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論　文　名	Identification of Vibrio campbellii as an emerging shrimp pathogen and development of a hemolysin gene-based multiplex PCR for the detection of *V.campbellii, V.harveyi* and *V.parahaemolyticus.* （エビの新興病原体としてのビブリオカンベリーの同定とビブリオカンベリー、ビブリオハーベイ及び腸炎ビブリオの鑑別検出法としてのヘモリシン遺伝子に基づくマルチプレックス PCR 法の開発）
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論文要旨

General introduction

Shrimp industry is growing rapidly after 80's, when there was a declining trend in the wild shrimp catch and the demand for shrimp was increasing in a staggering pace. In 1982, shrimp aquaculture contributed only about 5% of world shrimp supply; by 1994 this figure increased to 30%. According to recent statistical data, aquaculture now accounts for 50% of world shrimp supply. Globally, major earnings from shrimp farming were estimated about US $7 billion in 2000. India is one of the major shrimp producing countries having a gross production level of around 2 million tones of reared shrimps. In spite of an increasing trend of shrimp production in recent years, the growth curve shows relatively stationary phase. The main reason for this reduction in growth acceleration is mainly due to outbreak of some diseases, both of bacterial and viral origin. Slow down of growth not only hamper domestic economy, it

can also have indirect effects on the export trend. The major importers of Indian shrimp are EU, the US and Japan. Although export trend for the US and EU recently remains constant with 28 and 25%, respectively, the export to Japan has shown a declining trend with only 17.79% in recent year. Research on shrimp disease has a special importance in India because of its big impact in national economy.

Vibrios are the most important bacterial species contributing to the shrimp diseases mainly in the tropical countries including India. There were many reports about shrimp disease caused by different *Vibrio* spp. from long past. However, due to lack of accurate identification, causative agents of diseases were frequently identified as *Vibrio harveyi*-related organisms. Since proper identification of species is very important for appropriate prophylactic measures, attempts have been made by many researchers to identify disease-causing bacteria correctly. A hard task in the aquatic environment is to identify the appropriate organism responsible for the disease and to prove the pathogenic potential of the isolated strain. *Artemia* is an excellent model system for studying the pathogenic potential of many bacterial strains. As it is easy to culture and have very short life cycle, many contemporary studies have used *Artemia* model to examine the pathogenic mechanisms.

Recently, a bacterial disease outbreak was reported from a group of shrimp farms situated in the northern part of Chennai in south India. Disease symptoms were similar to vibriosis caused by different *Vibrio* spp. and there was no symptom of white spot disease generally caused by Systematic Ectodermal Mesodermal Baculovirus (SEMBV) in any of the pond throughout the culture. In the present study (1) bacteria were isolated from both diseased shrimp and pond environment of these affected shrimp farms and were identified systematically, (2) pathogenic potential of selected isolates were evaluated against *Artemia* model, (3) a simple multiplex PCR was finally developed to identify closely related *V. harveyi*, *V. campbellii* and *V. parahaemolyticus* simultaneously to reduce the ambiguity of identification problem.

Chapter 1. Isolation and identification of *Vibrio campbellii* as a shrimp pathogen

In the north coastal Chennai area, shrimp disease was observed in 3 farms (A, B, and C) consisting of 5, 6 and 8 ponds, respectively. The average size of the ponds was 0.5 to 1.0 hector (1 hector=10,000 m^2) with stocking density of 8-12 no./Sq.m (semi intensive type culture). During the months of November, 2004 to March, 2005 sudden shrimp disease broke out in these ponds. Disease symptoms included initial reduction in food uptake and arrested growth, followed by lethargic movement near the dyke during night and frequent death. Preliminary investigation revealed that the responsible bacteria might be pathogenic vibrios. Due to high phylogenetic similarity among marine vibrios, it is difficult to identify pathogenic strains. However, proper identification of strain is important for appropriate prophylactic measures.

In the present study a systematic screening was performed to identify the bacterial

group responsible for the disease outbreaks in the three shrimp farms. Initially a total of 88 isolates were biochemically identified. Biochemical tests revealed all the isolates were close to *V. harveyi*. Afterwards, 28 isolates were randomly selected and molecular identification was performed to improve the fidelity of identification. Initially species identification of these 28 isolates was done by sequencing of 16S rRNA and *pyr*H genes. Each *pyr*H gene sequence from all experimental strains was compared with published sequence data using genomic library (http://www.ncbi.nih.gov/BLAST) and 26 strains showed highest homology to published *V. campbellii* sequences with maximum score and lowest E value and remaining 2 strains showed closest match with published *V. harveyi* sequences. Based on the 16S rRNA and *pyr*H sequences, a maximum-parsimony phylogenetic tree was constructed with MEGA 4 software using neighbour-joining (NJ) program. Twenty-six strains formed a cluster with *V. campbellii* type strain (ATCC 25920^T) and the other two strains with *V. harveyi* type strain (ATCC 14126^T).

Ribotyping with *Bgl*1 was performed to evaluate the intra-specific variation in all strains identified as *V. campbellii* and *V. harveyi*. Interestingly, all 26 strains of *V. campbellii* were divided into 2 ribotype clones and other 2 *V. harveyi* formed a separate cluster. However pathogenic strains did not form any separate cluster in the present study.

Chapter 2. Virulence potential of *V. campbellii*

In recent years, scientists are trying to develop a consistent and repeatable experimental infection protocol for shrimp larvae. However, the infection studies with most shrimp species have had limited success to date, mainly due to the intrinsic variability and lack of reproducibility. There is an urgent need to develop a standardized protocol in order to evaluate the virulence of different *Vibrio* strains associated with shrimp mortality. *Artemia* spp., the brine shrimp, is an excellent model organism to study the mode of action of pathogenic bacteria, as it can easily be cultured under gnotobiotic conditions. So far *V. campbellii* is known as opportunistic pathogen but without any experimental proof. In the present study, attempt have been made to prove pathogenic potential of selected *V. campbellii* strain using *in vivo Artemia* model. Initially *Artemia* were hatched with autoclaved artificial seawater (ASW). After hatching, the healthy *Artemia* cysts were kept for 6 hrs and in each unit for challenge study 30 numbers of larvae were used. Bacterial concentrations were adjusted as 10^2, 10^5 and 10^8 $CFUml^{-1}$ in each tube and challenge experiments were conducted under sterile conditions. Dead larvae were collected after 24 hrs and bacterial strains were isolated and 16S rRNA gene sequence was performed. After 48 hrs, numbers of live *Artemia* in each tube were counted and average percentage of mortality (M_{obs}) with standard deviation (SD) value was calculated. All experiments were performed with three replicate per treatment to check the reproducibility. Among 28 strains, 8 were highly pathogenic, 4 were moderately pathogenic and 16 were found

to be non-pathogenic in this *Artemia* challenge model. LC_{50} values were determined for all strains except those which caused either 100% or 0% corrected mortality in any of the used concentration. This results were indirectly able to established that the disease was caused by selected pathogenic strains of *V. campbellii*.

To examine the *in vivo* colonization potential of *V. campbellii,* a highly pathogenic fluorescence-labeled strain IPEY54 and a non-pathogenic strain IPEY41 were fluorescently leveled initially. Fluorescent leveling was done by adding five hundred micro liter of DTAF solution (0.5 mg/ml) with 9.5 ml of bacterial suspension and mixture was incubated at 40°C under the dark condition with shaking (90 rpm) for 2 hrs. Subsequently fluorescently labeled bacteria (FLB) were inoculated at 10^5 CFU/ml in similar way as bacterial challenge study and after 24 hrs incubation FLB accumulation in the digestive tracts of *Artemia* were observed. *Artemia* challenged with fluorescently labeled pathogenic strain IPEY54 showed considerable patches of FLB clumps in the intestine of the larvae and no patch was observed when fluorescently labeled non-pathogenic strain IPEY41 was challenged against *Artemia* larvae. This result illustrated the *in-vivo* colonization of experimental strain (IPEY54) in the intestine of the *Artemia* larvae. Afterwards, colonization potentials of highly pathogenic strain IPEY54 and non-pathogenic strain IPEY41 were studied. A sharp increase of 84-fold for highly pathogenic strain (IPEY54) and a slight decrease of 0.1-fold for non-pathogenic strain (IPEY41) in cultivable counts were found in *Artemia* larvae after the bacterial challenge indicating higher colonization potential of the pathogenic *V. campbellii* strain.

Chapter 3. Development of a hemolysin gene-based multiplex PCR for the detection of *V. campbellii, V. harveyi* and *V. parahaemolyticus*

Simple and specific identification of disease-caused bacteria is essential for taking preventive and curative measures in aquaculture settings to enhance production and for better understanding of ecological and epidemiological events. Single, duplex or multiplex PCR based identification is a popular and accurate method to differentiate many closely related bacterial species. However success of this method depends on the selection of appropriate target gene, which should be species-specific, widely distributed and also very stable in the genome. Hemolysin is a widely distributed toxin among different *Vibrio* spp. Therefore, this gene was targeted for multiplex PCR. The *hly* gene sequences for some *V. harveyi* and *V. parahaemolyticus* strains already existed in GenBank data base (NCBI). However, no *hly* gene sequence of *V. campbellii* could be found in the NCBI database in the beginning of the present study. Therefore, complete *hly* gene sequence of *V. campbellii* was performed initially for developing species-specific primers. Then successfully developed a *hly* gene-based multiplex PCR which are highly sensitive and specific to differentiate the closely related species such as *V. campbellii*, *V. harveyi* and *V. parahaemolyticus*. Detection limit of all three-target species were

in between 10 to 100 cfu/PCR tube. The multiplex PCR was evaluated with 29 *V. campbellii*, 16 *V. harveyi* and 69 *V. parahaemolyticus* strains isolated from environment. Seventeen other *Vibrio* species, *Photobacterium damselae* and 9 clinical non-*Vibrio* species were also examined and this method was confirmed to specific.

Conclusions

The present study conducted a complete investigation of a shrimp disease outbreak in south India by detection of pathogen, prove the pathogenicity and finally development of a simple PCR based identification method to strengthen the future prediction system against this disease. Details are described as below:

- A bacterial disease with reduction of food consumption, lethargy and death was reported in three shrimp farms from Tamil Nadu near Chennai, south India. Biochemical identification followed by 16S rRNA and *pyrH* gene sequencing revealed dominance of mono-specific bacteria during disease outbreak and the responsible bacteria were *V. campbellii* (26 in numbers) and *V. harveyi* (2 in numbers).
- Ribotyping was able to differentiate *V. campbellii* from *V. harveyi* and all *V. campbellii* were divided into 2 clusters.
- *In vivo* pathogenic potentials of all 28 isolates revealed 8 were highly pathogenic, 4 were moderately pathogenic and remaining isolates were non-pathogenic against *Artemia*.
- Fluorescence labeling and co-infection revealed highly pathogenic strain produced fluorescent patches in the digestive tract with 84 fold increase in colonization potential in 24 hrs.
- A multiplex PCR based identification was developed for simultaneous detection of *V. campbellii*, *V. harveyi* and *V. parahaemolyticus*.

審査結果の要旨

エビ供給に関して養殖エビは、1982 年では世界全体で供給量の 5%でしかなかったが、1994 年には 30%まで、また最近の報告では 50%にまで増加している。この背景として、エビの乱獲による捕獲量の減少や需要の増加があり、エビ養殖産業は 1980 年以降、急速に成長している。インドは養殖エビを年間約 200 万トン生産しており世界で最も主要なエビの生産国の１つである。しかしながら、近年その増加傾向に歯止めがかかりつつ

あるが、その大きな理由として細菌やウイルスによる感染症が原因となっている。エビの輸出量の減少は輸出国の経済に影響を与えるのみでなく、輸入国にも多大の影響を与える。エビの病気に関する研究は、インド等のエビの養殖産業の盛んな国にとっては非常に重要な課題である。

熱帯地方の国々においてビブリオ属細菌はエビの病気の原因菌として最も重要な細菌の１つである。様々なビブリオ属細菌がエビの病気の原因となることが報告されている。しかしながら正確な菌種同定法が確立しておらず、多くの場合ビブリオハーベイ様細菌として処理されてきた。正確に菌種を同定することは、感染予防の対策を講じる上においても重要であり、多くの研究者によってビブリオ属細菌の正確な菌種同定法に関する研究が行われてきた。さらに、その菌種を同定した後、その菌が実際に病原性を示すかどうかを確認することも重要である。動物プランクトンの１種であるアルテミア（*Artemia salina*）は、培養が比較的容易でありライフサイクルが短いことからエビの病原性を調べる上において最も優れた感染実験のモデルとなる。近年、南インドのチェンナイ北部に位置する複数のエビの養殖場でエビの感染症が流行した。その病状からウイルス性によるものでなくビブリオ属細菌による可能性が示唆された。本研究では、発症エビとエビの感染症が発生した養殖池から細菌を分離し、細菌種を同定しビブリオカンベリーであることを明らかにした。さらに、単離菌の病原性についてアルテミアモデルを用いて解析し、エビの病気の原因となる近縁種のビブリオカンベリー、ビブリオハーベイ及び腸炎ビブリオの簡便迅速な PCR による検出法の開発を試みた。

第１章では、養殖場で発症したエビと養殖池の水から原因菌と思われる細菌 88 株を単離し、生化学的性状試験からビブリオ属細菌として同定した。その中から 28 株をランダムに選び、16S rRNA遺伝子と*pyrH*遺伝子を解析し、26 株はビブリオカンベリー、2 株はビブリオハーベイと同定した。*Bgl*II消化によるリボタイピングにより、ビブリオカンベリーは 2 種類のリボタイプに、ビブリオハーベイは１種類のリボタイプに分類されることを明らかとした。

第２章では、発症エビから分離したビブリオカンベリーが実際に病原性を有するか否かアルテミアモデルを用いて解析した。その結果、8 株は非常に強い病原性を示し、4 株は中程度の病原性を示し、他の 16 株は全く病原性を示さなかった。強い病原性を示したビブリオカンベリー1 株と病原性を示さなかったビブリオカンベリー1 株を蛍光標識し、アルテミアに投与したところ病原性を示さなかった株ではアルテミアの消化管内で蛍光は観察されなかったが、病原性の強い株では消化管内で強い蛍光が観察された。さらに病原性の強い株では、アルテミアの消化管内で菌数の増加が観察された。以上の結果より特定のビブリオカンベリーが、エビの消化管内に定着しエビの感染症を引き起こしている可能性を示した。

第３章では、ビブリオカンベリーのヘモリシン遺伝子の全構造を決定し、ヘモリシン遺伝子に基づくビブリオカンベリー、ビブリオハーベイと腸炎ビブリオを特異的に検出できるマルチプレックスPCR法を開発した。このマルチプレックスPCR法はPCRチューブあたり 10 個から 100 個の菌が存在すれば、これら 3 菌種を特異的に検出し、鑑別できることを明らかとした。

以上の結果は、ビブリオカンベリーがエビの病気の原因となることを示唆し、またアルテミアモデルを用いてその病原性を初めて明らかとした。さらに、ビブリオカンベリーのヘモリシン遺伝子の全構造を明らかにし、ヘモリシン遺伝子に基づくこれら３菌種の簡便・迅速なマルチプレックスPCR法による検出・鑑別法を開発した。これらの成果は、エビの新たな病原細菌の同定に留まらず、その鑑別検出法の開発も行い、獣医学の分野のみならず水産学の分野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。