (Chen et al., 2010). A review of 20 years of data collected by the Food and Drug Administration of the Department of Health between 1991 and 2010 determined that *V. parahaemolyticus* has played a leading role in foodborne outbreaks in Taiwan since 1995 (Cheng et al.,

2013). While illnesses attributed to *V. parahaemolyticus* has gradually decreased since its peak of 160 outbreaks in 1997, the pathogen still remains the most common bacterial etiological agent of foodborne outbreaks in Taiwan (Cheng et al., 2013).

Historically, *V. parahaemolyticus* infections were characterized by sporadic cases attributed to multiple diverse serotypes (Turner et al., 2013; Chowdhury et al., 2004). However, over a period of months in 1996, infections caused by strains belonging to an O3:K6 serotype and its related serovariants, now referred to as the pandemic clonal complex (CC3), emerged in India and subsequently disseminated around the world (Nair et al., 2007; Gonzalez-Escalona et al., 2008; Gavilan et al., 2013). Han et al. (2015) selected 218 clinical isolates, collected between 1990 and 2014 from different provinces within China, from the PubMLST database for analysis and found the *V. parahaemolyticus* population was highly diverse. The MLST scheme produced 137 STs, with ST3 being the most common, that were clustered into 6 CCs, including CC3, 6 doublets, and 91 singletons (Han et al., 2015). A study conducted in Thailand examining 101 isolates originating from clinical samples (n=15), healthy human carriers (n=18), various seafood products (n=52), and shrimp farm water (n=18) also found a high degree of genetic diversity within the *V. parahaemolyticus* population (Theethakaew et al., 2013). A total of 63 STs were identified, with ST251 being the most common, that were clustered into two clonal complexes (CC83 and CC233), two doublets, and 53 singletons (Theethakaew et al., 2013).

1.3.2 Incidence in Europe

In contrast to Asian countries, *V. parahaemolyticus* infections are less commonly reported in Europe (Baker-Austin et al., 2010). For example, in the United Kingdom only 57 cases of infections were reported between 2004 and 2005 and most of these were obtained via foreign travel to endemic regions (Baker-Austin et al., 2010; Wagley et al., 2008). Nonetheless, over the past 20 years, sporadic outbreaks have occurred. One of the first outbreaks, reported in 1973, involved consumption of locally caught and home prepared crabs in Britain (Hooper et al., 1974). In 1989, eight cases of acute gastroenteritis caused by *V. parahaemolyticus* and associated with fish and shellfish were reported in Spain (Molero et al., 1989). Much larger outbreaks have subsequently been reported including one that was associated with raw oyster consumption that sickened 64 individuals in Galicia, Spain in 1999 (Lozano-Leon et al., 2003). An outbreak affecting 44 patients associated with the consumption of shrimps imported from Asia occurred in France in 1997 (Robert-Pillot et al., 2004). Later, an outbreak that involved 80 illnesses among guests attending weddings in one restaurant located in A Coruña, Spain was reported in July 2004 (Martinez-Urtaza et al., 2005). More recently, a large *V. parahaemolyticus* outbreak affecting 100 passengers travelling on a food banquet cruise boat in August 2012 near Pontevedra, Spain was described (Martinez-Urtaza et al., 2016). This was the largest foodborne *Vibrio* outbreak reported in Europe that was linked to domestically processed shrimp. Moreover, the clinical isolates showed indistinguishable genetic profiles with hyper-virulent strains from the United States Pacific Northwest region but were never detected previously in Europe (Martinez-Urtaza et al., 2016). In particular, MLST analysis revealed the existence of ST36, the dominant MLST type in the Pacific Northwest, as well as strains sharing allelic profiles of six genes with ST417 (also endemic in the Pacific Northwest) among the outbreak strains (Martinez-Urtaza et al., 2016).

1.3.3 Incidence in North America

Historically in the US, *V. parahaemolyticus* has been primarily associated with sporadic disease; however, large outbreaks of gastroenteritis have occurred and it has been estimated that this pathogen causes approximately 35 000 cases, 100 hospitalizations, and 4 deaths each year (Scallan et al., 2011). *V. parahaemolyticus* was first identified as an etiological foodborne pathogen in Maryland, US in 1971 after three outbreaks of 425 gastroenteritis cases associated with the consumption of improperly cooked crabs (Molenda et al., 1972). Since then, intermittent *V. parahaemolyticus* outbreaks have been reported throughout the US coastal regions. Early on, post-cooking contamination of crustaceans (e.g. shrimp, crab, lobsters) was associated with outbreaks (Drake et al., 2007). In the late 1990s, there was a shift towards outbreaks linked to the consumption of raw oysters. Between 1973 and 1998, the US Center for Disease Control and Prevention reported approximately 40 outbreaks of *V. parahaemolyticus* (Daniels et al., 2000). Among them, four major outbreaks involved over 700 illnesses linked to the consumption of raw oysters in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions between 1997 and 1998. During the summer of 1997, a culture-confirmed outbreak involving 209 cases (including one death) were all attributed to the consumption of raw oysters harvested from the coastal water of California, Oregon, Washington, and British Columbia (Centers for Disease and Prevention, 1998). In 1998, two outbreaks of 43 cases in Washington and 416 cases in Texas were also associated with consumption of raw oysters (DePaola et al., 2000). Between July and September 1998, another small outbreak involving eight cases were reported in Connecticut, New Jersey, and New York as a result of eating oysters and clams harvested from Long Island Sound in New York (Centers for Disease and Prevention, 1999). According to the US Center for Disease Control and Prevention, *Vibrio* infections have shown an increase since 2001 and *V. parahaemolyticus* causes the highest number of seafood-associated bacterial gastroenteritis in the US (Mead et al., 1999). In July 2004, 14 passengers on board a cruise ship experienced gastrointestinal illness after eating raw oysters harvested from Alaskan waters

(McLaughlin et al., 2005). In 2006, 177 cases of *V. parahaemolyticus* infections were linked to the consumption of contaminated oysters harvested from Washington and British Columbia (Centers for Disease and Prevention, 2006). In Canada, a general upward trend of *V. parahaemolyticus* incidence rates has been observed (PHAC, 2014) and this same trend has been reported in BC (BCCDC, 2015). More recently, a total of 82 *V. parahaemolyticus* infections (including one hospitalization) occurred between May and September 2015; all the individuals (60 in BC, 19 in Alberta, 1 in Saskatchewan, and 2 in Ontario) reported consumption of raw shellfish (primarily oysters) (PHAC, 2015).

Lüdeke et al. (2015) tested 58 *V. parahaemolyticus* isolates (28 of oyster and 30 of clinical origin) representing different areas within the US as well as different serotypes and MLST analysis identified 41 different STs. Amongst the clinical isolates, ST36 (13.3%) and ST3 (10%) was most frequently observed while ST32 (10.7%), ST313 (7.1%), and ST676 (7.1%) were most common in oyster isolates (Ludeke et al., 2015). Two of the STs (ST3 and ST36) have been reported as part of clonal complexes CC3 and CC36, respectively, and have correlated with outbreaks in the US and Chile (Ludeke et al., 2015). Within the past 20 years, several large seafood-associated *V. parahaemolyticus* outbreaks in the PNW region have been attributed to strains with an O4:K12 serotype (Abbott et al., 1989; Paranjpye et al., 2012; Turner et al., 2013). Subsequently termed the Pacific Northwest complex (CC36) (Turner et al., 2013), these bacteria appear to be genetically and biochemically distinct and carry a significantly lower infectious dose compared to other toxigenic *V. parahaemolyticus* isolates (Turner et al., 2013). Turner et al. (2013) conducted a phylogenetic analysis of clinical and environmental *V. parahaemolyticus* isolates originating mainly from PNW and found of the 77 isolates analyzed by MLST, 24 STs were identified with ST3 (n=20) and ST36 (n=30) predominating. The authors concluded ST36 represents a genetically exclusive ST and suggested it is clonally related to the O4:K12 complex (Turner et al., 2013). Previously only reported in

the PNW, ST36 has since disseminated to the east coast of the US as well as Europe suggesting a recent transcontinental spread (Martinez-Urtaza et al., 2013; Martinez-Urtaza et al., 2016).

1.4 Virulence Factors

While *V. parahaemolyticus* has been recognized as a leading cause of seafood-derived infections, not all strains of this species are considered to be truly pathogenic (Nishibuchi and Kaper, 1995). A number of virulence factors are thought to play a role in the pathogenicity of *V. parahaemolyticus*. These include adhesins, toxins, two type III secretion systems (T3SS1 and T3SS2), and secreted effectors involved in attachment, cytotoxicity, and enterotoxicity (Broberg et al., 2011; Letchumanan et al., 2014).

While research into uncovering novel *V. parahaemolyticus* virulence factors continues, several major virulence factors have been characterized. Early investigations in Japan demonstrated that 96% of clinical *V. parahaemolyticus* strains produce a thermostable direct hemolysin; in contrast, only 1% of the environmental strains produce this hemolysin (FAO, 2011; Nishibuchi and Kaper, 1995). Later, a TDH-related hemolysin (TRH) was found in TDH-negative strains from clinical cases (Honda et al., 1988). At present, the World Health Organization considers *V. parahaemolyticus* strains producing TDH and TRH pathogenic to humans (Table 1.1).

Table 1.1. Two key virulence factors of *V. parahaemolyticus*

Name	Domain	Activity	Function
TDH	Thermostable direct hemolysin	Pore forming toxin	Cause cytotoxicity and enterotoxicity
TRH	TDH-related hemolysin	Pore forming toxin	Cause cytotoxicity and enterotoxicity

1.4.1 Thermostable Direct Hemolysin (TDH)

In early epidemiological studies, clinical strains of *V. parahaemolyticus* were observed to produce hemolysis on a blood agar medium (Miyamoto et al., 1969; Takeda, 1982). Subsequently, in 1965, Wagatsuma developed a specific culture medium to measure the hemolytic character of *V. parahaemolyticus* (Miyamoto et al., 1969). This medium, called Wagatsuma agar, is a high salt (7%) blood agar (defibrinated human or rabbit blood) that contains D-mannitol as the carbohydrate source. The β-hemolysis observed on Wagatsuma agar is termed the Kanagawa phenomenon (KP) and this phenotype has been used to identify pathogenic *V. parahaemolyticus* in seafood as well as patient samples. Sanyal and Sen (as cited in Takeda, 1974) conducted human volunteer feeding studies and showed ingestion of $2 \times 10^5 - 3 \times 10^7$ CFU of KP-positive *V. parahaemolyticus* can lead to the rapid development of gastrointestinal illness (Takeda, 1982). It has since been determined that the Kanagawa phenomenon is caused by the thermostable direct hemolysin protein (Nishibuchi and Kaper, 1995).

TDH consists of 165 amino acids and under aqueous conditions forms a tetrameric structure with a central pore 23 Å in diameter and 50 Å in depth (Hamada et al., 2007; Yanagihara et al., 2010). This hemolysin was named thermostable direct hemolysin based on its characteristics. Specifically, TDH was not inactivated by heating at 100°C for 10 minutes and the hemolytic activity was not enhanced by the addition of lecithin, suggesting direct activity on erythrocytes (Sakurai et al., 1973).

TDH is encoded by the *tdh* gene and was first cloned from a clinical KP-positive *V. parahaemolyticus* WP1 strain (Kaper et al., 1984). The probes derived from this cloned gene (designated as *tdh*1) were subsequently used to identify *tdh* genes in other *V. parahaemolyticus* strains. Researchers later discovered that the *V. parahaemolyticus* WP1 strain actually contained a second *tdh* gene, designated *tdh*2, that shared 97% homology with *tdh*1 (Nishibuchi and Kaper, 1990; Iida and Yamamoto, 1990). A

survey of KP-positive and KP-negative *V. parahaemolyticus* strains revealed that (i) all KP-positive strains contained two *tdh* gene copies, (ii) KP-intermediate (i.e. weak hemolysis) strains only had one *tdh* gene, and (iii) KP-negative strains either contained one copy of the *tdh* gene or did not have the *tdh* gene (Nishibuchi and Kaper, 1990). Construction of isogenic mutants defective in either *tdh*1 or *tdh*2 revealed that the KP phenotype and more than 90% of the total TDH protein production were attributable to expression of the *tdh*2 gene (Nishibuchi and Kaper, 1990; Nishibuchi et al., 1991). However, the *tdh*1 gene was not completely silent as it accounted for 0.5 – 9.4% of total TDH under various culture conditions (Nishibuchi et al., 1991).

1.4.2 TDH-Related Hemolysin (TRH)

V. parahaemolyticus strains that do not contain the *tdh* gene have been associated with outbreaks of gastroenteritis. In 1985, an outbreak of gastroenteritis occurred in the Maldives and was linked to KP-negative isolates of *V. parahaemolyticus*; these strains were shown to produce a TDH-related hemolysin which was similar but not identical to the TDH protein (Honda et al., 1988).

TRH also consists of 165 amino acids and exists as tetramer in solution (Ohnishi et al., 2011). This hemolysin, when injected intraperitoneally into mice, resulted in 30 to 40% lethality within 14 to 24 hours (Sarkar et al., 1987). In contrast to TDH, TRH is labile to heat treatment at 60°C for 10 minutes (Honda et al., 1988) and similar to TDH, TRH has been shown to induce Cl^- secretion in human colonic epithelial cells (Takahashi et al., 2000).

TRH is encoded by the *trh* gene and sequencing has revealed there is approximately a 69% nucleotide similarity between *trh* and *tdh* (Nishibuchi et al., 1989). In addition, there are multiple forms of the *trh* gene whose corresponding proteins differ in hemolytic activity (Kishishita et al., 1992). For example, a

*trh*2 gene product has been shown to be hemolytic to human and rabbit erythrocytes only and the hemolytic activities were relatively weak; in comparison, a *trh*1 gene product was shown to be hemolytic to human, rabbit, sheep, and calf erythrocytes (Kishishita et al., 1992).

1.4.3 Incidence of TDH and/or TRH

V. parahaemolyticus strains isolated from environmental samples often lack the *tdh* and/or *trh* genes (Deepanjali et al., 2005; Cruz et al., 2015; Robert-Pillot et al., 2004; DePaola et al., 2000). Nonetheless, studies from the US, Europe, and Asia have reported approximately a 0 – 10% prevalence rate of genes encoding for TDH and/or TRH toxins in environmental *V. parahaemolyticus* strains (DePaola et al., 2000; Cook et al., 2002a; Robert-Pillot et al., 2004; Ottaviani et al., 2010; Parthasarathy et al., 2016; Yu et al., 2013; Letchumanan et al., 2015a; Cook et al., 2002b).

Cook et al. (2002b) tested 370 lots of oyster shell-stock sampled at 275 different establishments including restaurants as well as retail and whole seafood markets, and found that *tdh* was detected in 9 out of 3429 (0.3%) *V. parahaemolyticus* cultures and 8 of 198 (4%) lots of oysters. A study conducted in South China tested 224 retail seafood samples and found that of the 150 *V. parahaemolyticus* strains isolated, 41% were *trh*-positive while 0% were *tdh*-positive (Xie et al., 2015). A similar recent study by Parthasarathy et al. (2016) examined 167 crustacean samples collected from different retail markets in and around Kolkata and Bhubaneswar, India and found that of 12% of the *V. parahaemolyticus* isolates recovered from crabs (n=82) and 12% of the isolates recovered from shrimps (n=85) carried the *tdh* gene. Surveys conducted in Italy reported a prevalence of *V. parahaemolyticus* isolates carrying the *trh* gene ranged between 6 % and 9% in environmental and shellfish samples (Ottaviani et al., 2010; Fabbro et al., 2010; Caburlotto et al., 2009). Furthermore, Ottaviani et al. (2013) tested *V. parahaemolyticus*

isolates recovered from different Italian areas and detected *trh* in 13% of the samples while *tdh* was not detected in any of the cultures.

1.5 Antimicrobial Resistance

The introduction of penicillin in the 1940s began an era of antibiotics and since then, hundreds of antimicrobial agents have been discovered, synthesized, and applied for clinical use, animal therapy, or growth promotion (Aarestrup and Wegener, 1999; WHO, 2014; Davies and Davies, 2010). Antibiotics can exert bactericidal or bacteriostatic effects by interfering with critical metabolic functions including cell wall biosynthesis, protein synthesis, as well as DNA replication and repair (Walsh, 2000). Classes of antibiotics, such as β-lactams, aminoglycosides, tetracyclines, quinolones, and sulfonamides target different metabolic functions.

Antimicrobial resistance is a broader term that includes resistance to drugs used to treat infections caused by bacteria, viruses, parasites, and fungi; however, within this thesis the terms antimicrobial and antibiotic are used interchangeably. Accordingly, antimicrobial resistance can be defined as the ability of a microorganism to resist the effect of an antibiotic and may be due to intrinsic bacterial resistance, a mutation of the bacterial genome, acquisition of genetic material, or a combination of these factors (Davies and Davies, 2010; Blair et al., 2015). Unfortunately, excessive and inappropriate use of antibiotics has resulted in the emergence of bacterial resistance to common antibiotics which can lead to failure of the available treatment options for common infections (WHO, 2014; Aarestrup and Wegener, 1999). As a result, antibiotic resistance is a global public health concern and a food safety problem. Food animals serve as a reservoir of resistant pathogens and resistance mechanisms that can spread from food animals to humans through the food chain and result in antibiotic resistant infections

in humans. Similarly, naturally-occurring bacteria, such as *V. parahaemolyticus*, can be reservoirs of resistance genes and may play a role in the evolution and spread of AMR in aquatic environments.

As the majority of *V. parahaemolyticus* infections are self-limiting, antimicrobial therapy is generally not required; although antibiotics may be prescribed in severe or prolonged illnesses (Butt et al., 2004; Elmahdi et al., 2016). Traditionally, *V. parahaemolyticus* was considered to be susceptible to most antibiotics of veterinary and human significance with documented ampicillin resistance (Zanetti et al., 2001; Han et al., 2007). More recent studies, conducted in different countries and aimed at characterizing AMR profiles, have reported that *V. parahaemolyticus* showed multiple antibiotic resistance or reduced susceptibility to one or more antimicrobial agents (Elmahdi et al., 2016; Odeyemi, 2016).

Jun et al. (2012) tested 24 *V. parahaemolyticus* strains isolated in Korea against 22 antibiotics and found that all the isolates were resistant to ampicillin and cefazolin with some isolates displaying resistance to more than 11 antibiotics. A similar study, conducted between June and October 2014 by Kang et al. (2016), tested oysters from four commercial harvesting areas off the west coast of Korea and recovered 71 *V. parahaemolyticus* isolates. All 71 isolates analyzed were resistant to ampicillin and vancomycin and approximately 50% of the isolates also exhibited resistance to cephalothin, rifampin, and streptomycin; in contrast, all isolates were sensitive to sulfamethoxazole/trimethoprim and chloramphenicol while reduced susceptibility was seen for erythromycin (85%) and kanamycin (72%) (Kang et al., 2016). A study characterizing *V. parahaemolyticus* strains arising from shrimp purchased in Malaysian supermarkets found of the 185 isolates analyzed, resistance to ampicillin (82%), amikacin (51%), cefotaxime (37%), kanamycin (28%), and gentamicin (11%) was observed; approximately 28% of

the isolates were resistant to three different antibiotics and one isolate exhibited resistance to 11 antibiotics (Letchumanan et al., 2015b).

A large scale investigation into the AMR of toxigenic and non-toxigenic *V. parahaemolyticus* strains isolated from shellfish and clinical samples in Italy was undertaken by Ottaviani et al. (2013). A total of 107 *V. parahaemolyticus* strains were analyzed including 87 isolates from bivalve shellfish collected between 2009 and 2011 along Italian coastal waters, 8 isolates from stool samples collected from patients who experienced gastroenteritis and *V. parahaemolyticus* was the only pathogen isolated, and 12 clinical strains from Japan (i.e. a non-European origin). While 62% of all strains were resistant to four different classes of antibiotics, no significant difference was observed in the distribution of multi-drug resistance with respect to pathogenic potential. All isolates were susceptible to chloramphenicol and doxycycline and resistant to ampicillin and amoxicillin. Resistance to ciprofloxacin was significantly ($p < 0.01$) more common in native clinical strains than those from bivalves and clinical strains of non-European origin (Ottaviani et al., 2013b).

Most studies investigating AMR in *V. parahaemolyticus* were conducted outside of North America (Elmahdi et al., 2016). Between 2005 and 2006, Han et al. (2007) examined 168 *V. parahaemolyticus* and 151 *V. vulnificus* isolates recovered oysters collected from the Louisiana Gulf Coast, retail seafood markets, as well as restaurants. Researchers found both organisms were susceptible to the majority of 8 antibiotics tested with only *V. parahaemolyticus* strains having reduced susceptibility for ampicillin (Han et al., 2007). Shaw et al. (2014) investigated AMR of 120 *V. vulnificus* strains and 77 *V. parahaemolyticus* strains isolated from Chesapeake Bay and Maryland coastal bay surface waters. The majority of isolates were susceptible to antibiotics recommended for treating *Vibrio* infections, although 98% of the *V. parahaemolyticus* strains displayed intermediate resistance to chloramphenicol and 68% were resistant

to penicillin (Shaw et al., 2014). Furthermore, in contrast to *V. vulnificus*, sampling location or month did not significantly impact *V. parahaemolyticus* patterns (Shaw et al., 2014).

1.6 Research Objectives

The overall objective of this research was to improve the understanding of the various types of *V. parahaemolyticus* strains that exist in the Canadian (primarily British Columbia) environment to better understand the risks potentially posed to consumers. Studies conducted to-date have demonstrated prevalence rates of pathogenic *V. parahaemolyticus*, as defined by the presence of *tdh* and/or *trh*, vary depending on source and geographic region. Moreover, AMR has been identified as a global threat with international commitments made to develop action plans to combat antibiotic resistance in various sectors. As such this project was undertaken to characterize the virulence profiles and population structure of *V. parahaemolyticus* circulating in Canada and gather data to fill the knowledge gap with regards to AMR for this pathogen in an effort to determine if differences in virulence profiles, population structure, and/or AMR patterns exist between source of isolation (clinical versus environmental), geographic areas, and/or isolation time points (years).

A library of *V. parahaemolyticus* isolates (n=600) recovered over a span of 14 years from oyster samples collected from Canadian coastal waters as well as clinical samples originating in BC were analyzed to address the following objectives:

1. Determination of the presence of the virulence genes *tdh* and *trh*
2. Determination of the population structure of a subset of *V. parahaemolyticus* strains using MLST
3. Determination of the resistance patterns to clinically relevant antibiotics

Chapter 2 – Virulence Potential of Canadian Isolates

2.1 Introduction

Vibrio parahaemolyticus is a natural inhabitant of temperate and tropical coastal waters and is the leading cause of seafood-associated gastroenteritis worldwide. Cases of illness are generally associated with the consumption of raw, undercooked, or improperly handled seafood. In Canada, factoring in under-reporting and under-diagnosis, an estimated 1800 cases of domestically acquired foodborne illnesses is attributed to *V. parahaemolyticus* (Thomas et al., 2013). Moreover, recent data show increased rates of *V. parahaemolyticus* infection in British Columbia (BCCDC, 2015).

V. parahaemolyticus is a diverse species and the majority of environmental strains are innocuous but small sub-populations are opportunistic human pathogens. The virulence of *V. parahaemolyticus* was initially attributed to the production of a thermostable direct hemolysin and subsequently a TDH-related hemolysin was discovered (Honda et al., 1988; Nishibuchi et al., 1985). Accordingly, strains of *V. parahaemolyticus* carrying *tdh* and/or *trh* genes, encoding for the respective pore-forming toxin, are considered to be pathogenic. This is corroborated by the prevalence of *tdh* and/or *trh* in clinical *V. parahaemolyticus* isolates and the relatively rare detection of these virulence markers in food and environmental isolates (FAO, 2011; Nishibuchi and Kaper, 1995). Environmental *V. parahaemolyticus* strains carrying *tdh* and/or *trh* have been reported to constitute 0 – 10% of the population and this frequency depends on the geographic location, sample source, and detection method used (Cook et al., 2002a; DePaola et al., 2000; Paranjpye et al., 2012; Martinez-Urtaza et al., 2008; Alam et al., 2002; Robert-Pillot et al., 2004; Parthasarathy et al., 2016; Ottaviani et al., 2010).

Strain delineation is an important element in understanding the epidemiology of disease and previous studies have shown that MLST can be used to track the source of human infection from animal and environmental reservoirs (Maiden, 2006; Cooper and Feil, 2004; Struelens and Brisse, 2013). The high genetic diversity of *V. parahaemolyticus* can complicate strain identification and epidemiological investigations. However, MLST has been successfully used to characterize diverse environmental and clinical *V. parahaemolyticus* global isolate collections as well as geographically restricted populations (Gonzalez-Escalona et al., 2008; Chowdhury et al., 2004; Yan et al., 2011; Johnson et al., 2009; Paranjpye et al., 2012; Turner et al., 2013; Ellis et al., 2012; Urmersbach et al., 2014). Furthermore, Banerjee et al. (2014) examined 100 *V. parahaemolyticus* clinical isolates collected across Canada (primarily BC) between 2000 and 2009 and developed a baseline for comparison for source attribution studies.

Following a 1997 *V. parahaemolyticus* outbreak in the Pacific Northwest where more than 200 illnesses were associated with eating raw oysters harvested from BC, Washington, Oregon, and California, the Canadian Food Inspection Agency established environmental indicator stations in six major oyster producing areas within BC. Analysis of oysters collected from these stations demonstrated an annual presence of *V. parahaemolyticus*. The data indicated when and to what extent the hazard of *V. parahaemolyticus* was present in a particular location, and using this information, the shellfish industry was required to employ adequate monitoring of specific lease sites where oysters were being harvested and destined for the raw consumption market (CFIA, 2012).

The primary objective of this study was to characterize, specifically with regards to the detection of *tdh* and/or *trh* genes, clinical and environmental *V. parahaemolyticus* strains isolated in Canada (predominantly BC). Following this, MLST analysis was conducted on a subset of the isolates to compare the phylogenetic relatedness of clinical strains to native environmental counterparts.

2.2 Materials and Methods

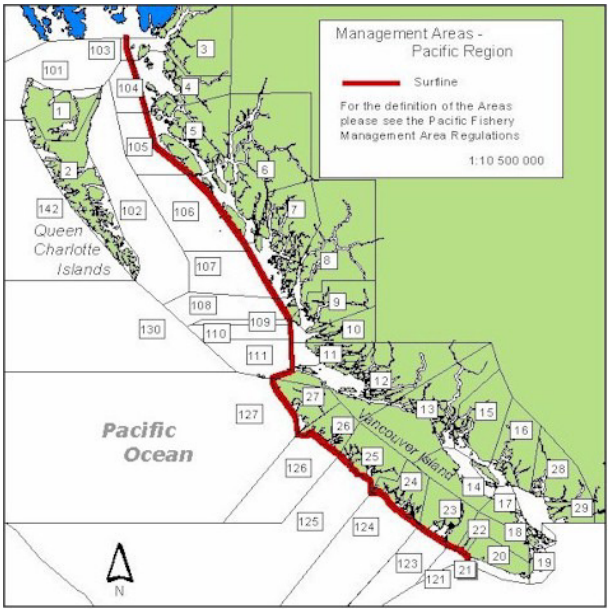
2.2.1 Bacterial Strains

A total of 600 *V. parahaemolyticus* isolates were examined in this study. The strain set consisted of clinical isolates (n=64) as well as environmental isolates (n=536). Human clinical *V. parahaemolyticus* isolates, defined as isolated from a patient source (e.g. stool or wound), were obtained from a BC medical laboratory between 2008 and 2011. Cultures were grown on thiosulphate citrate bile salts sucrose (TCBS) agar and genetically confirmed as *V. parahaemolyticus* using Health Canada's MFLP-23 method (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3-eng.php). In BC, enhanced surveillance of shellfish-related illnesses has been conducted since 1998 to identify and manage risks associated with the consumption of raw shellfish. Each case of shellfish-related illness, defined as any illness temporally associated with the consumption of shellfish, was interviewed by regional health authority staff using a standard questionnaire. In some circumstances, individuals who experience gastroenteritis symptoms elect to submit a specimen (e.g. stool) to a clinical laboratory for testing. Of the 64 clinical *V. parahaemolyticus* isolates tested, 22 were identified as cases that included shellfish as a potential food vehicle based on a cross-reference with available completed enhanced surveillance questionnaires (Table 2.1).

Table 2.1. Distribution of clinical *V. parahaemolyticus* strains isolated in British Columbia.

Year of Isolation	No. of Isolates	Shellfish Exposure
2008	11	7
2009	14	7
2010	18	0
2011	21	8
Total	**64**	**22**

Environmental strains were cultured from oyster samples collected from growing areas in BC and Atlantic Canada between 1998 and 2011 (Figure 2.1 and Table 2.2). Oysters, consisting of 10 – 12 animals, were analyzed using a 3-tube 4-dilution most probable number (MPN) method. Shellfish were aseptically shucked and the meat and liquor homogenized in a sterile blender for 1 minute. A 1 in 10 dilution was gravimetrically prepared using a 50 gram portion of homogenate and 450 grams of 2% sodium chloride (NaCl). Additional 10-fold dilutions were prepared and three 1 mL portions of each dilution were enriched in alkaline peptone water (APW). Following overnight incubation at 35°C, a 10 µL loopful from the top 1 centimetre of each APW tube was streaked onto TCBS agar and incubated overnight at 35°C. Typical colonies were confirmed as *V. parahaemolyticus* using MFLP-23.



© Department of Fisheries and Oceans Canada, Management Areas – Pacific Region, http://www.pac.dfo-mpo.gc.ca/fm-gp/maps-cartes/areas-secteurs/index-eng.html

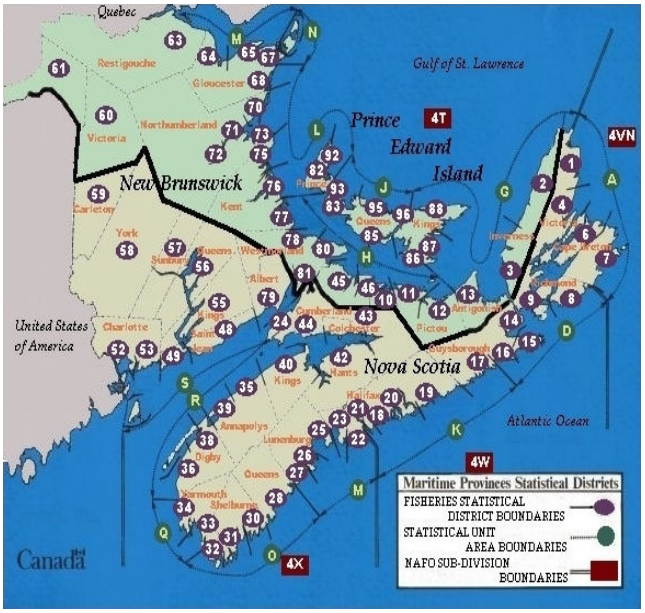


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Figure 2.1. Map of geographic locations sampled for *V. parahaemolyticus* in the Pacific and Atlantic regions of Canada.

Table 2.2. Distribution of environmental *V. parahaemolyticus* strains analyzed.

Province[a]	DFO[b] Statistical Area	DFO Sub-Statistical Area	Harvest Site	No. of Isolates	Year(s) of Isolation
BC	13	13-15	Gorge Harbour	26	1998 – 2001; 2007 – 2011
			Stove Islets	1	1999
	14	14-08	Denman Island	41	2008 – 2011
			Ship's Point	37	1999 – 2001
			Deep Bay	6	1999
			Fanny Bay	5	1998
	15	15-03	Twin Island	34	1998 – 2001
		15-04	Okeover Inlet	45	1999; 2004 – 2005; 2008 – 2011
		15-05	Mary Point	2	1999
			Desolation Sound	1	1999
	16	16-12	Sykes Island	61	2006; 2008 – 2011
		16-13	Dark Cove	44	1998 – 2001
	17	17-05	Coffin Point	47	2006 – 2011
		17-07	Ladysmith Harbour	133	1998 – 2005
	23	23-03	Ritherdon Bay	10	2000 – 2001
		23-06	Effingham Inlet	17	2004; 2006 – 2011
			Useless Inlet	3	1998; 2001
	24	24-09	Lagoon Inlet	5	2000
NB	NB-3B00		Baie de Caraquet	6	2005
	NB-4G00		Egg Island	1	2005
	NB-4H00		Baie Ste-Anne	1	2005
	NB-5F00		Richibuctou River	2	2005
	NB-6D00		Buctouche	3	2005
NS	NS-15		Eel Lake	2	2005
PEI	PEI-7C00		Orwell River	2	2005
	PEI-7F00		East River	1	2005
Total				**536**	

[a] BC is British Columbia; NB is New Brunswick; NS is Nova Scotia; PEI is Prince Edward Island
[b] DFO is Department of Fisheries and Oceans Canada

2.2.2 TDH and TRH Detection

A multiplex polymerase chain reaction (PCR) assay designated as MFLP-23 in Health Canada's Compendium of Analytical Methods was used to genetically confirm an isolate as *V. parahaemolyticus* and determine pathogenic potential. Specifically, the PCR procedure amplifies three DNA sequences –

the R72H fragment of the *V. parahaemolyticus* genome (species identifier), the *tdh* gene, and the *trh* gene (Robert-Pillot et al., 2002; Lee et al., 1995; Bej et al., 1999). Briefly, an isolated presumptive colony on TCBS agar was inoculated into 100 µL of sterile nuclease-free water. The cell suspension was vortexed, heated at 95°C for 5 minutes with constant shaking at 400 rpm (Eppendorf Thermomixer), and then snap-cooled in an ice bath for 10 minutes. A 2 µL aliquot of this crude lysate was used as template in the multiplex PCR method. PCR amplification was carried out using primers detailed in Table 2.3 with the following conditions: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of amplification consisting of a denaturation step at 94°C for 30 seconds, primer annealing at 58°C for 45 seconds, and extension at 68°C for 75 seconds, with a final extension step at 68°C for 7 minutes. Each reaction mixture (25 µL) contained final concentrations of 1X PCR Buffer, 2.5 mM $MgCl_2$, 0.252 mM dNTPs, 0.5 µM of VP-32 and VP-33 primers, 0.4 µM of TRH-L and TRH-R primers, 0.2 µM of TDH-L and TDH-R primers, and 2U of *Taq* DNA polymerase (ThermoFisher Scientific).

Table 2.3. Oligonucleotide primer sequences targeting sequences in the *V. parahaemolyticus* genome

Primer	**Sequence (5' to 3')[a]**	**Reference**
VP-33	TGC GAA TTC GAT AGG GTG TTA ACC	Robert-Pillot et al., 2002; Lee et al., 1995
VP-32	CGA ATC CTT GAA CAT ACG CAG C	
TRH-L	TTG GCT TCG ATA TTT TCA GTA TCT	Bej et al., 1999
TRH-R	CAT AAC AAA CAT ATG CCC ATT TCC	
TDH-L	GTA AAG GTC TCT GAC TTT TGG AC	Bej et al., 1999
TDH-R	TGG AAT AGA ACC TTC ATC TTC ACC	

2.2.3 Multi-Locus Sequence Typing

Bacterial DNA was purified using a Qiagen DNeasy kit and MLST analysis was conducted using a published *V. parahaemolyticus* protocol examining internal fragments of seven housekeeping genes (Gonzalez-Escalona et al., 2008). PCR amplification was performed using a Platinum High Fidelity *Taq*

DNA polymerase (Life Technologies, Inc., Ontario, Canada) and amplicons checked by gel electrophoresis (2% agarose) to ensure that only one product was amplified. For some strains, a PCR product was not generated for *recA*, *dnaE*, and/or *gyrB* using the primers originally published and listed in Table 2.4. In these cases, alternative published primers (Table 2.4) were successfully used to enable PCR amplification (Urmersbach et al., 2014; Theethakaew et al., 2013). Purification of PCR products and subsequent bi-directional sequencing using M13F and M13R primers was completed by Genome Quebec. Sequences were edited and the complementary fragments of each locus of the individual isolate were assembled, trimmed, and aligned using Geneious version 7.1.7 (https://www.geneious.com, Kearse et al., 2012). The consensus sequences were queried against the PubMLST database (https://pubmlst.org/vparahaemolyticus/) to determine allelic profiles and STs of each isolate. Sequences of new alleles and new allelic profiles were submitted to the PubMLST curator for assignment of new numerical identifiers. Global optimal eBURST (goeBURST) analysis of the resulting STs in the data set was performed using PhyloViz software (http://www.phyloviz.net/). For this study, to qualify as member of the same CC, a minimum of 6 of the 7 alleles used in the *V. parahaemolyticus* MLST scheme must be shared among isolates. Therefore, STs that differ by two alleles are termed double locus variants (DLV) and were not assigned as members of a CC while those that differ only at a single locus are single locus variants (SLV) and were included.

Table 2.4. Oligonucleotide primer sequences targeting seven housekeeping genes for MLST analysis

Primer	Target	Sequence (5' to 3')[a]	Reference
recA-1F	*recA*	GAA ACC ATT TCA ACG GGT TC	Gonzalez-Escalona et al., 2008
recA-1R		CCA TTG TAG CTG TAC CAA GCA CCC	
gyrB-1F	*gyrB*	GAA GGB GGT ATT CAA GC	Gonzalez-Escalona et al., 2008
gyrB-1R		GAG TCA CCC TCC ACW ATG TA	
dnaE-1F	*dnaE*	CGR ATM ACC GCT TTC GCC G	Gonzalez-Escalona et al., 2008
dnaE-1R		GAK ATG TGT GAG CTG TTT GC	
dtdS-1F	*dtdS*	TGG CCA TAA CGA CAT TCT GA	Gonzalez-Escalona et al., 2008
dtdS-1R		GAG CAC CAA CGT GTT TAG C	
pntA-1F	*pntA*	ACG GCT ACG CAA AAG AAA TG	Gonzalez-Escalona et al., 2008
pntA-1R		TTG AGG CTG AGC CGA TAC TT	
pyrC-1F	*pyrC*	AGC AAC CGG TAA AAT TGT CG	Gonzalez-Escalona et al., 2008
pyrC-1R		CAG TGT AAG AAC CGG CAC AA	
tnaA-1F	*tnaA*	TGT ACG AAA TTG CCA CCA AA	Gonzalez-Escalona et al., 2008
tnaA-1R		AAT ATT TTC GCC GCA TCA AC	
recA-F1	*recA*	CAT GCG CCT TGG TGA TAA	Theethakaew et al., 2013
recA-R1		CAG GTG CTT CTG GTT GAG	
dnaE-F1	*dnaE*	CGA GAT TCG TGT TGC GAT	Theethakaew et al., 2013
dnaE-R1		CTA GCG TCA TAC CCG GAT	
recA-up_rev	*recA*	ACG GAT TTG GTT GAT GAA GAT ACA	Urmersbach et al., 2014
recA-down_for		GGG TCT CCA AGC TCG TAT GC	
gyrB-up_rev	*gyrB*	CGA TTC AAC CGC TGA TTT CAC TTC	Urmersbach et al., 2014
gyrB-down_for		GCG GCA CTA ACA CGT ACG CTA AAC	

[a] M13F and M13R tailed primers were used (M13 primers sequences are listed at https://pubmlst.org/vparahaemolyticus/ and were attached to these target primers)

2.3 Results

2.3.1 Distribution of TDH and TRH Genotypes

All 600 isolates tested were positive for the presence of the R72H fragment and confirmed as *V. parahaemolyticus*. As summarized in Table 2.5, 30 of 64 (46.9%) clinical isolates and 491 of 536 (91.6%) environmental isolates were negative for both *tdh* and *trh;* in comparison, 20 (31.3%) clinical and 44 (8.2%) environmental isolates contained both the *tdh* and *trh* genes. *V. parahaemolyticus* isolates

possessing at least one virulence factor (*tdh* or *trh*) formed 21.9% (n=14) of the clinical strains and 0.2% (n=1) of the environmental strains tested.

Table 2.5. Distribution of *tdh* and *trh* genotypes for all *V. parahaemolyticus* isolates (n=600)

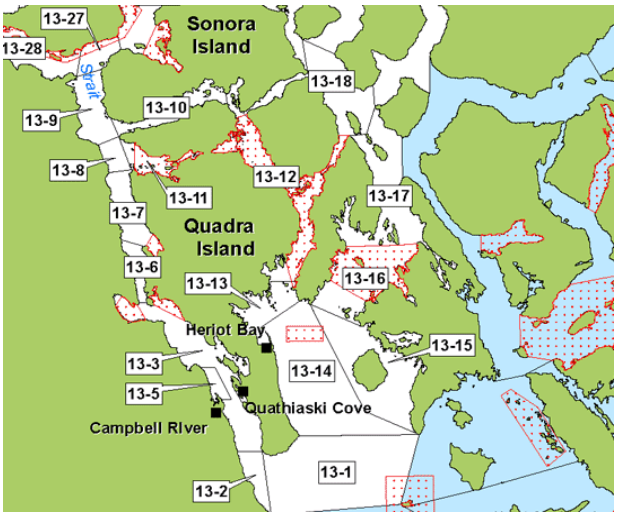
Toxin Gene Profile	**All Strains**		**Clinical Strains**		**Environmental Strains**			
					Pacific (BC)		Atlantic	
	N	%	n	%	n	%	n	%
tdh +, *trh* −	7	1.2	6	9.4	1	0.2	0	0.0
tdh −, *trh* +	8	1.3	8	12.5	0	0.0	0	0.0
tdh +, *trh* +	64	10.7	20	31.3	40	7.7	4	22.2
tdh −, *trh* −	521	86.8	30	46.9	477	92.1	14	77.8
Total	**600**	**100**	**64**	**100**	**518**	**100**	**18**	**100**

Environmental *V. parahaemolyticus* isolates were cultured from oysters harvested annually between May and October from 1998 to 2011. All 4 of the *tdh*-positive and *trh*-positive Atlantic isolates originated from oysters harvested from Baie de Caraquet, New Brunswick between August and September 2005. While the vast majority of BC environmental isolates were negative for *tdh* and *trh* and originated from 6 major oyster growing areas (Figure 2.2), 41 strains did possess at least one of these markers (Table 2.6). Specifically, 1 *tdh*-positive, *trh*-negative isolate originated from Coffin Point (Area 17-05) and the 40 *tdh*-positive, *trh*-positive isolates came from Gorge Harbour (Area 13-15; n=4), Okeover Inlet (Area 15-04; n=6), Sykes Island (Area 16-12; n=1), Coffin Point (Area 17-05; n=11), Ladysmith Harbour (Area 17-07; n=2), Ritherdon Bay (Area 23-03; n=2), Effingham Inlet (Area 23-06; n=10), and Lagoon Island (Area 24-09; n=4).

Table 2.6. Distribution of *tdh* and *trh* genotypes for BC environmental *V. parahaemolyticus* isolates (n=518)

Toxin Gene Profile	Area 13		Area 14		Area 15		Area 16		Area 17		Area 23		Area 24	
	n	%	N	%	n	%	n	%	n	%	n	%	n	%
tdh +, *trh* −	0	0	0	0	0	0	0	0	1	0.6	0	0	0	0
tdh −, *trh* +	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tdh +, *trh* +	4	14.8	0	0	6	7.3	1	1.0	13	7.2	12	40.0	4	80.0
tdh −, *trh* −	23	85.2	89	100	76	92.7	104	99.0	166	92.2	18	60.0	1	20.0
Total	**27**	**100**	**89**	**100**	**82**	**100**	**105**	**100**	**180**	**100**	**30**	**100**	**5**	**100**

A. Department of Fisheries and Oceans Canada Area 13 (n=11)



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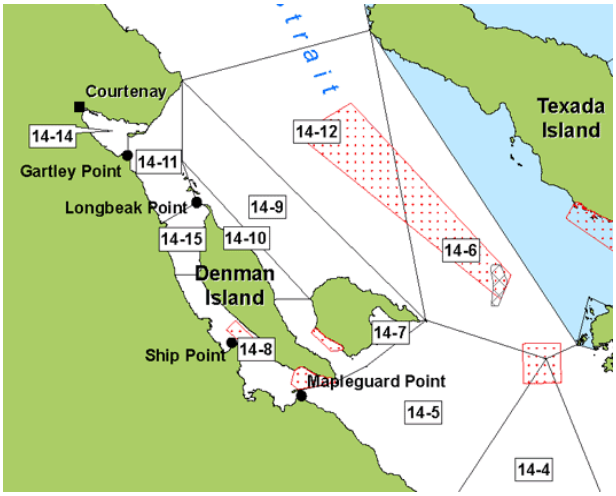
Area 13-15 Gorge Harbour (n=11)

Year	Sequence Type
1998	1205
1999	1565
2000	324, 1521
2001	762
2007	23
2008	1213
2009	135
2010	138
2011	417, 770

Figure 2.2. Geographic distribution of *V. parahaemolyticus* sequence types isolated from 6 major oyster producing areas in British Columbia including Area 13 (A), Area 14 (B), Area 15 (C), Area 17 (D), Area 16 (E), and Area 23 (F).

B. Department of Fisheries and Oceans Canada Area 14 (n=24)



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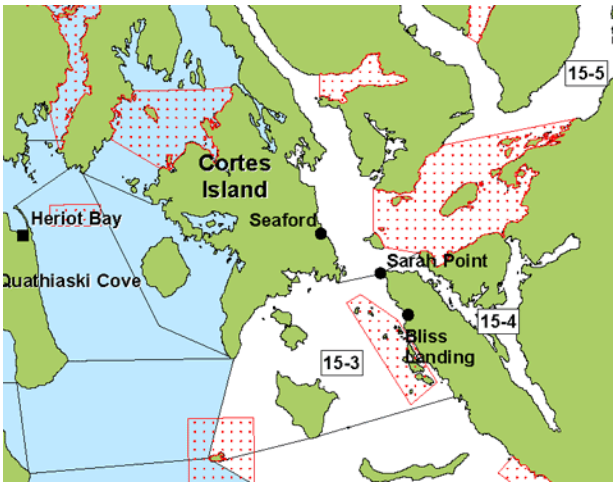
Area 14-08 Denman Island (n=15)

Year	Sequence Type
2008	137, 387, 1527
2009	1521, 1526
2010	1521
2011	322, 323, 1205, 1520, 1521

Area 14-08 Ship's Point (n=9)

Year	Sequence Type
1999	322, 794, 1205
2000	1205, 1521
2001	1205, 1529, 1563

C. Department of Fisheries and Oceans Canada Area 15 (n=17)



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Area 15-03 Twin Island (n=6)

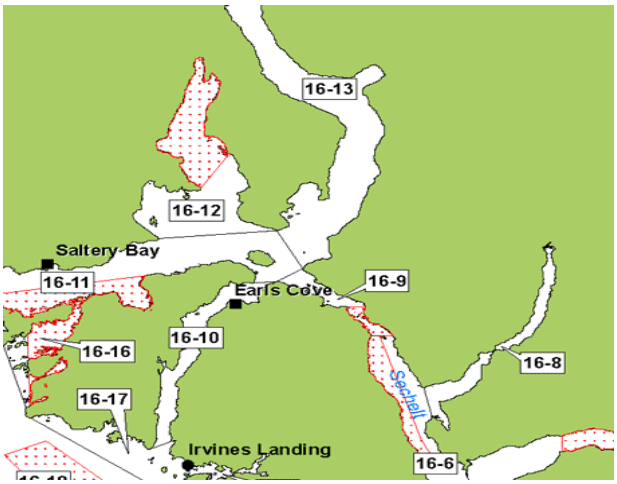
Year	Sequence Type
1998	135
1999	1564
2000	135, 762
2001	1189, 1346

Area 15-04 Okeover Inlet (n=11)

Year	Sequence Type
1999	1567
2004	34
2005	34
2008	137, 1556
2009	1213, 1528
2010	138, 1553
2011	138, 1552

Figure 2.2. Continued.

D. Department of Fisheries and Oceans Canada Area 16 (n=26)



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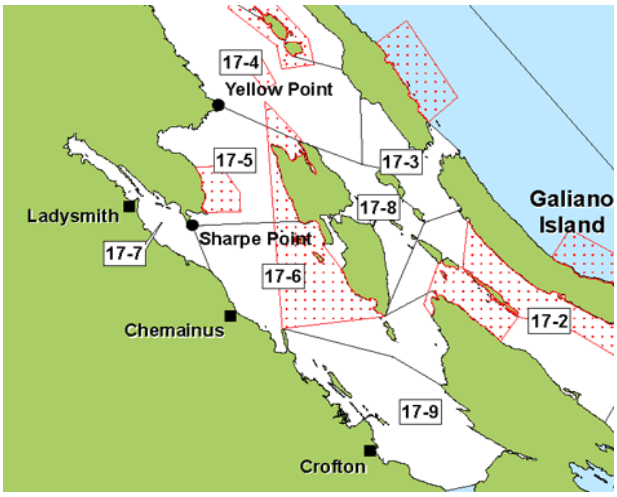
Area 16-12 Sykes Island (n=13)

Year	Sequence Type
2006	36
2008	141, 1189, 1528
2009	322, 1525, 1526
2010	1523, 1524
2011	1346, 1519, 1522

Area 16-13 Dark Cove (n=13)

Year	Sequence Type
1998	762, 1568
1999	1522
2000	1522
2001	135, 1203

E. Department of Fisheries and Oceans Canada Area 17 (n=41)



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Area 17-05 Coffin Point (n=21)

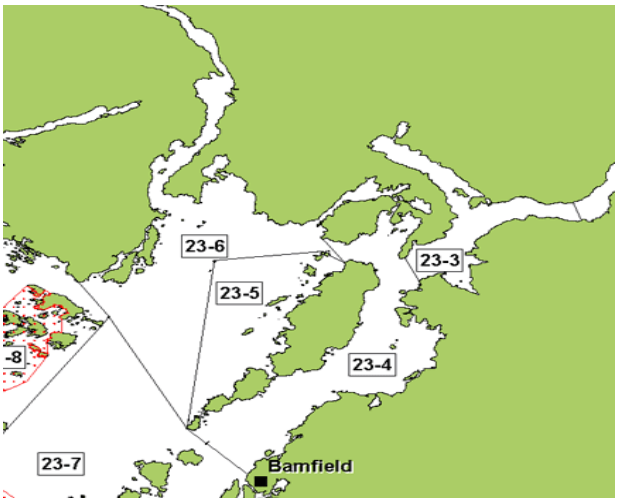
Year	Sequence Type
2006	23
2008	323, 1555
2009	1202, 1554
2010	137
2011	12, 23, 138, 322, 417, 1202, 1203, 1204, 1211, 1212

Area 17-07 Ladysmith Harbour (n=20)

Year	Sequence Type
1998	1039, 1568
1999	770, 1566
2000	137
2001	1203, 1562
2002	1203, 1561
2003	1203, 1521
2004	762, 1520
2005	470, 735, 1211

Figure 2.2. Continued.

F. Department of Fisheries and Oceans Canada Area 23 (n=11)



© Department of Fisheries and Oceans Canada, Fisheries Management Area 23 – Cape Beale, Ucluelet, http://www.pac.dfo-mpo.gc.ca/fm-gp/maps-cartes/areas-secteurs/23-eng.html

Area 23-06 Effingham Inlet (n=9)

Year	Sequence Type
2004	43
2006	15
2007	43
2008	43, 762
2009	36
2010	43, 1204
2011	324

Area 23-06 Useless Inlet (n=2)

Year	Sequence Type
1998	10
2001	1155

Figure 2.2. Continued.

2.3.2 Distribution of Sequence Types

Overall, a subset of 166 *V. parahaemolyticus* cultures were subjected to MLST analysis, which revealed that isolates belonged to 67 unique STs, 36 (54%) of which were new in comparison to PubMLST database entries (Table 2.7). Forty of the STs contained a single isolate while 27 STs included between 2 and 18 isolates. The most common STs amongst this Canadian data set were ST417 (n=18), ST43 (n=9), ST137 (n=8), ST1521 (n=8), ST36 (n=7), ST1205 (n=7), ST1203 (n=6), and ST1522 (n=6).

Table 2.7. Sequence types of the *V. parahaemolyticus* analyzed in the present study

	No. of Isolates	No. of STs	No. of New STs	STs Observed (Frequency)[a, b]
Clinical				
Shellfish Exposure	18	4	0	36 (3), 43 (3), 417 (11), 639 (1)
Unknown	13	6	0	3 (2), 36 (2), 43 (2), 332 (1), 417 (5), 637 (1)
Environmental				
Area 13	11	11	4	23 (1), 135 (1), 138 (1), 324 (1), 417 (1), 762 (1), 770 (1), **1205** (1), **1213** (1), **1521** (1), **1565** (1)
Area 14	24	13	7	137 (1), 322 (1), 323 (1), 332 (1), 387 (1), 794 (1), **1205** (6), **1520** (1), **1521** (6), **1526** (2), **1527** (1), **1529** (1), **1563** (1)
Area 15	17	14	8	34 (2), 135 (2), 137 (1), 138 (2), 762 (1), 1189 (1), **1213** (1), **1346** (1), **1528** (1), **1552** (1), **1553** (1), **1556** (1), **1564** (1), **1567** (1)
Area 16	21	16	9	36 (1), 135 (1), 141 (1), 322 (1), 762 (1), 1189 (1), **1203** (1), 1346 (1), **1519** (1), **1522** (6), **1523** (1), **1524** (1), **1525** (1), **1526** (1), **1528** (1), **1568** (1)
Area 17	41	25	13	12 (1), 23 (2), 137 (6), 138 (2), 322 (2), 323 (1), 417 (1), 470 (1), 735 (1), 762 (1), 770 (1), 1039 (1), **1202** (4), **1203** (5), **1204** (1), **1211** (2), **1212** (1), **1520** (1), **1521** (1), **1554** (1), **1555** (1), **1561** (1), **1562** (1), **1566** (1), **1568** (1)
Area 23	11	8	1	10 (1), 15 (1), 36 (1), 43 (4), 324 (1), 762 (1), 1155 (1), **1204** (1)
Atlantic	10	9	5	445 (1), 471 (1), 630 (1), 1189 (2), **1526** (1), **1557** (1), **1558** (1), **1559** (1), **1560** (1)

[a] Novel Sequence Type (ST) in bold

[b] Underlined ST3, ST36, and ST417 have good association with O3:K6, O4:K12, and O1:KUT serotypes, respectively

All novel STs were recovered from environmental isolates. In contrast, all clinical STs identified in this study matched previously identified STs in the MLST database (Table 2.8). In particular, two clinical

isolates (not necessarily associated with shellfish exposure) were typed as ST3. ST3 (representing the pandemic sequence type) corresponds to clinical isolates recovered from different countries within North America, South America, Asia, Europe, and Africa. Furthermore, it has also been observed in environmental isolates from Chile, China, Thailand, USA, and Mexico. Four STs (ST36, ST43, ST417, and ST639) had also been previously recovered from cases of disease in the US (Washington and Maryland) as well as the environments of the US and China. Of note, 3 STs reported in clinical isolates tested in this study were also identified amongst environmental isolates (Figure 2.3). Specifically, ST36 was recovered from Sykes Island (Area 16-12) in 2006 and Effingham Inlet (Area 23-06) in 2009. Similarly, ST43 was also isolated from oysters collected from Effingham Inlet in 2004, 2007, 2008, and 2010 while ST417 was found in 2011 in both Gorge Harbour (Area 13-15) and Coffin Point (Area 17-05).

Table 2.8. Geographic distribution in literature of Sequence Types (STs) identified among clinical *V. parahaemolyticus* strains isolated in BC between 2008 and 2011.

Sequence Type	Clinical Source	Environmental Source
ST3	Spain, Peru, India, Bangladesh, Japan, US, Thailand, Mozambique, Ecuador, Chile, China, Singapore, Korea, Mexico, Sweden, Canada	Chile, China, Thailand, US, Mexico
ST36	US, Canada	US
ST43	US, Canada	US
ST332	Canada	China
ST417	US, Canada	China
ST637	Canada	China
ST639	US, Chile, Canada	US

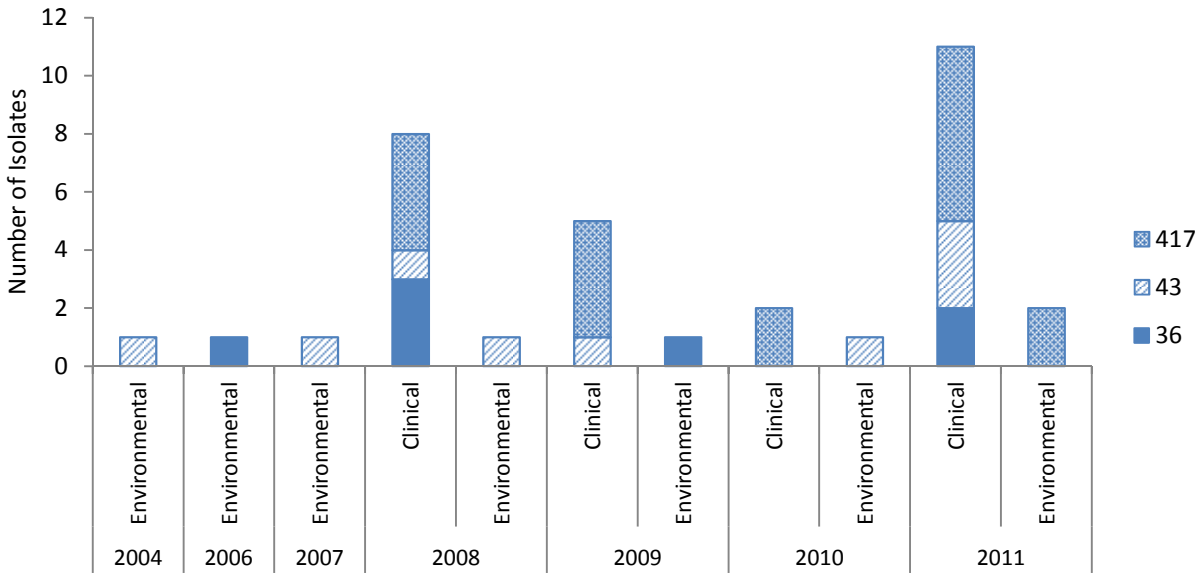


Figure 2.3. Distribution of ST36, ST43, and ST417 amongst Canadian *V. parahaemolyticus* isolates.

To visualize the clonal relationship between isolates the goeBURST algorithm was used. Accordingly, the 67 STs generated in this data set were separated into 1 clonal complex (CC34), 4 doublets (D1, D2, D3, and D4), and 56 singletons (Figure 2.4). CC34 consists of 6 environmental BC isolates. D1 is comprised of 8 isolates representing ST36 (5 clinical, 2 BC environmental) and a single-locus variant, ST639 (1 clinical). These two genotypes differ only in the *gyrB* locus. D2 corresponds to 3 environmental BC isolates representing ST470 and ST1346 where a difference in the *pntA* locus was observed. D3 consists of ST1202 (4 environmental BC isolates) and ST1525 (1 environmental isolate) which differ at the *recA* locus. D4 is comprised of 2 environmental strains that originated from Ship's Point, BC (Area 14-08) representing ST1529 and ST1563 which differ at the *pntA* locus. Based on the PubMLST database, the 67 STs detected were (where applicable) assigned to clonal complexes (CCs) based on their similarity to a central allelic profile. A total of 5 CCs were identified, including CC3 (comprised of ST3 (n=2)), CC34 (comprised of ST34 (n=2) and ST324 (n=2)), CC36 (comprised of ST36 (n=7)), CC155 (comprised of ST794 (n=1)), and CC412 (comprised of ST43 (n=9)).

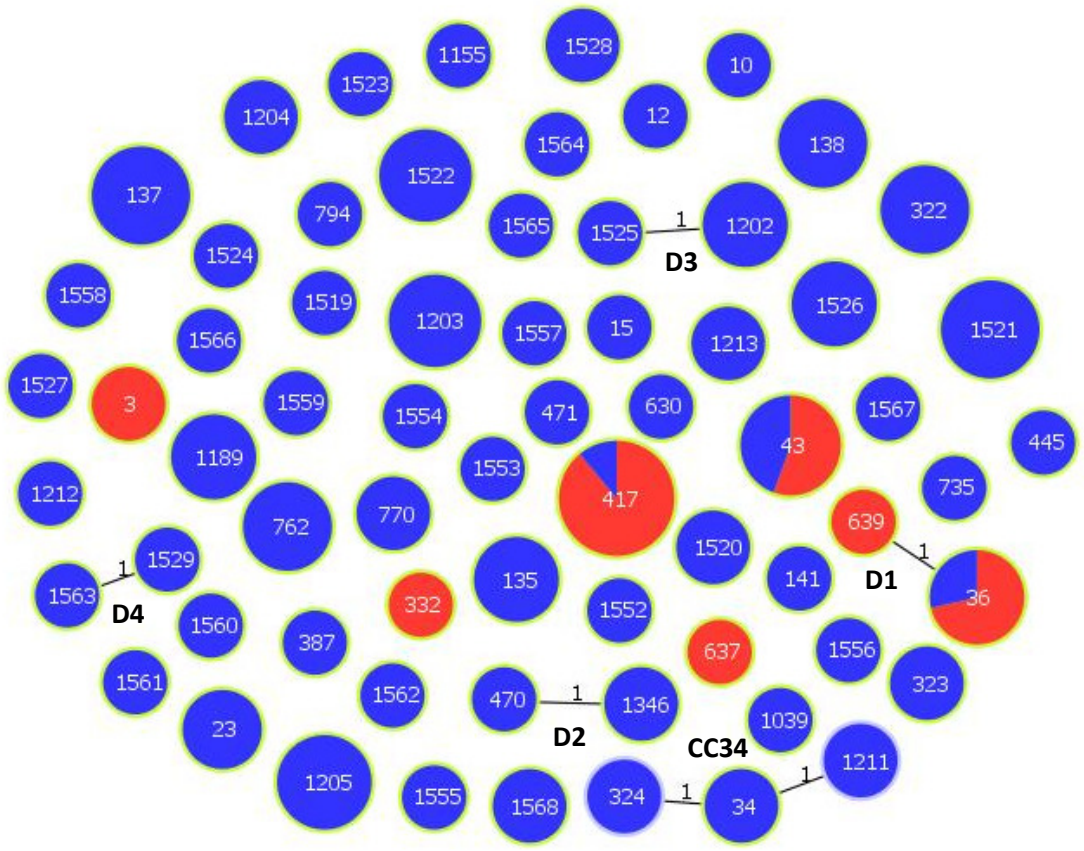


Figure 2.4. goeBURST analysis of 67 STs of *V. parahaemolyticus*. The analysis is based on allelic profiles of MLST data and displays linked and individual STs. Single-locus variants (SLVs) are illustrated by linkage lines among the nodes. Colour coding represents the source of isolation of each ST: red, clinical sample; blue, environmental sample. The frequency of each ST is indicated by the size of its node.

The goeBURST algorithm was also used to obtain a full minimum spanning tree (MST) or 'population snapshot' displaying all connections (with 0 of 7 loci shared) between STs (Figure 2.5).

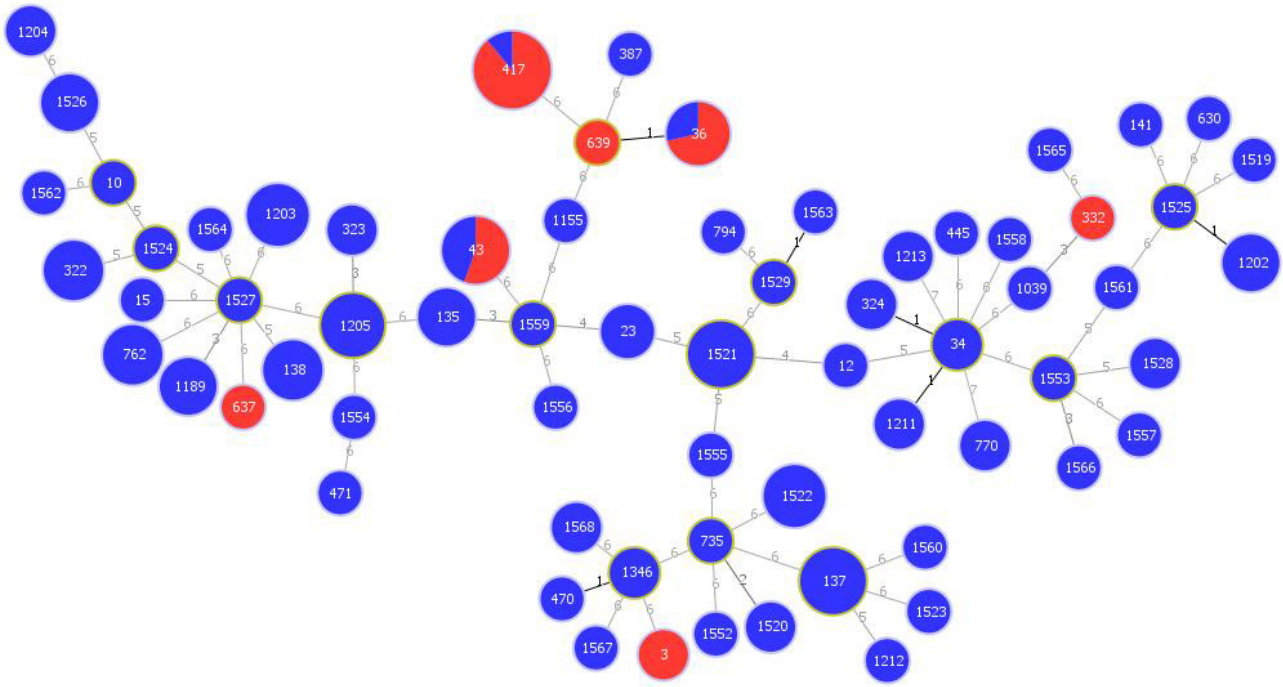


Figure 2.5. Full minimum spanning tree (MST) based on MLST profiles. The number of different alleles is indicated and all connections were drawn. SLVs are connected via black lines and all higher levels with grey lines. Colour coding represents the source of isolation of each ST: red, clinical sample; blue, environmental sample.

2.4 Discussion

Commonly found in marine environments throughout the world, *V. parahaemolyticus* can bio-accumulate in the gut of filter-feeding molluscan shellfish (e.g. oysters) and cause subsequent gastroenteritis in individuals who consume contaminated product. TDH and TRH are considered major virulence factors; accordingly, *tdh*-positive and/or *trh*-positive *V. parahaemolyticus* strains represent a public health risk. This study was undertaken to investigate the diversity and presence of established virulence markers (the *tdh* and *trh* genes) among 600 *V. parahaemolyticus* isolates. To our knowledge,

no other study has characterized such a large panel of *V. parahaemolyticus* strains, including environmentally-derived isolates collected over a decade from temperate Canadian waters.

In this study, of the 64 clinical isolates tested, 53% possessed the *tdh* and/or *trh* gene while 47% lacked both these virulence markers (Table 2.5) suggesting *tdh* and *trh* alone may not fully explain the pathogenicity of *V. parahaemolyticus*. Similar observations of clinical strains lacking *tdh* and *trh* have been reported by other researchers although the prevalence of such strains vary (Banerjee et al., 2014; Jones et al., 2012; Meador et al., 2007; Ludeke et al., 2015; Ottaviani et al., 2012; Hazen et al., 2015; Paranjpye et al., 2012). In particular, Paranjpye et al. (2012) analyzed clinical and environmental *V. parahaemolyticus* strains (n=149; 71 clinical and 78 environmental) isolated from the Pacific Northwest between 1997 and 2007 also reported the majority of clinical strains were positive for *tdh* and *trh* genes. Meanwhile, Jones et al. (2012) found that 27% (n=77) of clinical isolates collected from various parts of the US between 2006 and 2007 were *tdh*-negative and *trh*-negative. The authors noted that the clinical strains were isolated from different sources, including blood (5%), wounds (24%), stool specimens (33%), and "other" unknown origins (38%) and postulated that these *tdh*-negative and *trh*-negative strains were more opportunistic or have decreased potential virulence potential as "other" may refer to infections resulting from recreational water activities as opposed to seafood consumption (Jones et al., 2012). In contrast, Banerjee et al. (2014) identified only 4% (n=100) of the Canadian clinical *V. parahaemolyticus* isolates collected between 2000 and 2009 (2 from BC, 1 from Ontario, and 1 from New Brunswick) did not possess *tdh* or *trh* genes by PCR analysis. After whole genome sequencing (WGS), these *tdh*-negative and *trh*-negative clinically-derived strains were found to have 862 protein-coding genes that were not present in *tdh*-negative and *trh*-negative environmental isolates (Ronholm et al., 2015). The researchers observed several of these genes were highly homologous to genes from other enteric bacteria, and suggested that horizontal gene transfer may play an important role in the

ability of *tdh*-negative and *trh*-negative *V. parahaemolyticus* isolates to survive in the human gastrointestinal tract (Ronholm et al., 2015).

Multiple theories have been proposed to explain why *V. parahaemolyticus* strains lacking *tdh* and *trh* genes are isolated from clinical cases. These include the possibility of co-infection events, loss of virulence genes during infection, and the presence of novel and uncharacterized virulence factors. Bhoonpong et al. (2007) found that a single seafood sample could be contaminated by different strains of *V. parahaemolyticus,* including some of which appeared to be non-pathogens. Therefore, if an individual is co-infected with multiple *V. parahaemolyticus* strains (with at least one infecting strain carrying *tdh* or *trh*), it is possible a *tdh*-negative and *trh*-negative strain could be isolated from the sick patient. Furthermore, Bhoongpong et al. (2007) also examined the variability of *V. parahaemolyticus* isolates from single patients who presented with diarrhea at one hospital in Thailand. From each patient sample (n=63), 10 non-sucrose fermenting colonies on TCBS were randomly selected and tested and it was found that 21 samples (33%) contained heterogeneous populations of *V. parahaemolyticus* where isolates either contained or lacked one or both *tdh* and *trh* genes (Bhoopong et al., 2007). Therefore in a mixed *V. parahaemolyticus* infection, if only a single colony from a single patient sample is analyzed, it is possible an accidental isolation of an avirulent strain may occur. In contrast, there is also evidence to support the opinion that some *tdh*-negative and *trh*-negative isolates are able to cause clinical illness. Within the same study by Bhoophong et al. (2007), 30 isolates from 3 sick patients carried neither *tdh* nor *trh* genes when tested by PCR. Despite multiple culturing attempts, no *tdh*-positive or *trh*-positive *V. parahaemolyticus* strains or other enteric pathogens could be isolated (Bhoopong et al., 2007). Additionally, some studies have demonstrated that deletion of *tdh* and/or *trh* does not affect cytotoxicity towards cultured cells and only partially diminished the severity of enterotoxicity (Park et al., 2004a; Xu et al., 1994). This indicates that these hemolysins are not sole

contributors to pathogenicity and other virulence factors exist (Lynch et al., 2005; Park et al., 2004b). For example, two type III (T3SS1 and T3SS2) and type VI (T6SS1 and T6SS2) secretion systems may play a role in virulence. T3SS1 has been reported to be involved in cytotoxicity and essential for systemic infection while T3SS2 is associated with enterotoxicity in the rabbit ileal loop model and cytotoxicity in intestinal cell lines (Broberg et al., 2011; Wang et al., 2015). Meanwhile, T6SSs are purportedly necessary for the adhesion of *V. parahaemolyticus* to cells (Wang et al., 2015).

Amongst the 536 environmental isolates tested, the vast majority (92%) were negative for both *tdh* and *trh*, while 8% possessed one or both of these markers (Table 2.5). These findings are similar to other published studies (Paranjpye et al., 2012; DePaola et al., 2000; Cook et al., 2002a). Strains that were *tdh*-positive and/or *trh*-positive were observed on both Canadian coasts. Of the 18 Atlantic isolates tested in this study, 4 (22.2%) were *tdh*-positive and *trh*-positive which differs from findings published by Cox and Gomez-Chiarri (2012) who analyzed isolates derived from oysters collected from the coastal waters of Rhode Island and found *trh* was detectable in 43.8% (n=48) of the sample tested while *tdh* was non-detectable. It should be noted that direct comparisons is challenging as the Atlantic strains analyzed in this study were isolated in a short time frame (summer of 2005), and therefore may not be fully representative of the geographic environment, while isolates from research conducted by Cox and Gomez-Chiarri were collected over the summers of 2009 and 2010 (Cox and Gomez-Chiarri, 2012).

For *V. parahaemolyticus* isolates that originated from the Pacific Northwest in this study (n=518), none of the strains were *tdh*-negative and *trh*-positive while 1 strain was *tdh*-positive and *trh*-negative and 40 strains were *tdh*-positive and *trh*-positive. Strains positive for *tdh* and/or *trh* were found in various oyster growing areas including sites located in Department Fisheries and Oceans Canada Area 13, Area 15, Area 16, Area 17, Area 23, and Area 24 (Table 2.6). While the abundance of *tdh*-positive or *trh*-

positive *V. parahaemolyticus* may vary depending on the geographic or ecological niche, these strains have been found in various regions of the PNW since 1979 (Nolan et al., 1984; Kelly and Stroh, 1989; Abbott et al., 1989). Further, Kaufman et al. (2002) investigated the occurrence of hemolysin genes amongst isolates from infected human patients as well as oysters collected from the PNW between 1988 and 1997. The authors found all *V. parahaemolyticus* isolates from infected human patients from the 1997 PNW outbreak possessed *tdh* and *trh* genes and 72.3% of shellfish tested (n=11) also tested positive for both virulence markers (Kaufman et al., 2002). Similarly, *V. parahaemolyticus* strains (n=78) isolated from shellfish growing areas in the PNW of the United States (predominantly during 2007 from Hood Canal in Washington state) were examined and found that a large proportion were *tdh*-positive and *trh*-negative; nearly 60% of the isolates possessed the *tdh* gene (Paranjpye et al., 2012). More recent studies have reported *trh*-positive isolates have been predominantly detected in temperate regions of the world (Rodriguez-Castro et al., 2010; Ellingsen et al., 2008; Serracca et al., 2011); this was not seen in the present study. However, it should be noted that 58% of the environmental BC strains analyzed in this study were isolated in earlier years (1998 to 2005), when mostly *tdh*-positive or *tdh*-positive and *trh*-positive environmental *V. parahaemolyticus* have been reported (Kaufman et al., 2002; DePaola et al., 2003; Alam et al., 2003; Wagley et al., 2008; DePaola et al., 2000; Lozano-Leon et al., 2003).

To explore the extent of genetic diversity of *V. parahaemolyticus* found in Canada, a subset of isolates was subjected to MLST analysis. Overall, the data generated in this study supports other research that illustrated the *V. parahaemolyticus* population structure is highly diverse (Gonzalez-Escalona et al., 2008; Theethakaew et al., 2013; Urmersbach et al., 2014; Turner et al., 2013; Haendiges et al., 2015). A total of 67 unique STs were identified among the 166 isolates, and 36 (54%) of these were novel (Table 2.7). Certain STs are widely distributed across continents, while some predominate in specific regions.

In particular, ST3 has an international distribution having been isolated from clinical sources from 5 continents including North America, South America, Europe, Asia, and Africa (Table 2.8) while ST36 has historically been found in the Pacific Northwest (Han et al., 2014; Turner et al., 2013; Martinez-Urtaza et al., 2013). Not surprisingly, among the 31 BC clinical isolates tested in this study, ST3 and ST36 were recovered; ST43, ST332, ST417, ST637, and ST639 were also recovered (Table 2.8) and had previously been isolated from clinical cases primarily originating from the Pacific coast. As multiple STs have contributed to human infections in BC, *V. parahaemolyticus* gastroenteritis in this region is polyphyletic in nature. More recently, ST36 has shown to be responsible for large outbreaks that occurred in 2012 in the east coast of the United States as well as Spain and raising concerns of its spread across oceans and becoming endemic to other regions (Martinez-Urtaza et al., 2016; Haendiges et al., 2015).

Of the 135 environmental *V. parahaemolyticus* isolates tested by MLST, 63 unique STs were identified (Table 2.7). Of note, 36 novel STs were found and all were recovered from environmental isolates. In this study, 4 new alleles were discovered for *dnaE*, *dtdS*, and *pntA*, while 5 new alleles were found for *gyrB*, *pyrC*, and *tnaA*, and 6 new alleles were revealed for *recA*; these allelic variants gave rise to the new STs. The novel STs were recovered from strains that were isolated between 1998 and 2011 from various locations in Canada. Consistent with other MLST-based studies, the high degree of environmental diversity observed in this investigation, even from strains originating within a single geographic locality, is indicative of the poor representation of the genetic diversity of *V. parahaemolyticus* within the current PubMLST database (Theethakaew et al., 2013; Urmersbach et al., 2014). Generally, the PubMLST database reflects the diversity of strains analyzed thus far and may not represent the natural diversity of the *V. parahaemolyticus* population. As of January 2017, a total of 1712 unique STs have been identified encompassing 904 clinical strains and 1616 environmental isolates collected from Asia (58.1%), USA and Mexico (17.2%), Europe (9.8%), and South America (7.5%). Accordingly, continued

analysis of strains from different locations and sources may facilitate in garnering a more representative dataset.

Within this study, 3 STs (ST36, ST43, and ST417) that were recovered from clinical isolates were also recovered from environmental isolates (Figure 2.3) confirming the marine environment is a reservoir of potentially virulent *V. parahaemolyticus* strains. Further examination into STs that have been published by Banerjee et al. (2014) to have caused illness in Canada revealed that ST15, ST 141, ST324, and ST630 (in addition to ST36, ST43, and ST417) have also been isolated from environmental isolates. In particular, ST15 was recovered from Effingham Inlet (Area 23-06) in 2006, ST141 was recovered from Sykes Island (Area 16-12) in 2008, ST324 was recovered from Gorge Harbour (Area 13-12) in 2000 as well as Effingham Inlet in 2011, and ST630 was recovered from Baie de Caraquet in 2005. While MLST information for strains originating from the Atlantic coast of Canada and tested as part of this study was limited (n=10), ST1526 was isolated from oysters harvested Baie de Caraquet, New Brunswick in 2005 as well as oysters harvested from Denman Island, BC in 2009 and Sykes Island, BC in 2009. Similarly, ST1189 was isolated from oysters collected from Orwell River and East River (both in Prince Edward Island in 2005) as well as oysters collected from Twin Island, BC in 2001 and Sykes Island, BC in 2008. So far, it is unclear how these STs migrated from one coast to another.

The goeBURST algorithm was used to investigate the genetic relationships, such as identification and assignment of clonal complexes based on STs, among the 166 *V. parahaemolyticus* isolates. Overall, due to the high degree allelic diversity, there was limited ability in identifying closely related strains at the nucleotide level (Figure 2.4 and Figure 2.5). Despite the limitations, goeBURST analysis defined one clonal complex (CC34) within the strains tested in the present work; however 6 STs belong to 5 clonal complexes (CC3, CC34, CC36, CC155, and CC412) as defined by the PubMLST schema. When isolates are

more distantly related, such as the 56 singletons, minimal information can be gained about their relationships. While not conducted as part of this current study, other investigators have reported increased utility of goeBURST in revealing relationships among isolates if amino acid sequences were used (Urmersbach et al., 2014; Theethakaew et al., 2013; Han et al., 2015).

2.5 Conclusions

In summary, *tdh* and *trh* genes were detected in *V. parahaemolyticus* isolates recovered from clinical sources in BC as well as from oysters harvested from the coastal waters of Canada. Notably, a proportion of the clinical isolates tested lacked both these virulence markers highlighting the virulence potential of *V. parahaemolyticus* is more complex than historically thought. These *tdh*-negative and *trh*-negative clinical strains have demonstrated an ability to survive *in vivo* during illness but their role is unclear (e.g. causative agent, innocuous bystander, or active participant in a multi-strain infection). More discriminatory analyses, such as whole genome sequencing, of *tdh*-negative and *trh*-negative isolates may elucidate other factors and/or predictors of *V. parahaemolyticus* virulence that could be incorporated into diagnostic tests. MLST-based analysis of a subset of isolates demonstrated the *V. parahaemolyticus* population is diverse. A large proportion (54%) of the STs had not been previously identified in the PubMLST database and all the novel STs were recovered from environmental strains. In comparison, a smaller number of STs appear to be responsible for illnesses in BC. Furthermore, some of these STs were also recovered from oysters confirming the marine environment is a reservoir for virulent strains and suggests that a subset of *V. parahaemolyticus* isolates may be adapted to life in the marine environment as well as the human host.

Chapter 3 – Antimicrobial Resistance of Canadian Isolates

3.1 Introduction

Seafood is a nutrient rich part of a healthy diet and its consumption is associated with various benefits (Iwamoto et al., 2010). However, concomitant with nutritional benefits is the potential risk of eating contaminated seafood. In particular, bivalve molluscan shellfish filter-feed, thereby bio-accumulating naturally occurring microorganisms or anthropogenic contaminants from surrounding waters; additionally, the hazards posed by bioaccumulation can be further compounded by traditional consumption of certain shellfish in raw or undercooked dishes (Lees, 2000).

Vibrio parahaemolyticus is a naturally occurring marine organism with a worldwide distribution in estuaries and coastal environments. Consequently, *V. parahaemolyticus* bio-accumulates in the gut of filter-feeding shellfish, such as oysters, and has been the leading cause of seafood-borne illness in various parts of the world. While not all strains are considered to be pathogenic, currently the World Health Organization considers *V. parahaemolyticus* strains producing a thermostable direct hemolysin and/or a TDH-related hemolysin pathogenic to humans. In Canada, and particularly British Columbia, the rate of *V. parahaemolyticu*s infections has steadily increased since 2008 (BCCDC, 2015). While most *V. parahaemolyticus* infections are self-limiting, antimicrobial therapy may be prescribed for patients with severe or prolonged diarrhea or individuals with underlying medical conditions (Elmahdi et al., 2016; Butt et al., 2004; Han et al., 2007).

During the past decade, antimicrobial resistance has emerged in many species of microorganisms due to the increased use of antimicrobials in humans, agriculture, and aquaculture (Cabello, 2006; Mazel and

Davies, 1999). The role of the environment including water bodies such as rivers, streams, waste water effluents, and lakes may be important in facilitating the transfer of antibiotic resistance genes (Igbinosa et al., 2011; Lupo et al., 2012). Moreover, persistent low concentrations of antibiotics in the environment may result in the maintenance and selection of multi-drug resistant (MDR) bacteria (Gullberg et al., 2011).

Traditionally, *V. parahaemolyticus* was considered to be susceptible to most antibiotics of veterinary and human significance with documented ampicillin resistance (Zanetti et al., 2001; Han et al., 2007). More recent studies, conducted in different countries and aimed at characterizing AMR profiles, have reported MDR *V. parahaemolyticus* show reduced susceptibility to one or more antimicrobial agents and/or classes (Ottaviani et al., 2013b; Elmahdi et al., 2016; Hu and Chen, 2016; Kang et al., 2016).

Most studies investigating AMR in *V. parahaemolyticus* have been conducted outside of North America. In particular, information on *V. parahaemolyticus* AMR, including longitudinal data examining the dynamics of AMR patterns, in Canada is limited. Therefore, this study was undertaken to address this knowledge gap. Antibiogram profiles of toxigenic and non-toxigenic *V. parahaemolyticus* strains isolated between 1998 and 2011 from shellfish and clinical samples in Canada (predominantly BC) were determined.

3.2 Materials and Methods

3.2.1 Bacterial Strains

In total, 600 *V. parahaemolyticus* isolates were analyzed in this study. The strain set included 64 clinical isolates obtained from a BC medical laboratory between 2008 and 2011 (Table 2.1) and 536 isolates

recovered from oysters collected between 1998 and 2011. As summarized in Table 2.2, the environmental isolates were derived from oysters collected from several sites along the west coast of BC (97%) as well as the Atlantic coast (3%) where harvesting was permitted.

3.2.2 TDH and TRH Detection

All strains were genetically confirmed as *V. parahaemolyticus* and screened for the presence of the *tdh* and *trh* toxin genes as described in Chapter 2.2.2 of this thesis.

3.2.3 Antimicrobial Susceptibility Determination

AMR phenotypes were determined using a disc diffusion technique. Each *V. parahaemolyticus* isolate was resuscitated from -75°C onto tryptic soy agar supplemented with 1.5% sodium chloride (TSA-NaCl) and incubated overnight at 35°C. Following this, a single well-isolated colony was transferred to 10 mL of tryptic soy broth supplemented with 1.5% sodium chloride (TSB-NaCl) and incubated overnight at 35°C. Overnight TSB-NaCl broth cultures were then used to create 0.5 McFarland suspensions in 3 mL of TSB-NaCl. Each suspension was swabbed over the entire surface of a Mueller-Hinton agar plate, with swabbing repeated two times (rotating the plate 60° each time) to ensure even distribution of the inoculum. To ensure any excess surface moisture was absorbed, inoculated plates were left at room temperature for approximately 5 minutes prior to applying the antibiotic discs. Once antimicrobial impregnated test discs were applied, the plates (inverted) were incubated at 35°C for 16 – 18 hours. A panel of 20 antimicrobials recommended by the Clinical and Laboratory Standards Institute (CLSI) M45 document for infrequently isolated or fastidious bacteria (including *Vibrio* spp.) was used (Table 3.1) (CLSI, 2010). Zones of inhibition were measured to the nearest millimetre and each isolate was classified as resistant (R), intermediately resistant (I), or susceptible (S), according to the guidelines of

the CLSI. For each batch of *V. parahaemolyticus* testing, *Staphylococcus aureus* ATCC 25933, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 (using TSA and TSB where appropriate), were also processed in parallel as quality control strains as the zone of inhibition for these organisms are well established.

Table 3.1. Panel of 20 antibiotics recommended by the Clinical and Laboratory Standards Institute for infrequently isolated or fastidious bacteria (including *Vibrio* spp.) and used in the present study.

Antimicrobial Agent		Abbreviation	Concentration (μg)
Aminoglycosides	Gentamicin	GEN	10
	Kanamycin	KAN	30
	Streptomycin	STR	10
Cephems	Cefotaxime	CTX	30
	Ceftiofur	TIO	30
	Cephalothin	CEF	30
Folate Pathway Inhibitor	Trimethoprim/sulfamethoxazole	SXT	1.5/23.75
Lipopeptide	Polymyxin B	PMB	300
Macrolide	Erythromycin	ERY	5
Penicillins	Piperacillin	PIP	100
	Ampicillin	AMP	10
Phenicol	Chloramphenicol	CHL	30
Quinolones	Ciprofloxacin	CIP	5
	Enrofloxacin	ENR	5
	Nalidixic Acid	NAL	30
	Norfloxacin	NOR	10
	Oxolinic Acid	OA	2
Sulfonamide	Sulfisoxazole	SUL	250
Tetracyclines	Tetracycline	TET	30
	Oxytetracycline	T	30

3.2.4 Statistical Analysis

For each antibiotic, differences among *V. parahaemolyticus* strains exhibiting resistance were assessed using a Chi-squared (χ^2) test. In addition, the relationship between virulence gene profile (*tdh* and *trh*) and AMR was also examined using a χ^2 test for strains that displayed antibiotic resistance. When 20% or

more of the cells in a 2 x 2 contingency table had a value less than 5, Fisher's exact test was used instead of the χ^2 test. Results were considered significant when the *p*-value was less than 0.05. Data were analyzed using GraphPad Prism 7.0 (GraphPad software, La Jolla, California, USA).

3.3 Results

3.3.1 Overall Antimicrobial Resistance Patterns

Of the 600 isolates tested, all isolates were susceptible to CTX, NAL, NOR, and T while 91% and 29.2% of isolates exhibited resistance to AMP and CEF, respectively (Figure 3.1). Smaller percentages of the isolates were resistant to STR (4.3%), SUL (2.7%), PIP (2.3%), OA (0.5%), TIO (0.3%), GEN (0.2%), and KAN (0.2%).

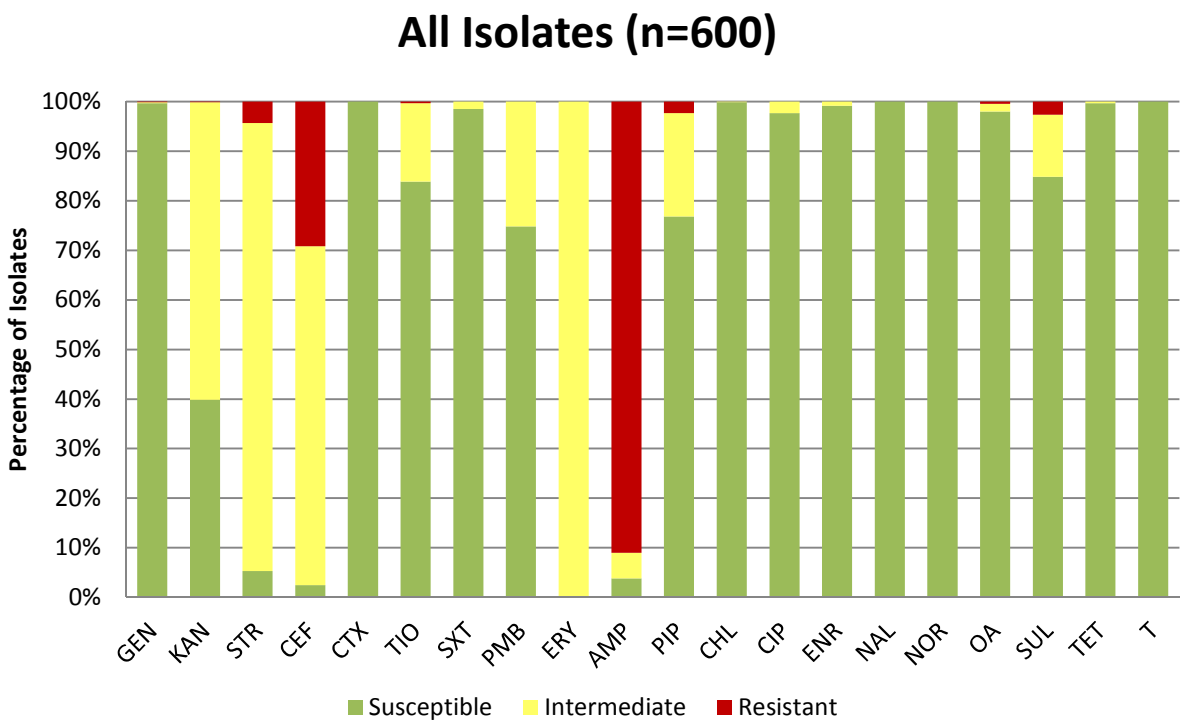


Figure 3.1. Antimicrobial resistance patterns of *V. parahaemolyticus* isolated in Canada between 1998 and 2011 (n=600).

Resistance in individual isolates ranged from 0 (pansusceptible) to 4 antibiotics. Overall, the prevalence of multiple drug resistance, defined as resistance to three or more antimicrobial agents in this study, was low (Table 3.2). Of 600 isolates, 3 (0.5%) were resistant to 4 antibiotics and 26 (4.3%) were resistant to 3 antibiotics. The majority of strains (60.3%) were resistant to 1 antibiotic.

Table 3.2. Distribution of multi-drug resistant phenotypes for all *V. parahaemolyticus* isolates (n=600)

Antimicrobial Resistance	**All Strains**		**Clinical Strains**		**Environmental Strains**			
					Pacific (BC)		Atlantic	
	n	%	n	%	n	%	n	%
0	43	7.2	9	14.1	34	6.6	0	0
1	362	60.3	53	82.8	292	56.4	17	94.4
2	166	27.7	2	3.1	163	31.5	1	5.6
3	26	4.3	0	0	26	5.0	0	0
4	3	0.5	0	0	3	0.6	0	0
5	0	0.0	0	0	0	0.0	0	0
Total	**600**	**100**	**64**	**100**	**518**	**100**	**18**	**100**

3.3.2 Antimicrobial Resistance Patterns Based on Isolation Source

The frequency of clinical and environmental isolates exhibiting resistance to each antibiotic (Table 3.3) was assessed. Resistance to AMP ($\chi^2 = 5.864$, $p = 0.0155$) and CEF ($\chi^2 = 18.211$, $p < 0.0001$) were significantly more common in environmental *V. parahaemolyticus* isolates than those from clinical sources; no significant differences were observed for the other antibiotics.

Table 3.3. Antimicrobial resistance of *V. parahaemolyticus* strains isolated from clinical and environmental samples (n=600).

Antibiotic[a]	Clinical Isolates (n=64)						Environmental Isolates (n=536)					
	S[b]		I[b]		R[b]		S[b]		I[b]		R[b]	
	n	%	N	%	n	%	n	%	n	%	n	%
GEN	64	100	0	0	0	0	534	99.6	1	0.2	1	0.2
KAN	50	78.1	14	21.9	0	0	189	35.3	346	64.6	1	0.2
STR	22	34.4	42	65.6	0	0	10	1.9	500	93.3	26	4.9
CTX	64	100	0	0	0	0	536	100	0	0	0	0
TIO	62	96.9	2	3.1	0	0	441	82.3	93	17.4	2	0.4
CEF	10	15.6	50	78.1	4	6.3	5	0.9	360	67.2	171	31.9
SXT	63	98.4	1	1.6	0	0	528	98.5	8	1.5	0	0
PMB	61	95.3	3	4.7	0	0	388	72.4	148	27.6	0	0
ERY	0	0	64	100	0	0	1	0.2	535	99.8	0	0
PIP	63	98.4	1	1.6	0	0	398	74.3	124	23.1	14	2.6
AMP	1	1.6	10	15.6	53	82.8	22	4.1	21	3.9	493	92.0
CHL	64	100	0	0	0	0	535	99.8	1	0.2	0	0
CIP	60	93.8	4	6.3	0	0	526	98.1	10	1.9	0	0
ENR	64	100	0	0	0	0	531	99.1	5	0.9	0	0
NAL	64	100	0	0	0	0	536	100	0	0	0	0
NOR	64	100	0	0	0	0	536	100	0	0	0	0
OA	64	100	0	0	0	0	524	97.8	9	1.7	3	0.6
SUL	58	90.6	6	9.4	0	0	451	84.1	69	12.9	16	3.0
TET	62	96.9	2	3.1	0	0	536	100	0	0	0	0
T	64	100	0	0	0	0	536	100	0	0	0	0

[a] GEN, gentamicin; KAN, kanamycin; STR, streptomycin; CTX, cefotaxime; TIO, ceftiofur; CEF, cephalothin; SXT, trimethoprim/sulfamethoxazole; PMB, polymyxin B; ERY, erythromycin; PIP, piperacillin; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; NAL, nalixidic acid; NOR, norfloxacin; OA, oxolinic acid; SUL, sulfisoxazole; TET, tetracycline; T, oxytetracycline

[b] S, susceptible; I, intermediate resistance; R, resistant

Discernable differences were observed in the distribution of multiple resistances with respect to the source of isolation (Table 3.2). Clinical isolates were resistant to no more than 2 antibiotics while environmental isolates displayed resistance for up to 4 antibiotics. A total of 9 different multi-drug antibiogram profiles were observed with isolates displaying resistance for up 3 different antibiotic classes (Table 3.4).

Table 3.4. Multi-drug resistance profiles observed**.**

Antibiogram Profile[a]	Clinical Isolates	Environmental Isolates
STR, CEF, AMP	0	12
CEF, AMP, PIP	0	6
CEF, AMP, PIP, OA	0	2
STR, CEF, AMP, PIP	0	1
GEN, CEF, AMP	0	1
CEF, AMP, SUL	0	4
AMP, PIP, OA	0	1
AMP, PIP, SUL	0	1
CEF, TIO, AMP	0	1
Total	**0**	**29**

[a] STR, streptomycin; CEF, cephalothin; AMP, ampicillin; PIP, piperacillin; OA, oxolinic acid; GEN, gentamicin; SUL, sulfisoxazole; TIO, ceftiofur

3.3.3 Antimicrobial Resistance Patterns Based on BC Geographic Location

Variations in antimicrobial resistance patterns were found amongst the environmental *V. parahaemolyticus* isolates that were examined. In general, *V. parahaemolyticus* strains isolated from 6 major oyster producing areas in British Columbia displayed resistance to AMP, CEF, and SUL (Table 3.5 and Figure 3.2). Analysis of resistance to each of these antibiotics between geographic areas of isolation determined the prevalence of AMP, CEF, and SUL resistance from each site was not significantly different from each other. Resistance to six additional antibiotics was also observed. Specifically, resistance to GEN (n=1, Area 14), KAN (n=1, Area 16), TIO (n=2, Area 17), OA (n=3, Area 16), PIP (n=2, Area 14; n=1, Area 15; n=11, Area 16), and STR (n=1, Area 13; n=3, Area 14; n=2, Area 15; n=5, Area 16; n=15, Area 17) was seen.

Table 3.5. Antimicrobial resistant environmental *V. parahaemolyticus* strains isolated from 6 major oyster producing areas of British Columbia.

Antibiotic[a]	Area 13 n=27		Area 14 n=89		Area 15 n=82		Area 16 n=105		Area 17 n=180		Area 23 n=30	
	n	%	n	%	n	%	n	%	n	%	n	%
GEN	0	0	1	1.1	0	0	0	0	0	0	0	0
KAN	0	0	0	0	0	0	1	1.0	0	0	0	0
STR	1	3.7	3	3.4	2	2.4	5	4.8	15	8.3	0	0
CTX	0	0	0	0	0	0	0	0	0	0	0	0
TIO	0	0	0	0	0	0	0	0	2	1.1	0	0
CEF	9	33.3	38	42.7	26	31.7	30	28.6	60	33.3	7	23.3
SXT	0	0	0	0	0	0	0	0	0	0	0	0
PMB	0	0	0	0	0	0	0	0	0	0	0	0
ERY	0	0	0	0	0	0	0	0	0	0	0	0
PIP	0	0	2	2.2	1	1.2	11	10.5	0	0	0	0
AMP	25	92.6	80	89.9	75	91.5	97	92.4	171	95.0	24	80.0
CHL	0	0	0	0	0	0	0	0	0	0	0	0
CIP	0	0	0	0	0	0	0	0	0	0	0	0
ENR	0	0	0	0	0	0	0	0	0	0	0	0
NAL	0	0	0	0	0	0	0	0	0	0	0	0
NOR	0	0	0	0	0	0	0	0	0	0	0	0
OA	0	0	0	0	0	0	3	2.9	0	0	0	0
SUL	2	7.4	1	1.1	5	6.1	2	1.9	4	2.2	2	6.7
TET	0	0	0	0	0	0	0	0	0	0	0	0
T	0	0	0	0	0	0	0	0	0	0	0	0

[a] GEN, gentamicin; KAN, kanamycin; STR, streptomycin; CTX, cefotaxime; TIO, ceftiofur; CEF, cephalothin; SXT, trimethoprim/sulfamethoxazole; PMB, polymyxin B; ERY, erythromycin; PIP, piperacillin; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; NAL, nalixidic acid; NOR, norfloxacin; OA, oxolinic acid; SUL, sulfisoxazole; TET, tetracycline; T, oxytetracycline

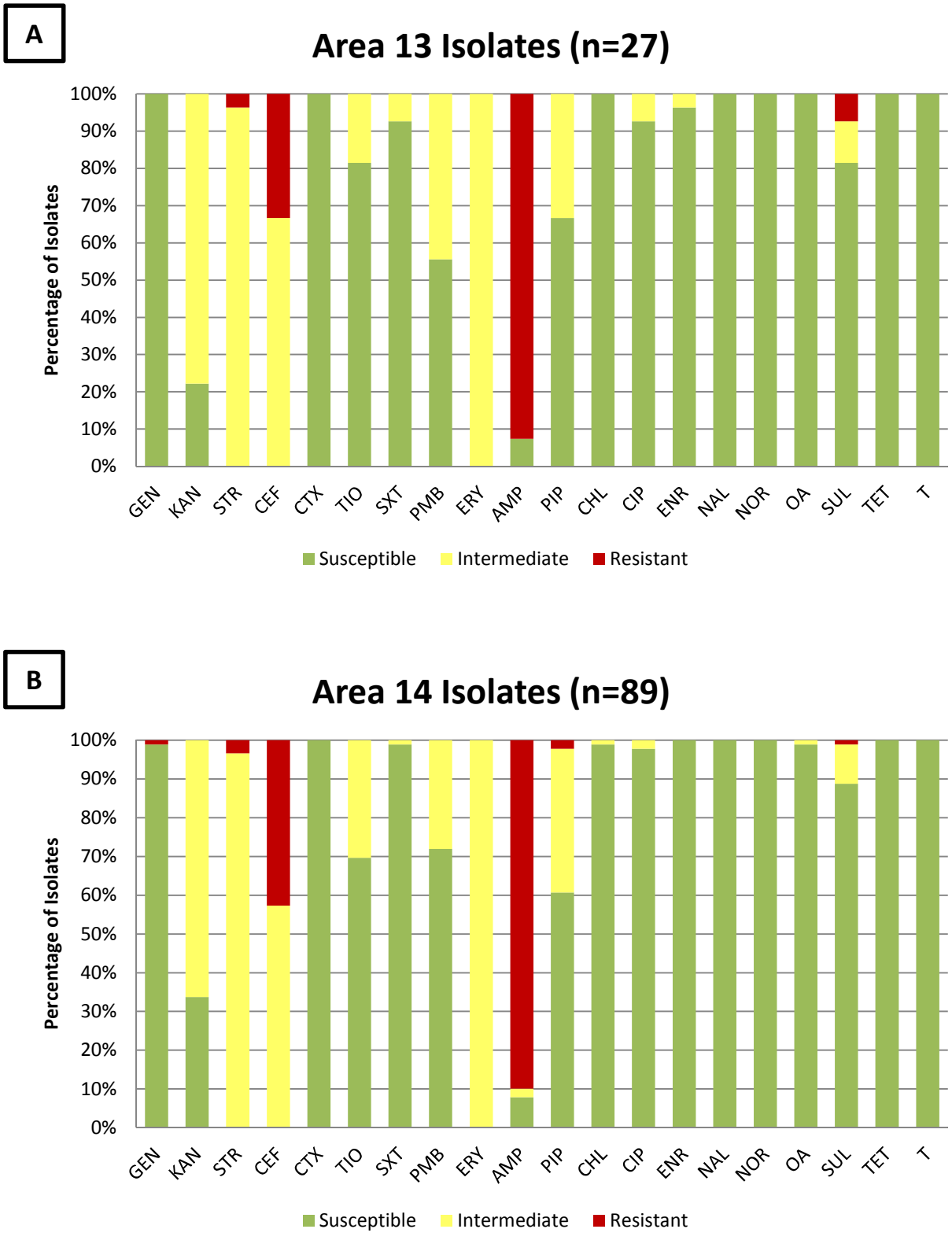
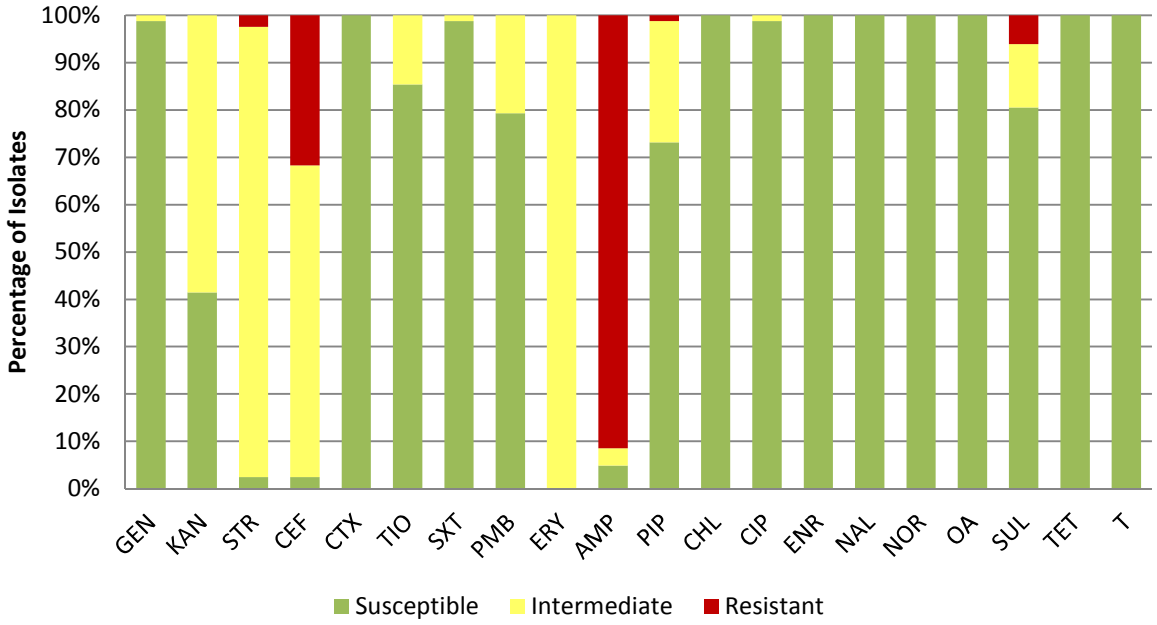


Figure 3.2. Antimicrobial resistance patterns of environmental *V. parahaemolyticus* isolated from 6 major oyster harvesting areas of British Columbia, including Area 13 (A), Area 14 (B), Area 15 (C), Area 16 (D), Area 17 (E), and Area 23 (F), between 1998 and 2011.

C



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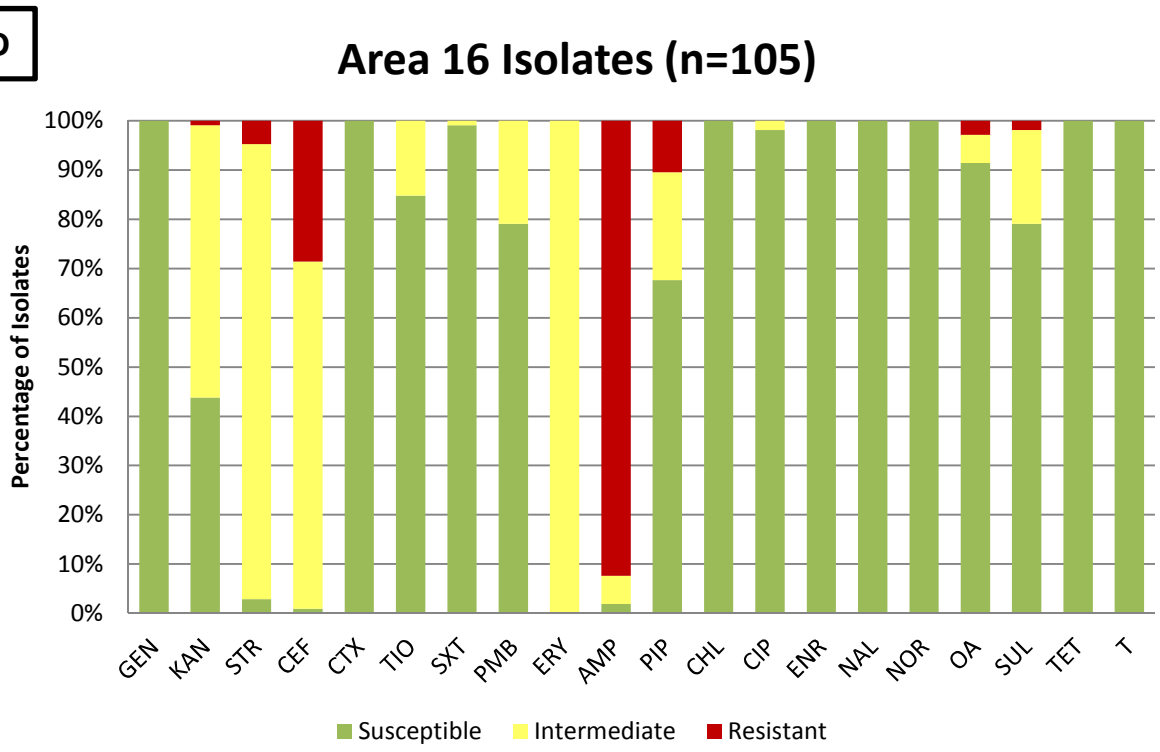

Figure 3.2. Continued.

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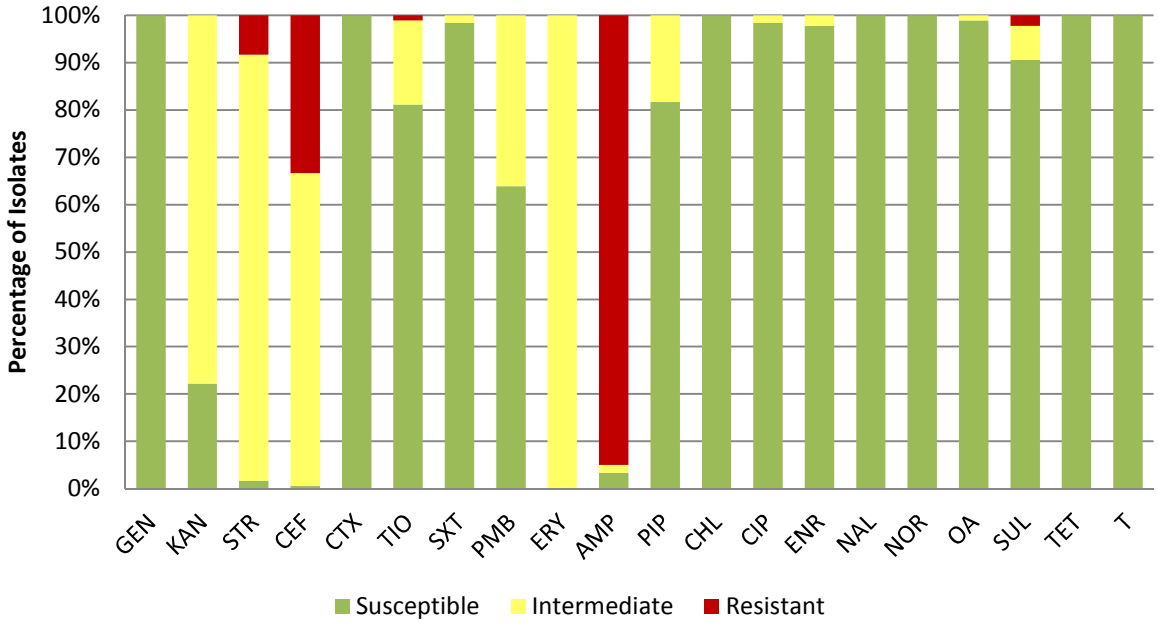

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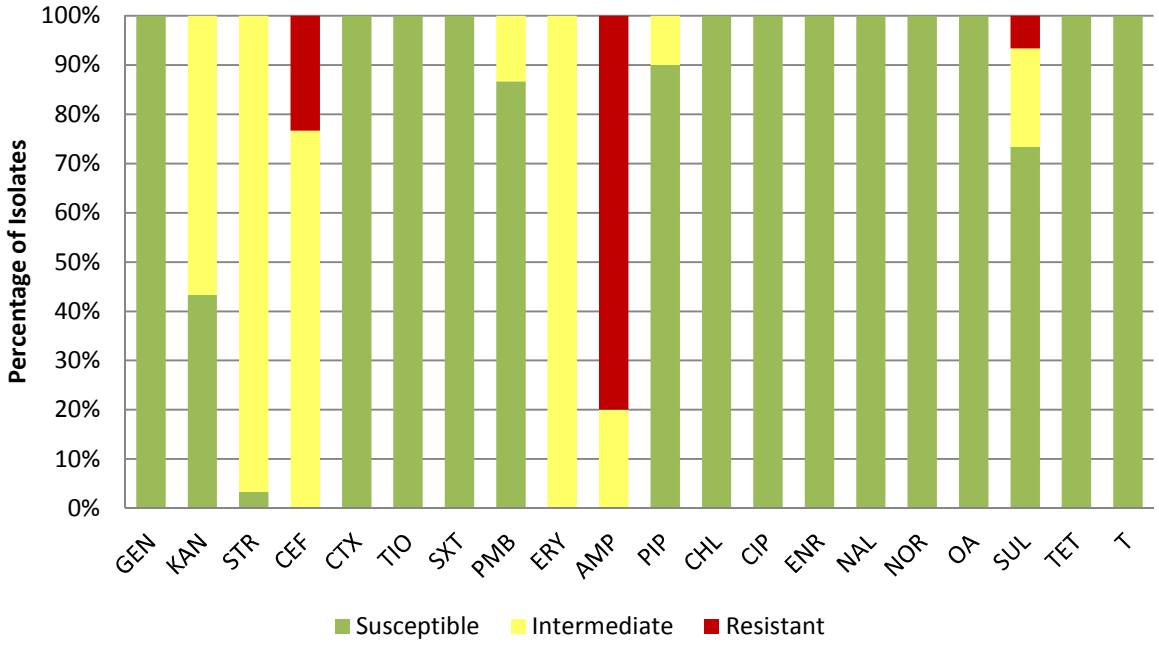


Figure 3.2. Continued.

As summarized in Table 3.6, isolates that displayed resistance to 4 antibiotics originated from Sykes Island (Area 16-12; n=2) and Dark Cove (Area 16-13; n=1) while isolates that were resistant to 3 antibiotics originated from Denman Island (Area 14-08; n=4), Twin Island (Area 15-03; n=3), Okeover Inlet (Area 15-04; n=1), Sykes Island (Area 16-12; n=3), Dark Cove (Area 16-13; n=4), and Ladysmith Harbour (Area 17-07; n=11).

Table 3.6. Distribution of multi-drug resistant phenotypes for environmental *V. parahaemolyticus* isolates originating from British Columbia (n=600)

Antimicrobial	**Area 13**		**Area 14**		**Area 15**		**Area 16**		**Area 17**		**Area 23**		**Area 24**	
Resistance	n	%	n	%	n	%	n	%	n	%	n	%	n	%
0	1	3.7	6	6.7	6	7.3	8	7.6	5	2.8	6	20.0	2	40.0
1	15	55.6	45	50.6	47	57.3	58	55.2	109	60.6	15	50.0	3	60.0
2	11	40.7	34	38.2	25	30.5	29	27.6	55	30.6	9	30.0	0	0
3	0	0	4	4.5	4	4.9	7	6.7	11	6.1	0	0	0	0
4	0	0	0	0	0	0	3	2.9	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	**27**	**100**	**89**	**100**	**82**	**100**	**105**	**100**	**180**	**100**	**30**	**100**	**5**	**100**

3.3.4 Antimicrobial Resistance Patterns of Isolates Possessing TDH and/or TRH Genes

Of the 600 *V. parahaemolyticus* isolates examined, *tdh* and/or *trh* were detected in 79 (13.2%) isolates (Chapter 2 Table 2.5). Overall, there were some discernable differences in antibiotic resistance phenotypes for *tdh*-positive and/or *trh*-positive isolates compared to *tdh*-negative and *trh*-negative strains (Table 3.7). Significantly more *V. parahaemolyticus* lacking *tdh* and *trh* were resistant to AMP (χ^2 = 63.514, $p < 0.0001$), CEF (χ^2 = 8.603, $p = 0.0034$), and STR ($p = 0.0369$).

Table 3.7. Antimicrobial resistance for *tdh*-positive and/or *trh*-positive *V. parahaemolyticus* compared to *tdh*-negative and *trh*-negative isolates.

Antibiotic[a]	***tdh*-positive and/or *trh*-positive Isolates (n=79)**						***tdh*-negative and *trh*-negative Isolates (n=521)**					
	S		I		R		S		I		R	
	n	%	n	%	n	%	n	%	n	%	n	%
GEN	79	100	0	0	0	0	519	99.6	1	0.2	1	0.2
KAN	56	70.9	23	29.1	0	0	183	35.1	337	64.7	1	0.2
STR	13	16.5	66	83.5	0	0	19	3.6	476	91.4	26	5.0
CTX	79	100	0	0	0	0	521	100	0	0	0	0
TIO	74	93.7	5	6.3	0	0	429	82.3	90	17.3	2	0.4
CEF	10	12.7	57	72.2	12	15.2	5	1.0	353	67.8	163	31.3
SXT	78	98.7	1	1.3	0	0	513	98.5	8	1.5	0	0
PMB	68	86.1	11	13.9	0	0	381	73.1	140	26.9	0	0
ERY	1	1.3	78	98.7	0	0	0	0	521	100	0	0
PIP	77	97.5	2	2.5	0	0	384	73.7	123	23.6	14	2.7
AMP	4	5.1	22	27.8	53	67.1	19	3.6	9	1.7	493	94.6
CHL	79	100	0	0	0	0	520	99.8	1	0.2	0	0
CIP	79	100	0	0	0	0	507	97.3	14	2.7	0	0
ENR	79	100	0	0	0	0	516	99.0	5	1.0	0	0
NAL	79	100	0	0	0	0	521	100	0	0	0	0
NOR	79	100	0	0	0	0	521	100	0	0	0	0
OA	77	97.5	2	2.5	0	0	511	98.1	7	1.3	3	0.6
SUL	69	87.3	10	12.7	0	0	440	84.5	65	12.5	16	3.1
TET	77	97.5	2	2.5	0	0	521	100	0	0	0	0
T	79	100	0	0	0	0	521	100	0	0	0	0

[a] GEN, gentamicin; KAN, kanamycin; STR, streptomycin; CTX, cefotaxime; TIO, ceftiofur; CEF, cephalothin; SXT, trimethoprim/sulfamethoxazole; PMB, polymyxin B; ERY, erythromycin; PIP, piperacillin; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; NAL, nalixidic acid; NOR, norfloxacin; OA, oxolinic acid; SUL, sulfisoxazole; TET, tetracycline; T, oxytetracycline

The resistant phenotypes of 79 isolates possessing *tdh* and/or *trh* are summarized in Table 3.8. Amongst this *tdh*-positive and/or *trh*-positive subset of isolates, resistance to AMP was frequently observed (71% of clinical isolates; 44% of environmental isolates). Clinical isolates possessing *tdh* and/or *trh* were resistant to no more than one antibiotic (AMP or CEF). However, 20% of *tdh*-positive and/or *trh*-positive *V. parahaemolyticus* isolated from oysters collected from the environment displayed resistance to two antibiotics. The 9 isolates originated from Gorge Harbour (Area 13-15), Okeover Inlet (Area 15-04), Coffin Point (Area 17-05), Ladysmith Harbour (Area 17-07), and Effingham Inlet (Area 23-06).

Table 3.8. Antimicrobial resistance patterns for *tdh*-positive and/or *trh*-positive *V. parahaemolyticus* tested in this study.

Antimicrobial Resistance	Antibiogram[a]	Clinical Isolates (n=34)	Environmental Isolates (n=45)
0	Not applicable	8	15
1	AMP	24	20
	CEF	2	1
2	AMP, CEF	0	9

[a] AMP, ampicillin; CEF, cephalothin

3.3.5 Longitudinal Antimicrobial Resistance Patterns

The 536 environmental *V. parahaemolyticus* cultures examined in this study were isolated over a 14 year span from 1998 to 2011. AMP resistance was ubiquitous, ranging from 43% of isolates in 2006 to 100% of the isolates in 2003 and 2004 (Figure 3.3). Similarly, CEF resistance was observed annually; a range of 14% to 52% of isolates displayed the resistant phenotype. Several antimicrobial agents (SUL [0 – 24%], STR [0 – 18%], TIO [0 – 2%], PIP [0 – 7%], and OA [0 – 3%]) had annual variations in the percentage of isolates showing resistance while gentamicin and kanamycin resistance was only seen in one year (Figure 3.3). As indicated previously, all isolates were susceptible to CTX, NAL, NOR, and T with intermediate susceptibility (but not resistance) observed for SXT, PMB, ERY, CHL, CIP, and ENR. The top three antibiotics that environmental isolates were commonly resistant to included AMP, CEF, and STR with fluctuations in the percentage of isolates displaying the resistant phenotype occurring over the years (Figure 3.4).

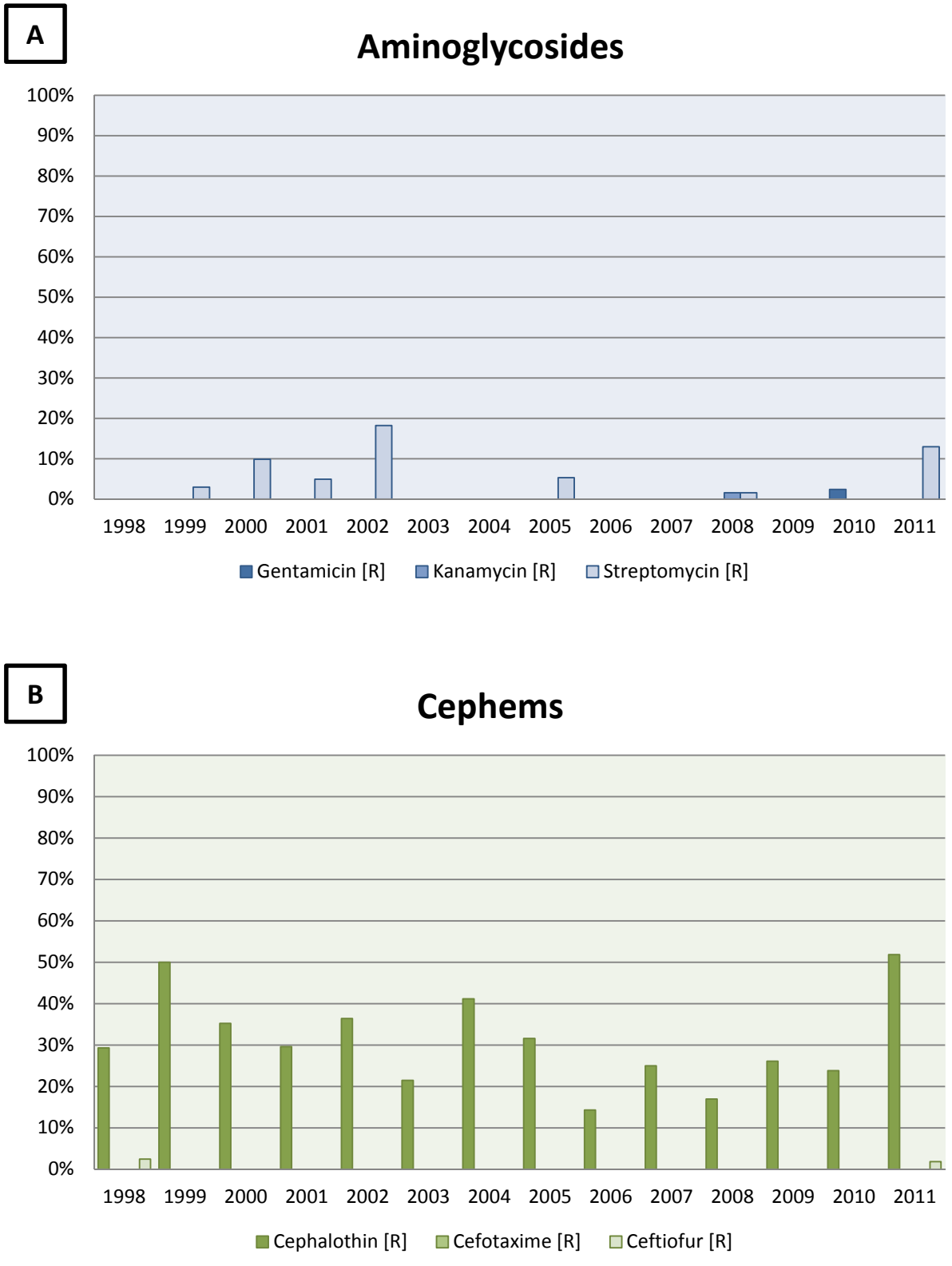
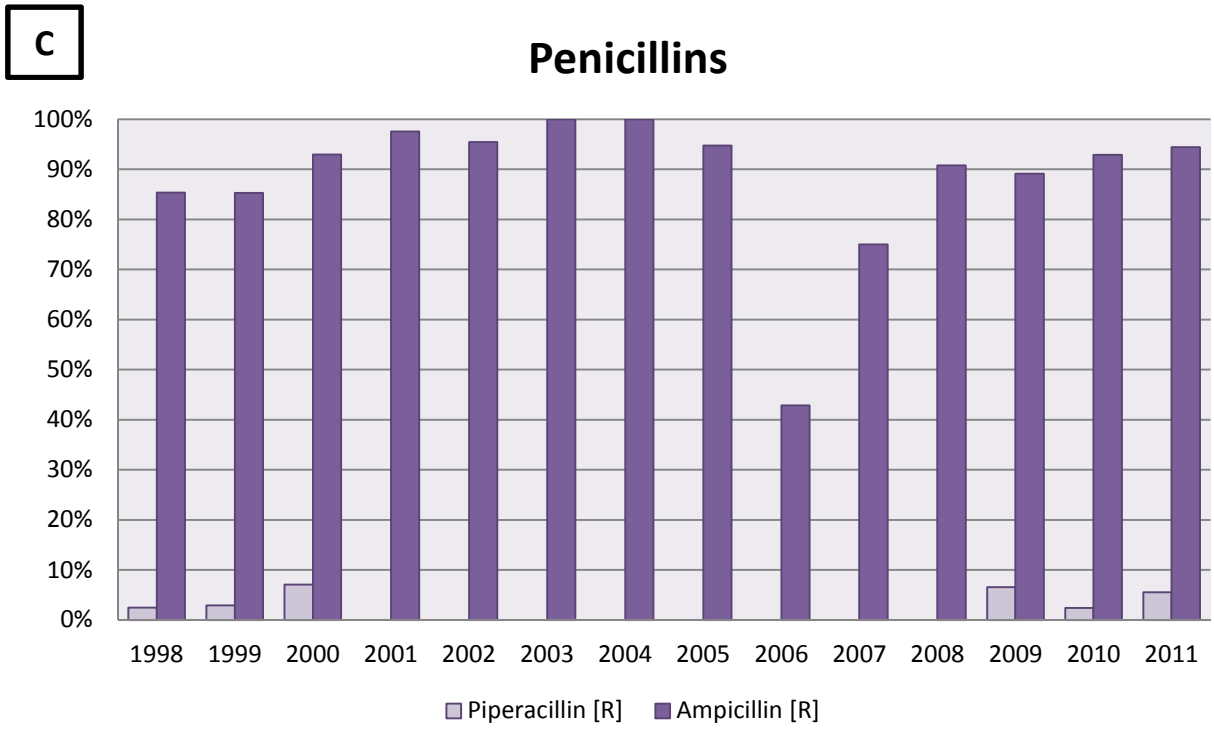
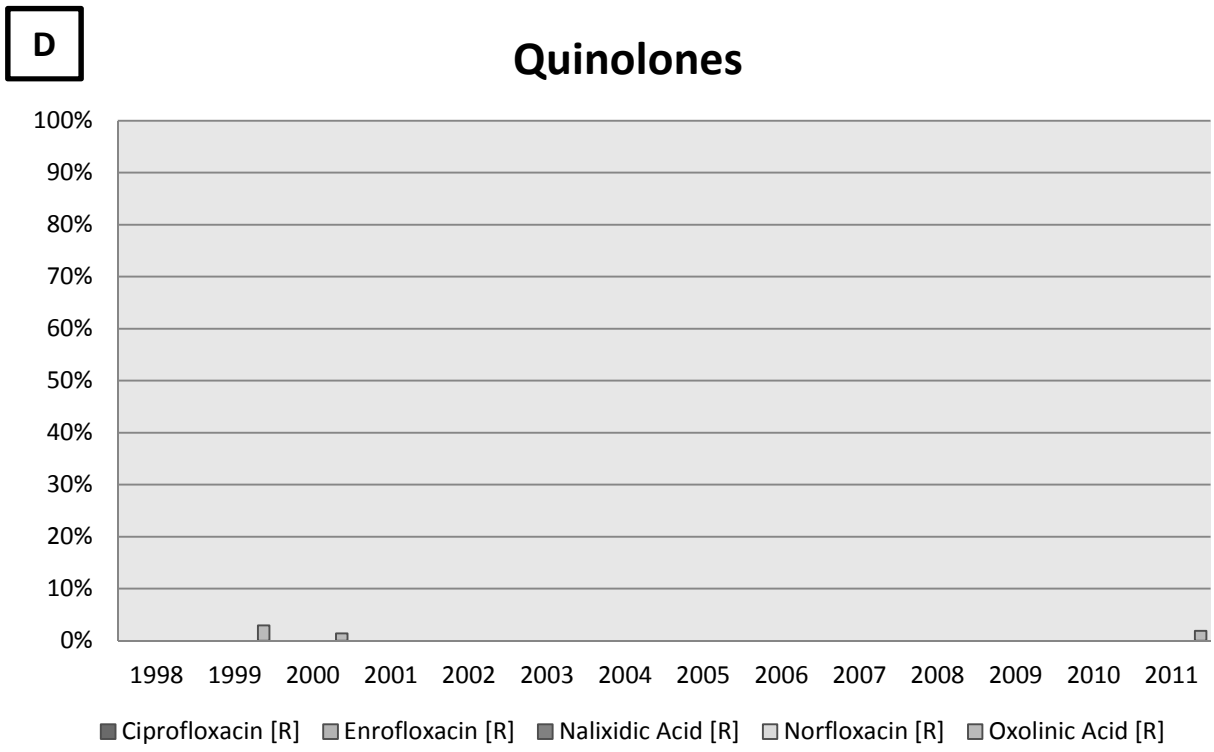


Figure 3.3. Percentage of *V. parahaemolyticus* isolated in Canada between 1998 and 2011 exhibiting antimicrobial resistance [R] grouped by antimicrobial classes including aminoglycosides (A), cephems (B), penicillins (C), quinolones (D), and sulfonamides (E).





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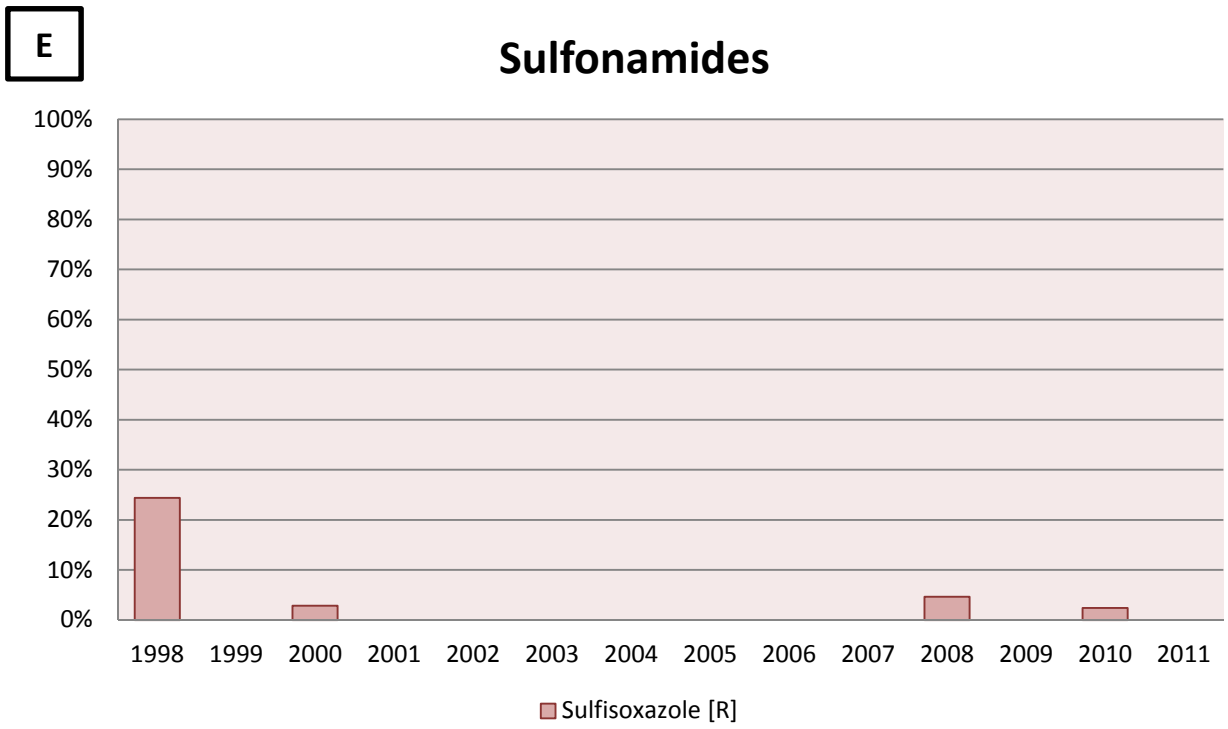

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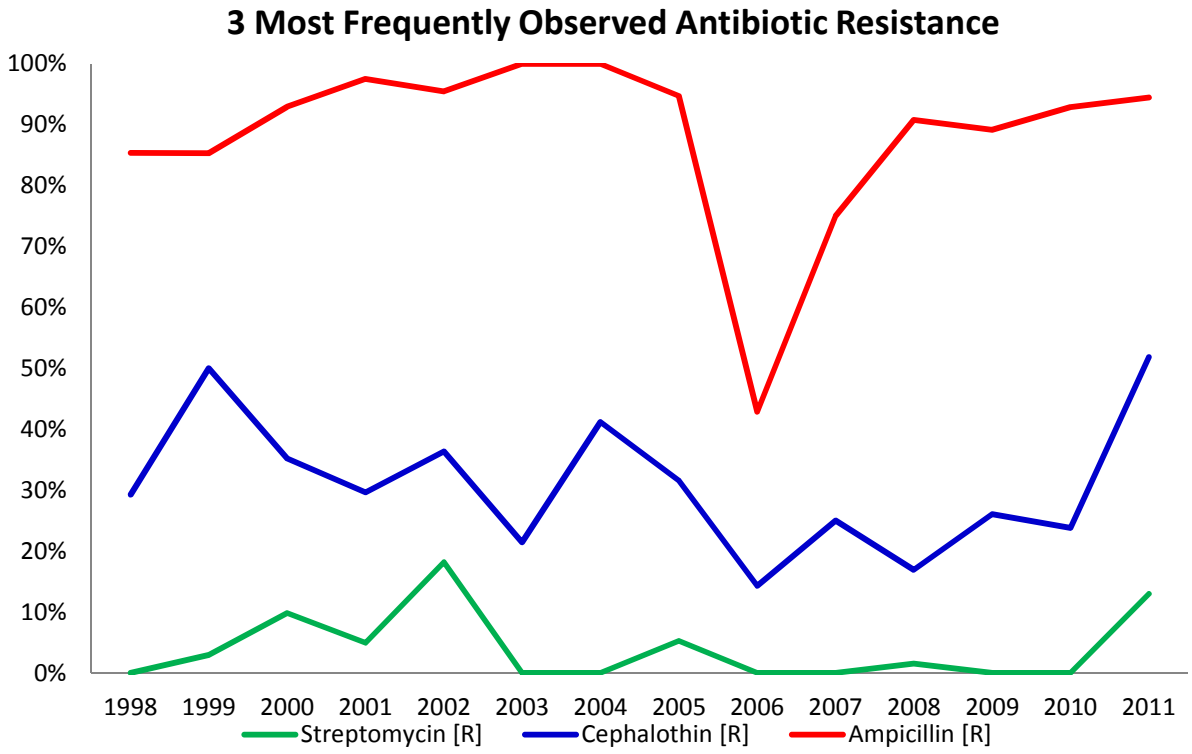
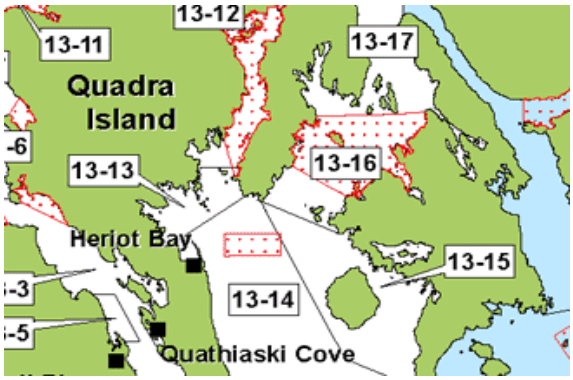


Figure 3.4. Percentage of *V. parahaemolyticus* isolated in Canada between 1998 and 2011 for the 3 most frequently identified antimicrobial resistance [R].

Three oyster growing areas in BC were consistently monitored over multiple years. These included Area 13-15, Area 14-08, and Area 17-07 (Figure 3.5). As such, AMR profile changes over time focusing on these specific areas were also examined (Figure 3.6). Time points were collapsed into two periods in an effort to have sufficient numbers to facilitate statistical analysis. While the data set is limited (n=27), isolates collected from Area 13-15 between 2007 and 2011 showed an increase in the incidence of resistance to STR (0% to 7.7%); however, a decrease was observed for CEF (50% to 15.4%) when compared to isolates collected between 1998 and 2001. Amongst the *V. parahaemolyticus* strains collected from Area 14-08 (n=89), those collected between 2008 and 2011 showed an increase in the prevalence of AMP resistance (81% to 100%) and STR (2.1% to 4.9%) while a decrease was observed for CEF (54.2% to 29.3%). In addition, resistance to GEN and PIP were only observed in strains collected between 2008 and 2011 in Area 14-08. Within Area 17-07, isolates were consistently resistant to AMP while decreases in the prevalence of resistance to CEF (27.7% to 38.2%) and STR (12.3% to 8.8%) were seen in the latter time period. Furthermore, resistance to TIO was seen in one strain recovered in 1998 while resistance to SUL was displayed in one isolate from 1998 and one isolate from 2000. As illustrated in Figure 3.7, variations in the occurrence of multi-drug resistance were detected. Isolates from Area 13-15 displayed resistance for up to 2 antibiotics in both time points with one isolate collected in 1998 that was susceptible to all 20 antibiotics examined in this study. In contrast, isolates that were resistant to 3 antimicrobials were only observed between 2008 and 2011 in Area 14-08. Amongst the Area 17-07 isolates, 11% of isolates collected between 1998 and 2001 compared to 6% of isolates collected between 2002 and 2005 were resistant to 3 antibiotics.



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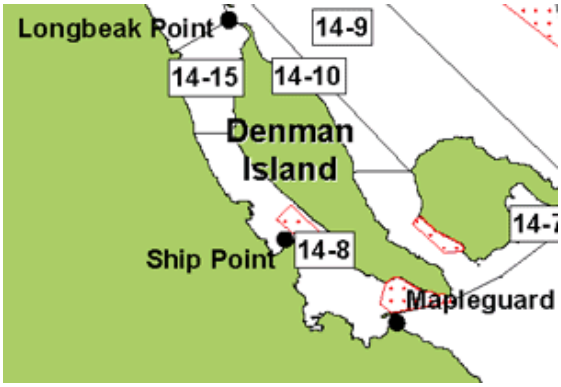
Area 13-15

Antibiotic[a]	1998 to 2001 n=14		2007 to 2011 n=13	
	n	%	n	%
AMP	13	92.9	12	92.3
CEF	7	50.0	2	15.4
SUL	1	7.1	1	7.7
STR	0	0	1	7.7

[a] AMP, ampicillin; CEF, cephalothin; SUL, sulfisoxazole; STR, streptomycin



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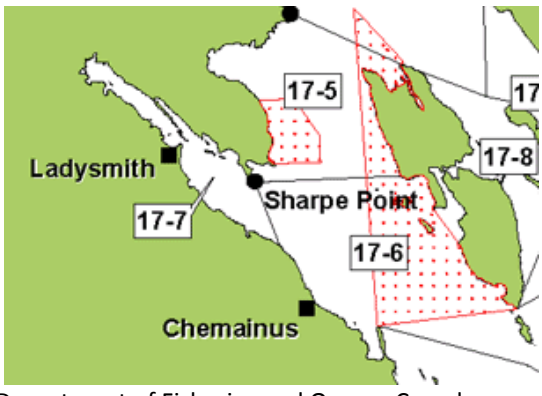
Area 14-08

Antibiotic[a]	1998 to 2001 n=48		2008 to 2011 n=41	
	n	%	n	%
AMP	39	81.3	41	100
CEF	26	54.2	12	29.3
GEN	0	0	1	2.4
PIP	0	0	2	4.9
SUL	1	2.1	0	0
STR	1	2.1	2	4.9

[a] AMP, ampicillin; CEF, cephalothin; GEN, gentamicin; PIP, piperacillin; SUL, sulfisoxazole; STR, streptomycin



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Area 17-07

Antibiotic[a]	1998 to 2001 n=65		2002 to 2005 n=68	
	n	%	n	%
AMP	64	98.5	67	98.5
CEF	18	27.7	26	38.2
TIO	1	1.5	0	0
SUL	2	3.1	0	0
STR	8	12.3	6	8.8

[a] AMP, ampicillin; CEF, cephalothin; TIO, ceftiofur; SUL, sulfisoxazole; STR, streptomycin

Figure 3.5. Antimicrobial resistance observed in *V. parahaemolyticus* isolated from oysters harvested from British Columbia Area 13-15, Area 14-08, and Area 17-07.

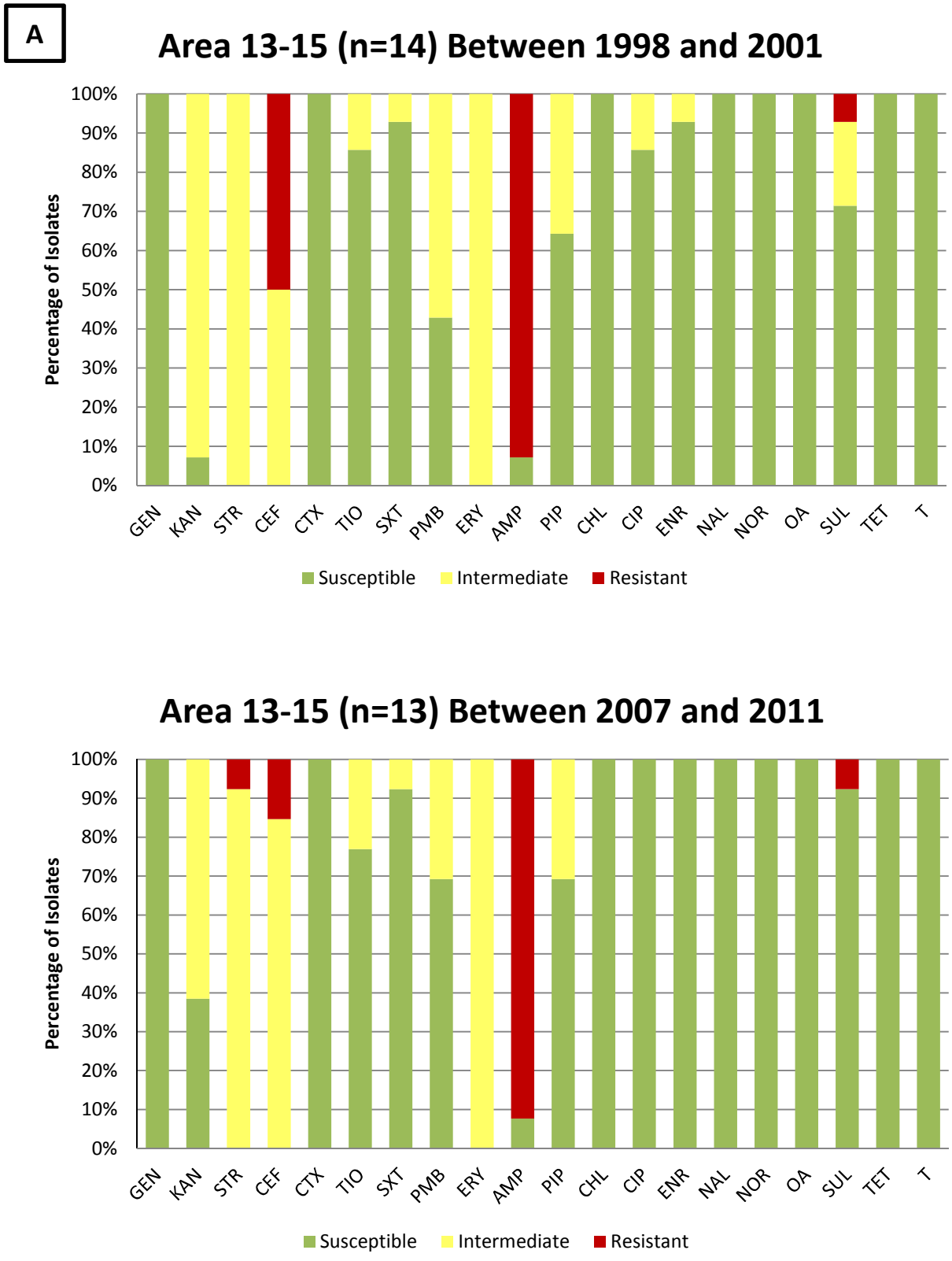
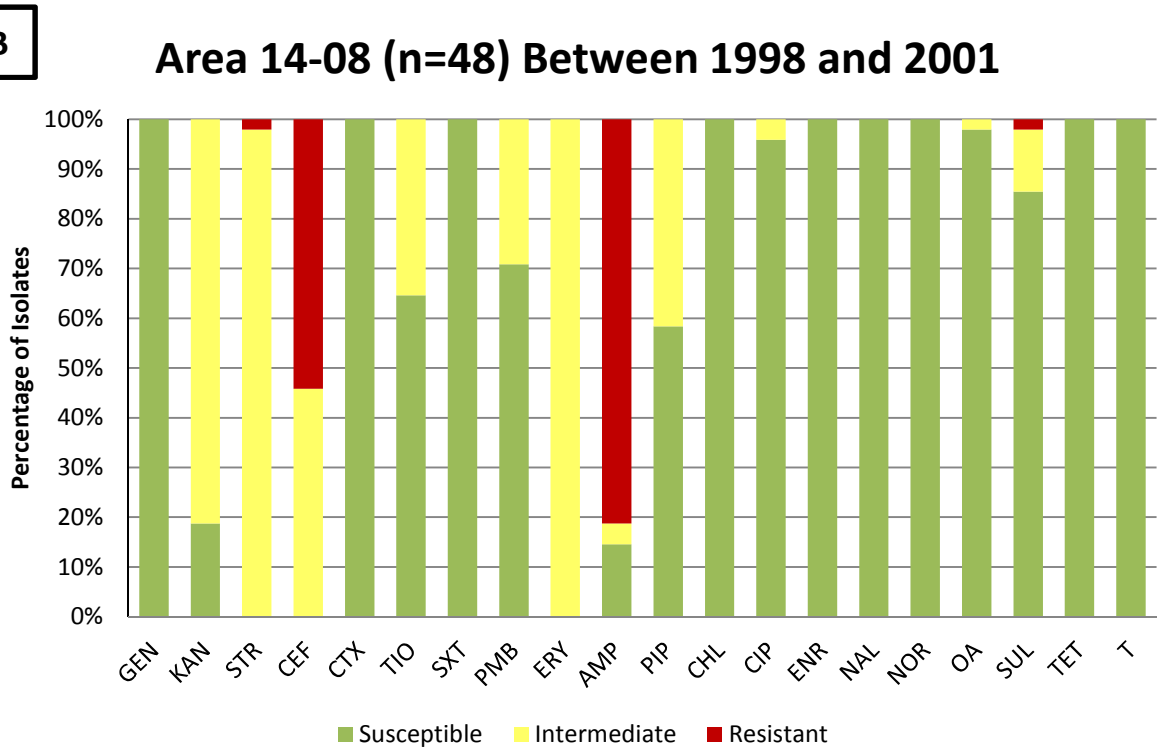
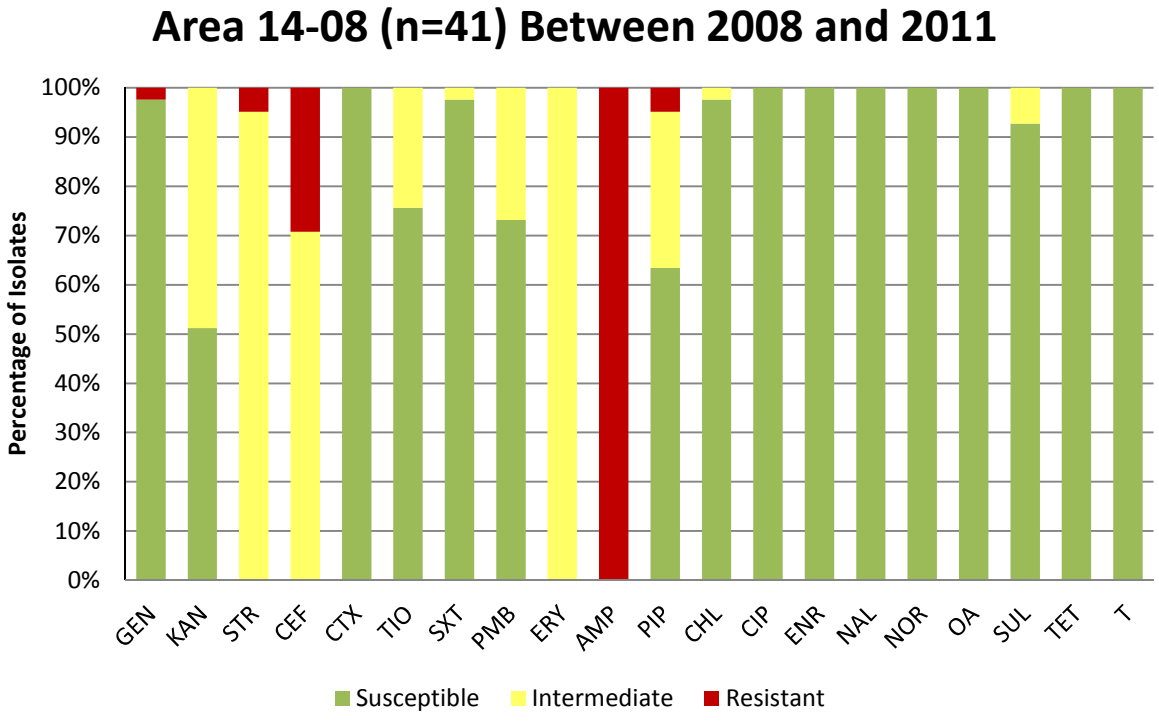


Figure 3.6. Antimicrobial resistance patterns of *V. parahaemolyticus* isolated from oysters harvested from Area 13-15 (A), Area 14-08 (B), and Area 17-07 (C) grouped by two time periods.

B





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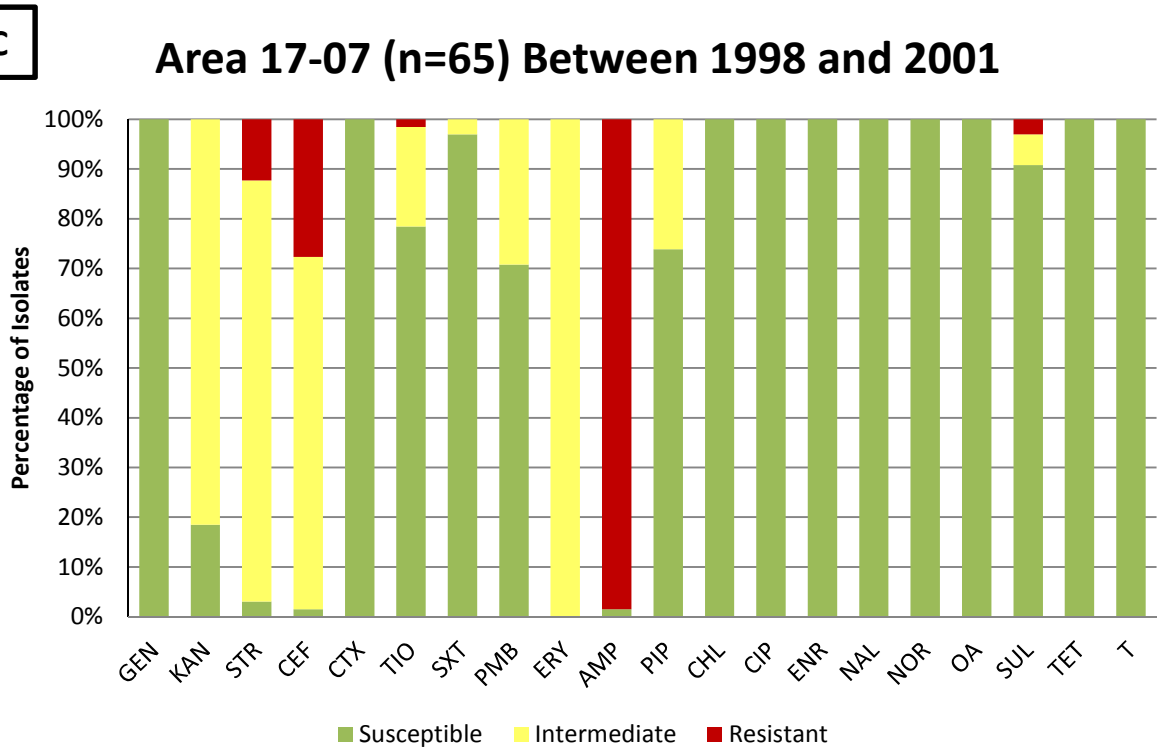

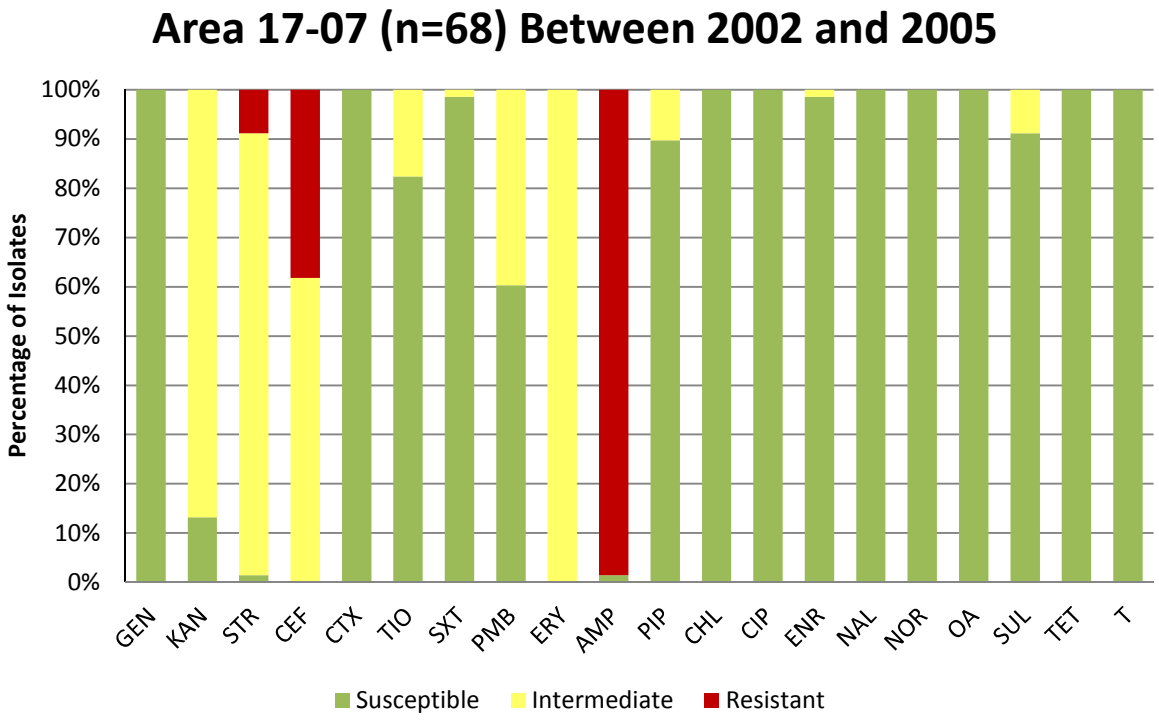

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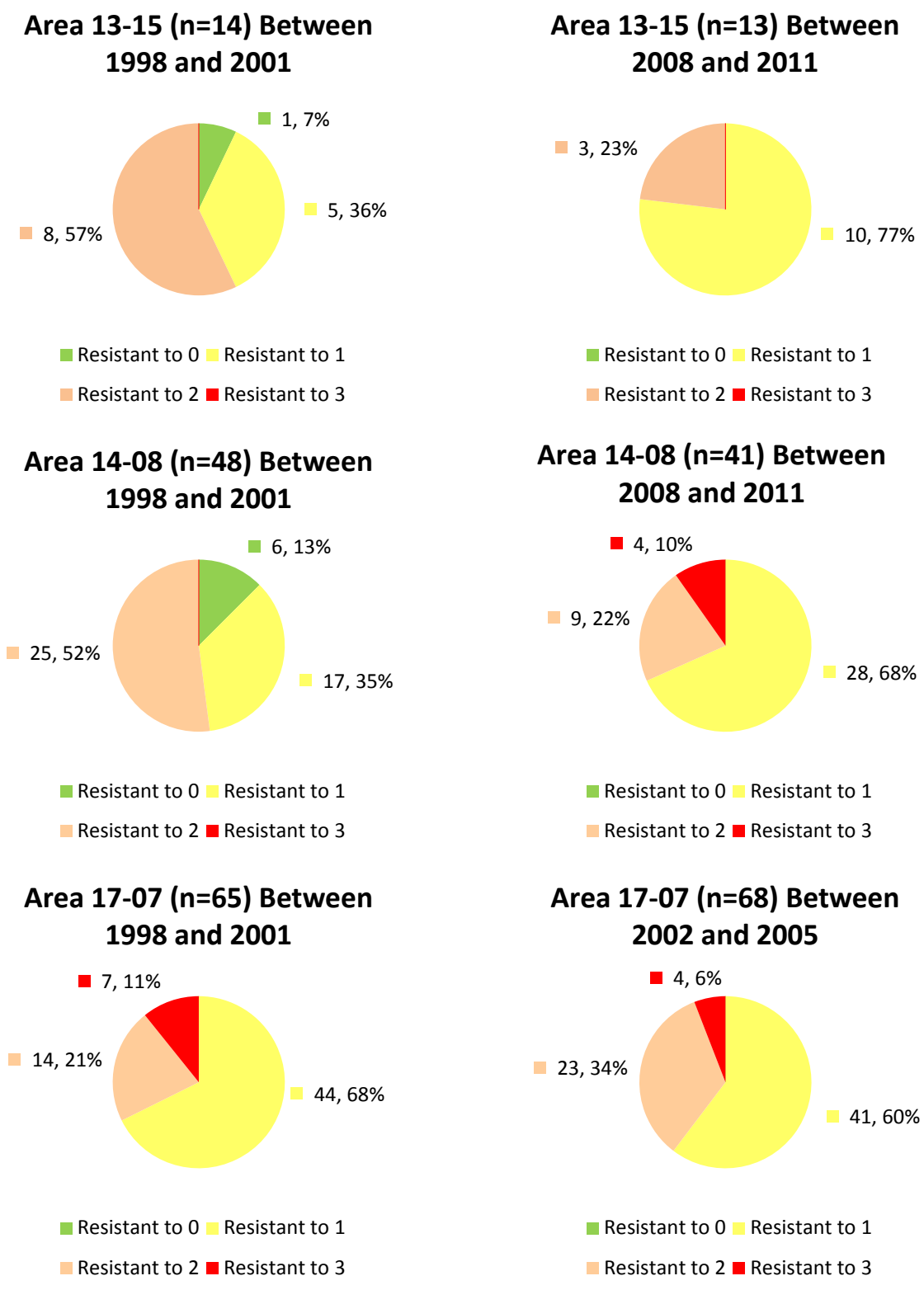

Figure 3.7. Percentage of multi-drug resistance observed in *V. parahaemolyticus* isolated from oysters harvested from Area 13-15, Area 14-08, and Area 17-07 grouped by two time periods

3.4 Discussion

In this study, susceptibility to 20 clinically relevant antibiotics used in human medicine and agriculture/aquaculture was determined for *V. parahaemolyticus* recovered from oysters harvested in Canada as well as clinical strains isolated in British Columbia. While antibiotic treatment is typically unnecessary for gastroenteritis, it may be needed for patients with underlying medical conditions and/or individuals suffering from wound infections and primary septicemia caused by *V. parahaemolyticus* (Daniels et al., 2000; Butt et al., 2004; Elmahdi et al., 2016). Treatment recommendations include tetracyclines (doxycline, tetracycline), fluoroquinolones (ciprofloxacin, levofloxacin), third-generation cephalosporins (ceftotaxime, ceftazidime, ceftriaxone), aminoglycosides (amikacin, apramycin, gentamicin, streptomycin), folate pathway inhibitors (trimethoprim-sulfamethoxazole), and erythromycin for pregnant women and children (Shaw et al., 2014; Elmahdi et al., 2016; Butt et al., 2004; PHAC, 2011).

In general, the AMR profiles of these Canadian isolates are similar to data published by researchers who analyzed *V. parahaemolyticus* isolated in other countries. Of the library screened here (n=600), most isolates were susceptible to antimicrobials recommended for treatment. Specifically, all isolates were either fully susceptible (CTX, NAL, NOR, and T) or exhibited reduced susceptibility (SXT, PMB, ERY, CHL, CIP, ENR, and TET) to the panel of antibiotics used in this study (Table 3.3 and Figure 3.1). In contrast, antibiotic resistance to AMP and CEF were prevalent across the library of isolates with resistance to STR (4.3%), SUL (2.7%), PIP (2.3%), OA (0.5%), TIO (0.3%), GEN (0.2%), and KAN (0.2%) seen less frequently.

Amongst this data set, resistance to AMP was seen in 91% of the isolates tested. This high resistance was not unexpected and is comparable to findings from studies completed in Europe and Asia. Ottaviani et al. (2013) conducted a large-scale study on antimicrobial susceptibility of *V. parahaemolyticus* found

in Italian shellfish and clinical samples (from Italy and Japan) between 2009 and 2011 and reported 100% (n=107) were resistant to AMP. Similarly, Kang et al. (2016) tested 71 *V. parahaemolyticus* recovered from oysters collected from the west coast of Korea in 2014 and found all the isolates were resistant to AMP while Letchumanan et al. (2015b) observed 82% (n=185) of *V. parahaemolyticus* isolated from shrimp sold in retail markets in Malaysia in 2014 were AMP resistant. In fact, high resistance rates to AMP have been observed as early as 1978 (Joseph et al., 1978) and some researchers have suggested this resistance pattern could be due to the fact that first generation antibiotics, including AMP, have been misused in the environment as part of aquaculture activities (Letchumanan et al., 2015b; Sudha et al., 2012). Other studies conducted in North America, which targeted areas along the US Atlantic coast, reported lower levels of AMP resistance in the aquatic environment. Specifically, Shaw et al. (2014) found *V. parahaemolyticus* isolated from surface waters in Chesapeake Bay (n=77) exhibited a lower prevalence (53%) of AMP resistance. Similarly, another study examined 350 *V. parahaemolyticus* strains recovered from water and sediment from the Atlantic coast of Georgia and South Carolina and found 142 (41%) were resistant to AMP (Baker-Austin et al., 2008). A study that focussed on examining *V. parahaemolyticus* and *V. vulnificus* isolated from oysters collected from the Louisiana Gulf coast, local retail markets, and restaurants between 2005 and 2006 found 56.5% (n=168) of *V. parahaemolyticus* isolates tested were resistant to AMP (Han et al., 2007). Variations in AMP resistance levels reported may be due to geographic differences or mechanisms mediating the resistant phenotype. For example, one study conducted by Pazhani et al. (2014) reported the AMP resistance observed was mediated by an efflux pump. The investigators found AMP resistance varied from moderate to high levels amongst the strains tested and the minimum inhibitory concentration of AMP for *V. parahaemolyticus* decreased considerably when an efflux pump inhibitor was added (Pazhani et al., 2014). Alternatively, Chiou et al. (2015) recently identified a novel class A carbenicillin-hydrolyzing β-

lactamase, $bla_{CARB-17}$, and found it to be responsible for the intrinsic penicillin resistance in *V. parahaemolyticus*.

A total of 29.2% of isolates tested in this study exhibited resistance to CEF. Resistance to this antibiotic has also been reported by other researchers; frequencies of observing this phenotype ranged from approximately 40% to 90% depending on the geographic area under study. For example, Ottaviani et al. (2013) found 43.7% (n=87) of shellfish collected between 2009 and 2011 from several sites along the Italian coast were CEF resistant while all (n=8) *V. parahaemolyticus* strains isolates from Italian stool cultures between 2007 and 2010 were CEF resistant. Similar CEF resistant levels were seen in studies carried out in Asia. Kang et al. (2016) reported 52.2% (n=71) of *V. parahaemolyticus* isolated from Korean shellfish displayed CEF resistance while Xie et al. (2015) found 56.7% (n=150) of isolates recovered from aquatic products (shrimp, freshwater fish, and saltwater fish) sold at retail markets in South China between 2013 and 2014. Detection of CEF resistance occurred much more frequently (90%, n=14) when Igbinosa et al. (2011) tested *V. parahaemolyticus* isolated from wastewater effluents from a peri-urban community in South Africa.

Antibiogram differences between clinical and environmental *V. parahaemolyticus* isolates were observed in this study. Clinical isolates were resistant to no more than two antibiotics with the vast majority (82.8%, n=64) resistant to only one antibiotic and 14.1% of strains were susceptible to all 20 antimicrobials (Table 3.2). In contrast, 3 environmental isolates from the west coast of BC displayed resistance to 4 antibiotics. Jun et al. (2012) also reported differences in AMR characteristics between clinical isolates and strains collected from seafood and environmental sources found in Korea. In particular, of the 24 *V. parahaemolyticus* tested (19 seafood, 3 environmental, and 2 clinical) the clinical isolates were resistant to more than 4 antibiotics compared to other isolates that were resistant to more

than 11 antibiotics (Jun et al., 2012). All isolates were tested against a panel of 22 antibiotics of which only 9 overlapped with this present study (Jun et al., 2012); however, specific AMR profiles were not published so further comparisons could not be made. Further examination of the clinical isolates tested in this present study found resistance to AMP, CEF, or both of these antibiotics occurred in 79.7%, 3.1%, and 3.1% of the tested cultures, respectively. In general, our findings indicate that frontline drugs recommended for treatment remain effective against *V. parahaemolyticus* of clinical origin in BC and mirror data reported by other investigators who examined AMR patterns of clinical isolates. While Ottaviani et al. (2013) tested a smaller number of strains (n=20), all isolates were resistant to AMP and CEF as well as amoxicillin with some isolates exhibiting resistance to other antibiotics such as STR, KAN, PMB, and ERY. A similar study examined 501 *V. parahaemolyticus* strains isolated from fecal specimens collected between July 2009 and June 2013 in southeast China and found most isolates were sensitive to common antimicrobial agents, with the exception of AMP (Chen et al., 2016). Additionally, while resistance to AMP and CEF were observed in both clinical and oyster-derived isolates in our study, it was significantly less common in clinical *V. parahaemolyticus* cultures.

Only a subset of the isolates (n=79) examined in this study possessed *tdh* and/or *trh* genes encoding for recognized virulence factors. Notably, resistance to AMP, CEF, and STR was significantly more common in *V. parahaemolyticus* lacking *tdh* and/or *trh*. In particular, *tdh*-positive and/or *trh*-positive isolates were resistant to no more than 2 antibiotics (Table 3.8). Baker-Austin et al. (2008) also found AMR was significantly less (5.8 antimicrobials per isolate) among virulent strains (n=15) compared to an average of 7.5 antimicrobials for isolates that were *tdh*-negative and/or *trh*-negative. In contrast, Ottaviani et al. (2013) did not observe any significant differences in AMR with regards to pathogenic potential. It is important to note that the strains examined in previous studies and in the present work were clearly isolated from different geographic environments and sources and observations were based on relatively

small libraries of *tdh*-positive and/or *trh*-positive strains. Accordingly, broad conclusions about the association between virulence factors and antibiotic resistance would be premature. However, the results of the present work on Canadian isolates are suggestive of trend that warrants further investigation. Interestingly, a few studies conducted on other bacteria have examined the relationships between virulence and AMR (Banerjee and Anupurba, 2015; Da Silva and Mendonca, 2012; Beceiro et al., 2013). In some cases, increased resistance has been associated with decreased virulence and fitness. For example, it has been hypothesized that the expression of vancomycin resistance is biologically costly for enterococci, and in scenarios where survival necessitates the acquisition of vancomycin resistance the fitness cost is reduced by the loss of virulence plasmids (Banerjee and Anupurba, 2015). However, there is also evidence showing the opposite whereby increased resistance has been accompanied by increased virulence (Beceiro et al., 2013).

Environmental *V. parahaemolyticus* isolates analyzed in this study (n=536) displayed resistant phenotypes to AMP, CEF, STR, SUL, PIP, OA, TIO, GEN, and KAN (Table 3.3). As mentioned above, resistance to AMP and CEF were significantly more common in environmental *V. parahaemolyticus* isolates than those from clinical sources while no significant differences were observed for the other antibiotics. In addition, differences in AMR patterns observed among isolates collected from 6 major oyster producing areas in BC (Table 3.5 and Figure 3.2) were not statistically significant. With the exception of AMP and CEF, where 92.0% and 31.9% of environmental isolates were respectively resistant, the prevalence of resistance to other antimicrobials was lower. Specifically, resistance to STR, SUL, PIP, OA, GEN, and KAN were observed in less than 5%, for each antibiotic, of environmental isolates examined. Few studies have investigated AMR of *V. parahaemolyticus* isolated from oysters collected from the aquatic environment. Of the similar research that has been published, Ottaviani et al. (2013) found of the 87 strains tested (all isolated from bivalve shellfish collected from sites along the Italian

coast where harvesting was permitted), 32.2%, 20.7%, 3.1%, and 1.1% were resistant to ERY, STR, KAN, and OA, respectively. Lopatek et al. (2015) examined 64 isolates recovered from shellfish (mussels, clams, oysters, and scallops) sold in Polish markets; 70.3% exhibited STR resistance and 10.9% displayed GEN resistance. In Asia, Jun et al. (2012) found of the 24 strains tested (19 were derived from shellfish including Pacific oysters available in Korean markets) nearly 80% of isolates were PIP resistant and over 55% were GEN resistant. Similarly, Hu and Chen (2016) examined 208 *V. parahaemolyticus* isolated from crustaceans and other shellfish available in Shanghai fish markets and reported 77.9% were resistant to STR with less than 10% of shellfish derived isolates resistant to GEN and KAN. Resistance to PIP has also been found in *V. parahaemolyticus* recovered from retail shellfish sold in Shanghai (Yu et al., 2016) as well as Atlantic surface waters of Chesapeake Bay and Maryland Coastal Bays (Shaw et al., 2014). Liu et al. (2009) found 47.3% (n=38) of *V. parahaemolyticus* of seafood origin were resistant to SUL (Liu et al., 2009) while OA resistance has been reported in *V. parahaemolyticus* isolated from farmed fish in Korea (Son et al., 2005).

The more frequent display of resistance to β-lactams (AMP, CEF) and to a lesser extent aminoglycosides (STR, GEN, KAN) observed among environmental *V. parahaemolyticus* may be associated with use of these antimicrobials in treating human infections and/or other applications (Cabello, 2006; Lupo et al., 2012; Allen et al., 2010; Baquero et al., 2008). In aquatic environments, bacteria from various sources (e.g. hospitals, wastewater, etc.) mix, providing opportunities for exchange or acquisition of antimicrobial resistance genes (Baquero et al., 2008; Allen et al., 2010). Furthermore, antimicrobial agents are released into marine environments from aquaculture as well as sludge treatment facilities, pharmaceutical production facilities, and agriculture at concentrations that may apply selective pressure and subsequently promote the evolution and spread of organisms with AMR (Gullberg et al., 2011; Allen et al., 2010). Of the isolates tested in this study, 180 originated from oysters harvested from Ladysmith

Harbour (Area 17-07) which is situated in proximity to a wastewater treatment plant (Table 3.5 and Figure 3.5). Similar to environmental strains isolated from other oyster producing areas in BC, resistance to AMP (95.0%), CEF (33.3%), STR (8.3%), and SUL (2.2%) were observed. However, one isolate exhibited resistance to TIO (a third generation cephalosporin); resistance to TIO was also seen in one isolate collected from the Coffin Point which is adjacently located in Area 17-05. Igbinosa et al. (2011) conducted a similar investigation determining antibiogram profiles of *V. parahaemolyticus*, *V. fluvialis*, and *V. vulnificus* isolated from wastewater final effluents in a peri-urban community of South Africa. The authors found all isolates (n=58) were resistant to ERY and CHL and amongst the *V. parahaemolyticus* isolates (n=14), 80% were resistant to CIP and NAL while 90% were resistant CEF and NOR while 55% were AMP resistant (Igbinosa et al., 2011). The lower level of AMP resistance reported by Igbinosa et al. (2011) may be attributed to exposing isolates to 25 µg for this antibiotic compared to the 10 µg used in the present work. Okoh and Igbinonsa (2010) conducted a similar study in a rural community in South Africa and found among the 52 isolates analyzed (consisting of *V. vulnificus*, *V. parahaemolyticus*, *V. fluvialis*, and *V. metschnikovii*), all exhibited resistance to 5 antibiotics including AMP and sulfamethoxazole (Okoh and Igbinosa, 2010).

A critical public health concern is the number of pathogens with resistance to clinically important antibiotics, particularly if they become multi-drug resistant. Of the 600 *V. parahaemolyticus* isolates examined, the prevalence of MDR (defined as resistance to 3 or more drugs in this study) was low and only seen in environmental isolates from Area 14, Area 15, Area 16, and Area 17 (Table 3.2 and Table 3.6). While 3 strains exhibited resistance to 4 antibiotics in the present work, much higher levels of MDR have been reported by other researchers. Baker-Austin et al. (2008) found 24% of the 350 *V. parahaemolyticus* isolates collected from water and sediment samples in Georgia and South Carolina demonstrated resistance to 10 or more antibiotics. Similarly, Letchumanan et al. (2015b) reported

approximately 28% of the isolates recovered (n=185) from shrimp sold in Malaysian markets were resistant to three different antibiotics with one isolate exhibiting resistance to 11 antibiotics. Kang et al. (2016) found a vast majority (78.4%, n=71) of the isolates recovered from Korean raw oysters were resistant to 3 or more antibiotics. Likewise, Hu and Chen (2016) reported 74.5% of 208 *V. parahaemolyticus* isolated from shellfish sold in Shanghai were resistant to 3 or more antimicrobial agents and noted that a higher incidence of MDR isolates were observed in shellfish samples compared to those recovered from crustaceans. While the prevalence of MDR *V. parahaemolyticus* of the Canadian isolates tested in this study is much lower (4.8% of all strains; 5.4% of environmental strains), direct comparisons to other studies are difficult as the panel of antibiotics used in each investigation was different.

Extremely few longitudinal studies examining the dynamics of AMR patterns in *V. parahaemolyticus* have been published; to the best of our knowledge this project represents the first one examining *V. parahaemolyticus* found in the Canadian environment. Overall, resistance to AMP and CEF were observed annually while variations in the percentage of isolates exhibiting resistance was noted for SUL, STR, TIO, PIP, and OA; moreover, resistance to GEN and KAN were only observed in one year (Figure 3.3). Most isolates displayed either singular resistance or a combination of resistance to AMP, CEF, and STR (Table 3.4 and Figure 3.4). As illustrated in Figure 3.4, AMP resistance was generally seen in more than 80% of the isolates analyzed for each year with the exception of 2006 (46%) and 2007 (75%); it should be noted that only 7 and 4 isolates, respectively, were tested for each of these years. Accordingly, the lower prevalence of AMP resistance for these two time points could be an artifact of the smaller sample size. Furthermore, while the occurrence of intermediate resistance has been relatively consistent, decreases in zones of inhibition have emerged for specific antibiotics (e.g. ERY, KAN), and with sustained selective pressure, these AMR phenotypes may become resistant.

AMR patterns for three oyster growing areas that were consistently monitored over multiple years were further evaluated to assess changes over time in these specific geographic sites (Figure 3.5 and Figure 3.6). Within Area 13-15, the percentage of isolates exhibiting AMP and SUL resistance was relatively stable at approximately 92% and 7%, respectively; in contrast, CEF resistance decreased from 50.0% to 15.4% and STR resistance was observed in one isolate recovered in 2008. Changes in AMR patterns were also seen in isolates recovered from Area 14-08. In particular, latter years recovered GEN resistant and PIP resistant isolates and also evidenced a significant increase in the frequency of resistance to AMP (p = 0.0032) and a significant decrease in CEF (p = 0.0201). While variations in the incidence of SUL and STR resistance were also observed, the differences were not statistically significant. For isolates recovered in shellfish harvesting areas near the Ladysmith wastewater treatment plant found in Area 17-07, resistance to AMP was found in more than 98% of the strains tested from both time points while decreases were noted for CEF and STR resistance. Additionally, SUL resistance was seen in 2 isolates recovered from 1998 and 2000 and one isolate from 1998 exhibited resistance to TIO. The occurrence of MDR *V. parahaemolyticus* was not observed in Area 13-15 for either time period; however, in Area 14-08, MDR isolates were only recovered in more recent years while isolates that were susceptible to all antimicrobial agents used in this study were only observed in an earlier time period (Figure 3.7). For isolates collected from Area 17-07, MDR resistance was observed in both time periods and none of the isolates were susceptible to all antibiotics assayed.

3.5 Conclusions

V. parahaemolyticus isolated from human clinical samples originating from BC as well as oysters collected from Canadian coastal waters were susceptible to the majority of antimicrobials tested, including frontline drugs recommended for treatment. However, resistance to AMP, CEF, STR, SUL, PIP,

OA, TIO, GEN, and KAN were observed in this data set. In addition, discernable differences in susceptibilities between clinical and environmental isolates were noted; clinical isolates were resistant to no more than two antibiotics while a small number of environmental isolates were resistant to 4 antimicrobials. Furthermore, while AMP and CEF resistance was ubiquitous and seen annually through a 14 year period, resistance to other antibiotics was intermittently detected. Reasons for the differences and changes observed are not currently understood and probable causes for resistance to specific antibiotics remain a matter of speculation. Nonetheless, to our knowledge this study represents one of the largest surveys of antimicrobial resistance of *V. parahaemolyticus* recovered from Canada. As such, these data can serve as a baseline against which future studies can be compared to evaluate whether susceptibilities change over time.

Chapter 4 – Conclusions and Future Directions

4.1 Conclusions

In the present study, the virulence profiles and AMR patterns for a library of 600 *V. parahaemolyticus* isolates recovered over a span of 14 years from oyster samples collected from Canadian coastal waters and clinical samples originating from BC were characterized. In general, differences with respect to virulence potential and AMR were observed between clinically-derived isolates compared to environmentally-derived strains. This was further corroborated by observed sub-typing differences determined by MLST analysis on a subset of *V. parahaemolyticus* isolates.

The presence of virulence markers *tdh* and/or *trh* was found in 53.1% and 8.4% of clinical and environmental isolates, respectively. The prevalence of *tdh* and/or *trh* in environmental isolates tested in this study is consistent with other published reports, while the proportion of clinical isolates lacking both these markers highlights the virulence potential of *V. parahaemolyticus* is more complex than historically thought. Although, most *V. parahaemolyticus* isolates examined in this study were susceptible to the majority of antibiotics tested, including frontline drugs recommended for treatment, resistance was observed for clinically relevant antibiotics including AMP, CEF, STR, SUL, PIP, OA, TIO, GEN, and KAN. Furthermore, discernable AMR differences between clinical and environmental isolates were seen; clinical isolates were resistant to no more than two antibiotics while 0.6% of environmental isolates displayed resistance to four antimicrobials. Additionally, AMP and CEF resistance was ubiquitous and seen annually through the 14 year period, while resistance to other antibiotics were intermittently detected. Differences in AMR patterns were also noted in three BC oyster growing areas that were consistently monitored over multiple years. The occurrence of MDR *V. parahaemolyticus*

(defined as resistant to 3 or more antibiotics in this study) was not evidenced in Area 13-13 between 1998 and 2011 but was observed in Area 17-07 between 1998 and 2005; whereas in Area 14-08, MDR isolates were only recovered in more recent years (between 2008 and 2011). Finally, MLST analysis demonstrated the *V. parahaemolyticus* population structure is diverse with differences once again noted between clinically-derived isolates and environmentally-derived strains. Over 50% of the STs had not been previously identified and all the novel STs, which may have occurred as a result of genetic exchanges, were recovered from environmental isolates. In contrast, a smaller number of STs appear to be responsible for illnesses in BC; some of these STs (e.g. ST 36, ST43, ST417) were also recovered from oyster-derived strains confirming the marine environment is a reservoir for virulent strains and suggests that a subset of *V. parahaemolyticus* strains may be adapted to life in the marine environment as well as the human host.

Therefore, considering the objectives and the knowledge gaps identified at the beginning of this study:

1. Virulence profiles and population dynamics of *V. parahaemolyticus* strains found in Canada were investigated and observed to differ between clinical and environmental sources.
2. *V. parahaemolyticus* recovered from the Canadian environment possess resistance to clinically relevant antibiotics which may have been drained into Canadian estuaries.
3. Antimicrobial resistance (AMR) profiles of *V. parahaemolyticus* strains isolated from Canadian sources (clinical versus environmental), including geographic area, and/or different years were found to be different, and induces new knowledge and scientific challenges.

4.2 Future Directions

In this study, a proportion of clinical *V. parahaemolyticus* isolates tested lacked recognized virulence markers *tdh* and *trh*. These *tdh*-negative and *trh*-negative strains demonstrated an ability to survive *in vivo* during illness, and suggest other factors may be playing a role in causing infection. For example, the T3SS of *V. parahaemolyticus* has been investigated as a potential indicator of strain virulence as correlations of *tdh* with T3SSα and *trh* with T3SSβ has been reported (Noriea et al., 2010; Park et al., 2004b). Furthermore, T6SS has been described as necessary for the adhesion of *V. parahaemolyticus* to cells (Wang et al., 2015). Additionally, capsule formation, iron acquisition, and flagellar motility have been described as necessary for host infection and investigations into these other cellular processes may provide insight into the virulence mechanisms of *V. parahaemolyticus* (Broberg et al., 2011; Wang et al., 2015). Therefore, applying more discriminatory analyses such as whole genome sequencing, on *tdh*-negative and *trh*-negative isolates, may provide useful insight into fundamental attributes that promote *V. parahaemolyticus* virulence and potentially reveal more reliable predicators that could subsequently be incorporated into diagnostic tests.

With regards to AMR, the library of *V. parahaemolyticus* isolates characterized thus far serves as a prelude and contributes to the Government of Canada's federal framework for action to combat AMR and use in Canada. As only AMR phenotypes were determined in this study, future studies should investigate and determine the underlying mechanisms mediating antibiotic resistance.

The data generated in this project also hinted at a trend that *V. parahaemolyticus* isolates lacking *tdh* and/or *trh* may be more susceptible to certain antibiotics. As such, further investigations exploring the

interplay between virulence and AMR would be valuable in providing more comprehensive risk assessments for this organism.

Lastly, this study demonstrated *V. parahaemolyticus* is genetically diverse and polyphyletic with evidence suggesting some infections were caused by a subset of strains. Therefore, there is promise in monitoring particular lineages and/or STs of concern. For example, using whole genome analysis of 295 *V. parahaemolyticus* genomes, a multiplex PCR assay was recently developed to identify ST36 strains and may be useful in more targeted monitoring in natural environments (Whistler et al., 2015). Moreover, future work examining conditions that have enabled certain strains to establish residency in specific geographic regions would undoubtedly improve the understanding of the various types of *V. parahaemolyticus* that exist in the environment and pose a higher risk to public health.

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