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論　文　名	Analysis of prevalence, genetic diversity, biological activity and expression of cholix toxin in *Vibrio cholerae* non-O1/non-O139 (Non-O1/non-O139 コレラ菌が産生するコリックス毒素の遺伝子の分布と多様性および生物活性と毒素産生性に関する解析)
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論文要旨

Introduction: *Vibrio cholerae* is a Gram-negative, curved rod shaped bacterium. Till date >200 'O' serogroups of this species have been reported. Among them, *V. cholerae* O1 or O139 serogroup possessing two major virulent genes, *ctx* (encodes cholera toxin, CT) and *tcpA* (encodes toxin-coregulated pilus, TCP) are known as causative agent of cholera. Strains other than O1 or O139 serogroups are referred to as "non-O1/non-O139 *V. cholerae*". The *V. cholerae* non-O1/non-O139 generally does not carry *ctx* and *tcpA* genes but some of them can be potentially virulent in human, causing sporadic cases of diarrhea and extra-intestinal infections. The unsuccessful attempts to correlate genotypes of *V. cholerae* non-O1/non-O139 isolates with their virulence phenotypes in rabbit and mouse models suggest the presence of additional virulence factors and thus the search for virulence factors in *V. cholerae* non-O1/non-O139 is still ongoing.

Recently, cholix toxin (ChxA) has been reported in *V. cholerae* non-O1/non-O139 strains, which have been characterized as eukaryotic elongation factor 2-specific ADP-ribosyltransferase toxins. The *chxA* gene encodes a 666-amino acid (aa) residue protein (70.7 kDa, 634 aa mature protein) including a 32 amino acid residue leader sequence. ChxA consists of three structural domains for receptor binding, translocation and catalysis. Studies on the toxigenic properties of ChxA are in earlier

stage. There is little information about the abundance and genetic diversity of *chxA* gene among *V. cholerae* strains and their pathogenic mechanisms.

In the present study, the occurrence and genetic diversity of *chxA* gene was extensively investigated in clinical and environmental *V. cholerae* strains belonging to O1, O139 as well as non-O1/non-O139 serogroups. Furthermore, the virulence properties of ChxAs were also evaluated by cytotoxicity, rabbit ileal loop (RIL), and mouse lethality assays. Our findings illustrate the potential of ChxA as an important virulence factor among *V. cholerae* non-O1/non-O139 strains.

Chapter 1: Prevalence and genetic diversity of cholix toxin in *V. cholerae*

A total of 765 *V. cholerae* strains including O1 (*n*=485), O139 (*n*=84) and non-O1/non-O139 (*n*=196) were screened for the presence of *chxA* gene by colony hybridization. The present study has revealed the presence of *chxA* gene among a large number (53 out of 196, 27%) of *V. cholerae* non-O1/non-O139 strains of clinical and environmental origins. All O1 and O139 strains tested were negative for this gene. By sequencing the entire *chxA* gene of 53 strains, we further identified 2 variants of *chxA* gene designated as *chxA* II (*n*=16) and *chxA* III (*n*=4) beside prototype *chxA*, representing *chxA* I (*n*=33) previously reported. We have also observed high sequence diversity in *chxA* genes (29 subtypes among 53 sequences). The deduced amino acid sequences of ChxA revealed that the receptor binding domain (RBD) of ChxA II and catalytic domain (CD) of ChxA III were diverse when compared to that of ChxA I (or prototype sequence). 'O' antigen determination revealed that these 53 strains belonged to 27 diverse serogroups. Majority of *chxA* gene-positive strains (43 out of 53) did not have other potential virulence genes tested but all were positive for *rtx* and *hly* which encodes accessory virulence factors and are ubiquitously present in *V. cholerae*. The presence of *chxA* gene among a large number of genetically and serotypically divergent non-O1/non-O139 strains isolated from patients as well as environment and the absence of any other important virulence genes illustrates the potential of ChxA as an important virulence factor in *V. cholerae* non-O1/non-O139.

Chapter 2: Determination of cholix toxin's biological activity

The recombinant ChxAs (rChxA) were used for the analysis of biological activities. The cytotoxicity assay clearly demonstrated high cytotoxic effect of rChxA I on HeLa and Y1 cells but no effect by rChxA II. Interestingly, both the toxinotypes have similar growth inhibitory effect on CHO and Caco-2 cells. On the other hand, both the toxinotypes showed cytotoxicity on Vero, Int-407, Hep-2 and NIH-3T3 cells. rChxA III failed to cause cytotoxicity to any cell lines tested. The hallmark catalytic residues

for ADPRT activity are conserved in all the ChxA sequences. *In silico* structural analysis of ChxA toxinotypes suggest that they are nearly identical with no significant differences. However, the RBD of ChxA II has an altered β-jellyroll chain which resulted in altered conformation of this domain. Therefore, the variations in ChxA II induced cytotoxicity may be due to the altered RBD. To confirm this, we performed the competition assay using HeLa cells between ChxA toxinotypes. rChxA I-induced cytotoxicity was suppressed by rChxA III but not by rChxA II, indicating that ChxA I and III possibly share a common receptor on HeLa cells whereas ChxA II may not bind to the same receptor. However, the growth inhibition effect of ChxA I and II on CHO and Caco-2 cells and cytotoxicity to other cell lines are independent of their diversity at RBD or holotoxin level. This illustrates the probable presence of multiple cell receptors on these cell lines or presence of an alternative but common mechanism of ChxA to invade host cells.

The failure of ChxA III to cause cytotoxicity could not be explained by *in silico* structural analysis or competition assay. When analyzed carefully, it was found that ChxA III has an altered furin binding site with serine to glycine substitution. Although the significance of this amino acid substitution in furin binding site is not established, this mutation hampered the *in vitro* proteolysis of ChxA III and thus may repress cellular activation. This could explain the reason for the failure of ChxA III to induce cytotoxicity in the tested cell lines.

It was observed in this study that all the three ChxA toxinotypes failed to cause fluid accumulation by RIL assay, suggesting that ChxA could not be associated with enterotoxicity at least in animal model. It was also revealed that intravenous injection of rChxA I or II in mice resulted in their deaths, probably due to damage to the critical organs. Indeed, the occurrence of severe coagulation necrosis of the hepatocytes after injection of rChxA in mice suggests that liver could be the primary target of the ChxA. The rChxA III was not evaluated in mouse lethality assay as they failed to demonstrate any biological activity.

Chapter 3: Development of Bead-ELISA and quantification of cholix toxin in *V. cholerae*

The analysis of the biological activity of rChxA in chapter 2 clearly revealed that ChxA is an important virulence factor in *V. cholerae* non-O1/non-O139. However, further understanding the role of ChxA in the infectious process requires a detailed analysis of the molecule and its expression level. In this study, we developed the bead-ELISA for ChxA quantification and analyzed the expression level in selected *V. cholerae* strains. Anti-ChxA IgG was obtained after immunizing the rabbits with

rChxAI and Fab' fragment of IgG was conjugated with horseradish peroxidase and used in bead-ELISA. The standard detection range of the bead-ELISA developed was 0.2 to 5.0 ng ml^{-1} using purified rChxA I, II and III as a control respectively. The ChxA production levels were measured in alkaline peptone water (APW: vibrio selective broth), Luria Bertani broth (LB broth: commonly used bacteriological broth) and AKI broth (reported for higher CT production). The production level of ChxAs varied from strain to strain. When analyzed in APW, among the strains from ChxA I, Vc129 and C44 produced 1542 and 1114 ng ml^{-1} toxin respectively whereas Vc102 produced only 4 ng ml^{-1}. All other strains produced toxin in the range of 60 to 600 ng ml^{-1}. The strains from ChxA II also expressed varied levels of ChxA ranging from 98 to 1692 ng ml^{-1}. Vc67 and Vc75 from ChxA II were the highest producer with 1692 and 1517 ng ml^{-1} toxin respectively. The strains from ChxA III produced very low levels of toxin (5 to 15 ng ml^{-1}). The representative strains produced nearly similar level of ChxA regardless of medium except one strain which produced low levels of ChxA (56 ng/mL) in APW produced higher amounts in LB broth (~1.6 µg/mL), indicating that regulatory mechanisms of ChxA production may be different. The reason for this variation could not be established in this study. Further studies are required to see if the ChxA production level is correlated with the severity of extra-intestinal infection caused by *V. cholerae* non-O1/non-O139 in animal models.

Conclusions

In conclusion, *chxA* is prevalent among large proportion of *V. cholerae* non-O1/non-O139 strains and possess significant genetic diversity. This study reports the existence of at least three *chxA* toxinotypes (*chxA* I, II and III) including novel variants *chxA* II and III. Among them ChxA II and III posses highly diverse RBD and CD respectively, which may be attributed to their varied virulence patterns. None of the ChxA toxinotypes can cause enterotoxicity in rabbit. ChxA I and II can cause extensive damage to internal organs of mice especially liver, which is lethal. The production level of ChxA varied from strain to strain with majority of strains producing 200 to 400 ng/mL of ChxA. This study suggests that ChxA may be associated with extra-intestinal infections rather than enterotoxicity at least in the animal model.

審査結果の要旨

コレラ菌は O 抗原の違いに基づき現在 200 種以上の血清型が知られている。その中でコレラの原因となるのは O1 と O139 で、それ以外の血清型を non-O1/non-O139 コレラ菌と呼ぶ。Non-O1/non-O139 コレラ菌は通常、散発性下痢症や敗血症等の腸管外感染症の原因となる。O1 と O139 コレラ菌の主要な病原因子として、コレラ毒素と定着繊毛が知られているが、non-O1/non-O139 コレラ菌の病原因子は十分明らかとなっていない。

近年、non-O1/non-O139 コレラ菌が真核細胞の蛋白合成を阻害し細胞を致死させる新たな毒素としてコリックス毒素（ChxA）を産生することが報告された。これまでに、ChxA は 634 個のアミノ酸残基からなる分子量約 71 kDa の蛋白毒素であること、緑膿菌の産生するエキソトキシン A やジフテリア菌の産生するジフテリア毒素と同様、真核細胞の伸長因子2を ADP-リボシル化する ADP-リボシルトランスフェラーゼであること、レセプターへの結合部位、毒素の細胞内挿入部位、酵素活性を持つ触媒部位という3つのドメインで構成されていることなどが報告されている。しかしながら、*chxA* 遺伝子の分布や多様性、ChxA の病原性については十分明らかとなっていない。本研究では、*chxA* 遺伝子の分布及び病原性について明らかにすることを目的に、1）患者及び環境由来のコレラ菌における *chxA* 遺伝子の分布と多様性、2）ChxA の細胞及び動物レベルでの病原性、3）ChxA の定量系を構築し、各種コレラ菌が産生する ChxA の産生能を調べた。

第1章では、*chxA* 遺伝子の分布と多様性ついて解析した。インドやバングラデシュの下痢症患者及び環境から分離された 485 株の O1、84 株の O139、及び 196 株の non-O1/ non-O139 コレラ菌を含む合計 765 株のコレラ菌について *chxA* 遺伝子の分布を調べたところ、53 株（27%）の non-O1/non-O139 コレラ菌からのみ *chxA* 遺伝子が検出された。53 株の *chxA* 遺伝子の全塩基配列を調べたところ3つのクラスターに分かれること、推定アミノ酸配列の相同性からプロトタイプと相同性の高い ChxA I に加え、ChxA II と ChxA III という新たな2つのバリアントの存在を見いだした。ChxA I と比べ、ChxA II はレセプター結合部位のアミノ酸残基に多様性があり、ChxA III は触媒部位に多様性があることを見いだした。また、*chxA* 遺伝子が陽性となった non-O1/non-O139 コレラ菌のほとんどは、これまでに報告された他のコレラ菌の病原遺伝子を保有していなかったことから、ChxA が発症に関与する病原因子として重要な役割を果たしている可能性を提唱した。

第2章では、プロトタイプを含む3つの ChxA バリアントの生物活性について調べた。8種類の培養細胞に ChxA を作用させたところ、細胞は ChxA タイプにより異なる細胞感受性を示した。ChxA I と ChxA II は一部の細胞を除き致死活性あるいは細胞増殖抑制活性を示した。しかしながらウサギ腸管ループ試験では両毒素とも腸管毒性は示

さなかった。一方、ChxA III は細胞毒性も腸管毒性もどちらも示さなかった。マウスに静脈内注射するとChxA I、ChxA II は致死活性を示し、 それはChxA I よりもChxA II の方が活性は強かった。死亡したマウスを解剖したところ肺及び肝臓に対する毒性が認められ、特に肝臓は凝固壊死を示しており ChxA の主たる標的組織であることが示された。

第3章では、ChxA を定量するための Bead-ELISA を構築し、各種コレラ菌が産生するChxA を定量した。構築したBead-ELISA では、200 pg/mL から5.0 ng/mL の範囲で3つのChxA バリアントを定量できた。この系を用いて各種コレラ菌が産生するChxA を定量したところ、菌株間で ChxA の産生量に大きな差があることがわかった。その差は0.004～1.7 μg/mL であったが、使用する培地によっても発現量が大きく異なり、さらにある培地で産生量が少ない場合でも別の培地では産生量が約 29 倍も多くなるなど、ChxA の発現調節機構は菌株間で大きく異なることを明らかとした。

以上の結果は、*chxA* 遺伝子はnon-O1/non-O139コレラ菌特異的に存在し、またChxA には新たな2種のバリアントがあることを明らかとした。ChxA I とChxA II では細胞感受性が異なり、腸管毒性は示さず肝毒性によってマウスを致死させること、また、ChxA IよりもChxA II の毒性がより強いことを明らかとした。ChxA の産生量は菌株間あるいは培地によっても大きく異なり、ChxA の発現調節機構も菌株間で異なることを明らかとした。これらの成果は non-O1/non-O139 コレラ菌の腸管外感染症の病原因子に対する新しい知見を提供したものであり、獣医学領域のみならず医学領域においても多大な貢献をするものと考えられる。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。