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論文名	Ecological and genetic fitness of <i>Vibrio</i> populations in estuary, mangrove and ocean with particular focus on pathogenic <i>Vibrio cholerae</i> (汽水域、マングローブ生息域及び海水域におけるビブリオ属細菌、特に病原性コレラ菌の生態と遺伝学的適合性)	
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論文要旨

Introduction

Vibrio spp. are Gram negative bacteria living in coastal and marine environment where they interact with virtually all kind of aquatic organisms. They play an important role in nutrient turnover including degradation of chitinous exoskeleton of plankton, shrimp, etc. Until now 80 *Vibrio* species are known. However, some *Vibrio* spp. can cause diseases in aquatic organisms as well as human. Among them the most important species is *V. cholerae* causing cholera which affects 2-3 million people each year. Besides, *V. parahaemolyticus* and *V. vulnificus* can cause tens of thousands of gastroenteritis, necrotizing fasciitis and septicemia each year.

Cholera is a devastating diarrheal disease in tropical coastal countries. The cholera toxin (CT) secreted by *V. cholerae* is the main cause of cholera. CT is encoded by a bacteriophage (CTX phage) which is integrated into bacteria through the toxin correlated pilus (TCP). Among 209 serogroups of *V. cholerae* only O1 and O139 serogroups have epidemic potential while others (non-O1 non-O139) can cause sporadic diarrhea. The O1 serogroup can be divided into classical and El tor biotypes. The classical biotype caused the previous 6 cholera pandemics (1817-1923) while the El tor biotype is the cause of the current 7th pandemic since 1961.

Historical and genetic data indicates Bengal delta as the pivotal base of global cholera

spread. Importantly, the Bengal delta coast is a cyclone and monsoon prone area, and also considered as the most vulnerable region due to predicted climate change and sea level rise. *Vibrio* spp. are also considered as emerging pathogens with possible link to global climate change. Nevertheless, there is no detail information on *V. cholerae* and other vibrios in ocean as well as tropical coastal environment (estuary and mangrove) of cholera endemic region like Bengal delta.

The objectives of the present study were 1) to gather detail information about the abundance and distribution of *Vibrio* spp. in coastal habitats (estuary and mangrove) as well as ocean, 2) to check the impact of climatic factors (salinity, cyclone and monsoon) on *Vibrio* population, 3) to identify the role of various nutrients and other physico-chemical factors favoring vibrios' abundance, and 4) to understand the toxigenic potential, genetic diversity, and antimicrobial resistance among coastal *V. cholerae* near the cholera endemic zone of Bengal delta.

Chapter 1. Comparative abundance of *Vibrio* population in coastal and oceanic habitats and development of a multiplex PCR assay for detection of pathogenic species

Vibrio abundance was determined by culture of direct or enriched samples on selective TCBS agar. Coastal expeditions were conducted in 14 and 22 sites along 30 and 90 km transects of Karnaphuli estuary beside a cholera endemic city and the world's largest Sunderban mangrove, respectively, in Bangladesh. Estuarine samples were collected during pre-cyclone, post-cyclone and post-monsoon conditions. Estuarine *Vibrio* population (10^3 - 10^8 CFU L⁻¹) increased after cyclone at all salinity zones (0-20 PSU, practical salinity unit) while monsoon rainfall reduced *Vibrio* abundance. Occurrence of *Vibrio* spp. was observed throughout the Sunderban mangrove (10^1 - 10^3 CFU L⁻¹), higher abundance near sea (3-5 PSU). Sampling was also done in the high saline Can Gio Mangrove (20-22 PSU) in Mekong delta, Vietnam where *Vibrio* population showed higher abundance (10^5 CFU L⁻¹) during low tide than high tide indicating probable role of nutrients.

Oceanic expedition was done from ~50°N to ~25°S (Germany to South Africa) in tropical and sub-tropical Atlantic. Samples were collected from surface (26 stations) and waters at great depths (7 stations) reaching seafloor (>4000 m). *Vibrio* abundance was comparatively less in ocean (10^1 - 10^4 CFU L⁻¹) than coastal zone. A gradual decrease in cultivable *Vibrio* numbers from surface to seafloor was noticeable. Interestingly, *Vibrio* spp. could be isolated at great depths. Oceanic *Vibrio* population showed a general increasing trend with rise in temperature (>20°C).

To understand the distribution of *Vibrio* populations as 'plankton-associated' or 'free-living', aquatic samples were fractionated using 55 µ net for zooplankton and 20 µ net for phytoplankton. In Atlantic samples, *Vibrio* population was predominantly present in <20 µ fraction. Similarly, pathogenic *V. cholerae* O1 abundance in coastal samples was 1-2 log higher in <20 µ fraction (free-living) than plankton fractions. The predominant occurrence of *Vibrio* population as 'free-living' indicated greater role of nano-particles, sediment and

dissolved nutrients.

Pathogenic *Vibrio* spp. can co-exist in aquatic samples. In this study, a simple and rapid multiplex PCR assay was developed to detect *V. cholerae* and *V. parahaemolyticus* and *V. vulnificus*. The PCR assay could amplify as low as 10 cells of the 3 species indicating its excellence. The assay showed 100% specificity and sensitivity when evaluated with a large number of target ($n=430$) and closely related *Vibrio* species ($n=59$) as well as other important bacteria ($n=47$) from various countries. Among Bangladesh isolates *V. cholerae* (15-50%) and *V. parahaemolyticus* (12-18%) while in Vietnam mangrove *V. parahaemolyticus* (66%) and *V. vulnificus* (13%) dominated. In contrast, these pathogenic species were absent in oceanic samples indicating their habitat preference towards the coastal region nearby human activities.

Chapter 2. Role of various physico-chemical parameters and nutrients in regulating *Vibrio* population in coastal and oceanic habitats

Physico-chemical parameters e.g., temperature, salinity, turbidity, etc. were measured by fieldmeters. Standard procedures were followed for spectrophotometric determinations of inorganic N, P and Si using Nutrient Analyzer. Organic nutrients including particulate organic C (POC) and N (PON) and dissolved organic C (DOC) and N (DON) were measured after catalytic oxidation followed by non-dispersive infrared detection and gas chromatography, respectively.

Higher *Vibrio* abundance at low tide in Vietnam mangrove coincided with increase in suspended particulate matter or turbidity and POC. In Karnaphuli estuary, rise in salinity and turbidity also favored *Vibrio* abundance. Microcosm experiments showed that coastal sediment having organic matter harbor most vibrios (>85%) and provide better survival capacity. Later a regression model was developed to predict coastal *Vibrio* abundance based on salinity and turbidity and a significant correlation was found between observed and predicted values.

As a probable nutritional substrate of suspended particulates for *Vibrio* spp., chitin concentration was determined fluorometrically using fluorescently labeled wheat germ agglutinin that has high affinity to chitin. Higher concentration of chitin was observed in <20 μ fraction than phyto- and zooplankton fractions at all salinity levels. In pre-cyclone normal condition a good correlation was observed between chitin and *V. cholerae* O1 in <20 μ fraction.

In contrast to coastal zone, oceanic *Vibrio* population had good correlation with the concentration of dissolved organic matter like DOC ($r=0.68$, $p<0.0005$) but no correlation with POC. This might be due to very low concentration of POC or particulate matters. *Vibrio* abundance in the Atlantic increased with temperature but their fluctuations could be better explained with variations in DOC or DON rather than temperature alone. The gradual decrease in *Vibrio* number with depth coincided with decrease in DOC but not POC. Therefore, dissolved organic matter likely provide majority of nutrient needs of *Vibrio*

populations in oceanic habitat.

Chapter 3: Genetic diversity, antimicrobial resistance and toxigenic potential of *Vibrio cholerae* strains in coastal habitat

V. cholerae strains from Bangladesh coast were screened for pathogenic genes by colony blot hybridization. Abundance of toxigenic genes was higher in Karnaphuli estuary near cholera endemic city where only 43% strains ($n=96$) were non-toxigenic in comparison to 62% strains ($n=50$) in Sunderban mangrove. Cytotoxic cholera toxin (*ctxA*) gene dominated among 40 and 32% strains of these habitats. The cholera toxin (*ctxA*) gene positive O1 strain which has epidemic potential was present among 6% Karnaphuli strains. Besides, *ctxA* gene was also present in some non-O1 non-O139 strains (1-2%) in both habitats.

To understand the genetic diversity of coastal *V. cholerae*, pulsed field gel electrophoresis (PFGE) technique was applied. Among 50 strains from Sunderban mangrove 26 pulsotypes existed, indicating high diversity. The genetic diversity of *V. cholerae* was even higher in Karnaphuli estuary: among 96 strains 61 pulsotypes existed. Some pulsotypes occurred in several distantly located sites indicating higher ecological fitness. The toxigenic genes distribution was independent of pulsotypes, e.g., among 7 *ctxA* positive strains 4 types and 7 patterns existed, etc.

The antimicrobial resistance patterns of the coastal *V. cholerae* strains were checked by disc diffusion method. Interestingly, *V. cholerae* strains in both Karnaphuli estuary and Sunderban mangrove showed resistance to all kinds of commonly used antimicrobials against cholera. More than 25% strains of both habitats were multi-drug resistant (MDR) including some estuarine (8%) and mangrove (14%) strains showing resistance against 7 or more antimicrobials. Importantly, 75% of strains having epidemic potential (*ctxA* and *tcpA* positive) had become MDR.

The CT production capacity of coastal *V. cholerae* strains were checked by Bead-ELISA. Most strains belonging to epidemic O1 serogroup were moderate CT producer (50-150 ng/ml) and two O1 strains showed high CT production (500-1200 ng/ml). All these O1 strains were MDR.

In the recent decade, El Tor variant strains producing classical type CT has replaced the typical El Tor strains in causing cholera epidemics. PCR scanning and sequencing of selected genes indicated that most toxigenic O1 strains were El Tor variant type. However, typical El Tor strain also existed in coastal habitat. Interestingly, one *ctx*-negative pre-El Tor strain also existed. The *ctx*-positive non-O1 strains had environmental type CTX phage possessing classical *ctx* gene.

Conclusion

Estuary and mangrove near cholera endemic zone are favorable habitats for *Vibrio* spp. Climatic factors like cyclone and intrusion of saline water can instigate rise in coastal *Vibrio* populations. The newly developed multiplex PCR assay can be very useful in screening of pathogenic *Vibrio* spp. Turbidity, suspended particulate matter, chitin and POC

have positive influence on coastal *Vibrio* while in oceanic habitat temperature and dissolved organic matter play important roles. A large number of coastal strains can be MDR. Coastal environment also favor genetic diversification in pathogenic *Vibrio* spp. However, the genetic diversity and abundance of toxigenic genes in *V. cholerae* is higher in estuary nearby cholera endemic city than pristine mangrove. Presence of toxigenic genes among genetically diverse and MDR coastal strains pose a great threat to nearby human. Therefore, *Vibrio* spp. can adopt diversified strategies for survival, spread and infection to attain greater ecological fitness.

審査結果の要旨

ビブリオ属細菌は、多くの海洋生物と深く関わりながら汽水域や海水域に生息するグラム陰性桿菌であり、甲殻類の殻のキチンの分解等様々な栄養物の代謝回転に重要な役割を担っている。現在、ビブリオ属には **80** 菌種が知られているが、いくつかの菌種は海洋生物やヒトに病原性を示す。特にコレラ菌はコレラの原因となり多くのヒトの命を奪っている。一方、腸炎ビブリオやビブリオバルニフィカスも胃腸炎や壊死性筋膜炎、敗血症の原因となる。コレラは熱帯・亜熱帯地方で重要な下痢性疾患である。コレラ菌の主要な病原因子として、コレラ毒素 (**CT**) や **TCP** と呼ばれる定着因子がある。現在までに **209** 種類の **O** 血清型が報告されているが、コレラの原因となるのは **O1** と **O139** のみである。**O1** 血清型にはさらに古典型とエルトール型の **2** つの生物型がある。第1次から第6次の世界大流行では古典型が、**1961** 年から今日まで続いている第7次はエルトール型が関わっている。コレラの疫学的、遺伝学的解析からベンガルデルタ地域がコレラ流行の発祥地として考えられている。ベンガルデルタ地域はサイクロンやモンスーンが多発し、気候変動、海面上昇等の影響を最も受ける地域であることが懸念されている。このような状況にも関わらず、ベンガルデルタ地域を含む熱帯・亜熱帯地域における汽水域、マングローブ生息域や海水域におけるコレラ菌やその他のビブリオ属細菌の分布に関する詳細な情報は無い。

本研究では、1) 汽水域、マングローブ生息域や海水域におけるビブリオ属細菌の分布に関する情報を集め、2) これらの水域における塩濃度やサイクロンの発生、モンスーンの有無等がビブリオ属細菌の分布に及ぼす影響を調べ、3) ビブリオ属細菌が好む栄養条件や物理化学的因子について明らかにし、4) ベンガルデルタ地域で分離されるコレラ菌の病原性、遺伝的多様性や薬剤感受性について調べることを目的とした。

第1章では、沿岸と遠洋におけるビブリオ属細菌の分布を比較し、病原性ビブリオ **3** 菌種を検出できる **Multiplex PCR** を開発し分離したビブリオ属細菌の菌種を同定した。バングラデシュのコレラ流行地に近いスンドラバンマングローブ生息域やカルナプリ汽水域において、サイクロン前・後及びモンスーン後のビブリオ属細菌の分布について調べたところ、全ての水域でサイクロンの後、ビブリオの菌数が増加したがその後の雨で菌数は減少した。ベトナムにおけ

る調査でもバングラデシュと同様、マングローブ生息域の高塩濃度の水域でビブリオの菌数が多かった。大西洋における調査では沿岸地域と比べ、ビブリオの菌数は少なかったが、海水を粒子径に基づき分画したところ **20 μm** 以下の画分でビブリオの菌数が最も多かった。ヒトに病原性を示すコレラ菌、腸炎ビブリオ及びビブリオバルニフィカスを検出できる特異的なマルチプレックス **PCR** を開発し、マングローブ生息域のビブリオの菌種を調べたところバングラデシュでは **15-50%**がコレラ菌、**12-18%**が腸炎ビブリオ、ベトナムでは **66%**が腸炎ビブリオ、**13%**がビブリオバルニフィカスであった。一方、海水域ではこれら **3** 菌種のビブリオ属菌は検出されなかった。

第2章では、様々な物理化学的因子や栄養物が沿岸及び遠洋におけるビブリオ属細菌の分布に及ぼす影響について調べた。ベトナムのマングローブ生息域で引き潮の時にビブリオ属細菌の検出率が高かったのは、海水中の浮遊粒子数あるいは濁度が増加して、その結果、粒子性の有機炭素量が増加したことに起因したと考えられる。カルナプリ汽水域では、塩濃度や濁度とビブリオの菌数で相関が見られた。サイクロン前では、粒子径 **20 μm** 以下の画分でキチン濃度と **O1** コレラ菌の菌数で相関が見られた。遠洋ではビブリオの菌数と可溶性の有機炭素濃度と相関があったが、浮遊性の有機炭素とは相関が無かった。遠洋では海底に行くほどビブリオの菌数は減少したが、それは可溶性の有機炭素の減少と関係していると考えられた。

第3章では、沿岸地域で分離したコレラ菌の遺伝学的多様性、薬剤感受性及び病原因子について解析した。病原遺伝子はコレラの流行地に近いカルナプリでの分離菌で多く検出された。コリックストキシン遺伝子は **40%**、コレラ (**ctx**) 毒素遺伝子は **6%**で検出された。**PFGE** で遺伝学的多様性を解析したところ、カルナプリ、スンダラバンの両分離株とも非常に高い多様性を示した。カルナプリ、スンダラバンの両分離株ともコレラの治療に一般的に用いられている抗菌薬の全てに対して耐性を示した。**25%**以上の菌株が多剤耐性菌であり、特に、病原性のコレラ菌の **75%**が多剤耐性菌であった。**CT** 産生性の **O1** コレラ菌 **7** 株のうち、**2** 株が **CT** 高産生株であった。また、**CT** 陽性の **O1** コレラ菌の多くは、近年問題となっているエルトールバリエントと呼ばれる古典型の **CT** を産生するエルトール型 **O1** コレラ菌であったが、エルトール型 **O1** コレラ菌も存在した。

以上の結果は、環境中、特に沿岸域や遠洋にも多くのビブリオ属細菌が存在し、その分布は様々な物理化学的な要因、特に塩濃度、濁度と可溶性の有機物が関係していることを明らかとした。沿岸部のマングローブ生息域は病原性ビブリオを含むビブリオ属細菌に適した生息場所であり、中でもコレラ菌はスンダラバンマングローブ生息域やカルナプリ汽水域で多く分離された。これらの成果は、コレラ菌を含むビブリオ属細菌の環境中での生態と遺伝学的適合性に関して新しい知見を提供したものであり、獣医学の分野のみならず環境微生物学領域においても多大な貢献をすると考えられる。従って、最終試験の結果と併せて、博士(獣医学)の学位を授与することを適当と認める。