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論　文　名	Comparative analysis of cytolethal distending toxin (*cdt*) genes, and biological activities of CDTs in *Campylobacter helveticus* and *Campylobacter upsaliensis* （カンピロバクター・ヘルベティカスとカンピロバクター・ウプサリエンシスの細胞膨化致死毒素［*cdt*］遺伝子と CDT の生物活性の比較）
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論文要旨

Introduction

Campylobacter is considered as one of the most prevalent zoonotic pathogens causing human gastroenteritis worldwide. Although the poultry and livestock animals are the main source of infection, pets are also well-recognized carriers of *Campylobacter* spp., and possess an important risk factor for human campylobacteriosis. *C. upsaliensis* and *C. helveticus* are the major campylobacters commonly found in dogs and cats, both sick and healthy animals. Considering zoonotic pathogens to humans, *C. upsaliensis* is considered to be third predominant emerging *Campylobacter* next to *C. jejuni* and *C. coli* and campylobacteriosis in humans associated with their healthy household pets have been reported. Although information about *C. helveticus* in clinical sample is relatively rare, this species also has been detected from diarrheal patients. Thus, *C. helveticus* might be associated with human disease, which has been so far possibly underestimated. On the other hand, very little information is known about the pathogenic mechanisms and virulence factors of both C. *upsaliensis* and *C. helveticus*. Cytolethal distending toxin (CDT) is known to contribute to as a potential virulence factor for certain *Campylobacter* species, for example, cytotoxicity, invasiveness, and persistent colonization of *C. jejuni in vivo*. Recently, *cdtB* gene has been detected from *C. helveticus* strain from healthy cat in Japan by PCR targeting a conserved region in *cdtB* genes of *C. jejuni*, *C. coli* and *C. fetus*.

CDT-like effect was also observed in HeLa cells when cultured with the bacterial cell lysate of *C. helveticus,* and accordingly, particular strains of the bacterium might harbor entire *cdt* genes, and produce biologically active CDT. Subsequently, a *cdtB* gene-based PCR-RFLP assay was established for the detection of 7 *Campylobacter* species and this assay yielded differential RFLP patterns in *C. upsaliensis* strains isolated from healthy dogs in Japan. The objective of this study was to divulge into more details of the genetic variations among the *cdt* genes in *C. helveticus* and C. *upsaliensis*, and the biological activities of, *C. helveticus* (CheCDT) and *C. upsaliensis* CDT (CuCDT), in comparison to other campylobacters.

Chapter 1: Determination and analysis of *cdt* gene cluster in *C. helveticus* and *C. upsaliensis*

Culture followed by PCR-based screening of fecal samples from healthy cats in Japan detected the occurrence of *cdtB*-like gene in *C. helveticus* strain CAT and the gene sequence was highly homologous to the *cdtB* genes of certain *Campylobacters*. The bacterial cell lysate of the isolated *C. helveticus* strain caused cell distention in HeLa cells. To check whether this type of *C. helveticus* strain harbor the complete *cdt* genes cluster, indicating their as biologic potential, nucleotide sequences of the entire *C. helveticus cdt* (Che*cdt*) gene cluster and its flanking region were determined and analyzed. All *C. helveticus* strains, isolated from both diarrheic and healthy cats including ATCC 51209 strain (n=9), were observed to harbor the complete *cdt* genes cluster with the conservation of putative amino acid residues essential for DNase I activity in the CdtB. Among genus *Campylobacter*, the deduced amino acid sequences of CheCDT were highly homologous to those of CuCDT.

With relation to the findings of a recent study showing existence of at least3 subtypes of Cu*cdt* genes (RFLP patterns Cu-I, Cu-II, and Cu-III) in *C. upsaliensis* by the *cdtB* gene-based PCR-RFLP assay, it is worthy to check whether the genetic variation could be either due to mutation in *cdtB* gene sequence or presence of the *cdt* gene variant. Therefore, in this study the entire *cdt* genes of 3 *C. upsaliensis* strains, ATCC 43954 (Cu-I), 40-1 (Cu-II) and 9-1 (Cu-III) were sequenced. Comparative genetic analysis revealed all these strains harboring *cdt* gene cluster with subtle differences among *cdt* genes of each representative strain at both nucleotide and amino acid levels. However, this of genetic variation may not be enough to consider each of the PCR-RFLP patterns representing different sub-types.

In summary, *C. helveticus* harbored *cdt* gene cluster which was found to be ubiquitously conserved among *C. helveticus* strains isolated from both diarrheic and healthy cats. On the other hand, *C. upsaliensis* analyzed in this study most likely harbored identical *cdt* gene variant although they showed different RFLP patterns.

Chapter 2: Evaluation of the biological activities of *cdt* gene-products from *C. helveticus* and *C. upsaliensis*

C. helveticus harbored the *cdt* gene cluster and CDT-like effect was observed in cells cultured with bacterial cell lysate of the isolate. To see if the cytotoxic effect was due to CDT, biological activities of *cdt* gene-products were evaluated. CDT effects including cell distention, cell cycle arrest and γH2AX were observed in cells cultured with

reconstituted rCheCDT holotoxin as well as bacterial cell lysate from strain CAT. The cytotoxic effects of this strain could be neutralized by antiserum against rCheCdtA and rCheCdtC. Additionally, bacterial cell lysate of other *C. helveticus* strains (*n*=8) also caused cell distention in HeLa cells which was neutralized by the anti-rCheCdtC serum.

In case of *C. upsaliensis*, although Cu*cdt* genes seem to be identical, its gene-products may possess different biological activities or may have the same. Therefore, cytotoxic effects were examined in HeLa cells with bacterial cell lysate of 3 representative strains. All strains demonstrated CDT effects which were completely neutralized by anti-rCuCdtC serum. Interestingly, CDT activity titer produced by *C. upsaliensis* was much higher than that of the other *Campylobacters*. The toxin titer among 3 representative strains is variable (1,024, 8,192 and 65,536 in Cu-II, I and III, respectively). When compared for amino acid sequences, in CdtC, arginine at position 69 was replaced with threonine in strain 99-1, showing the highest titer, when compared with the other two representative strains. Furthermore, in case of CdtA, 7 amino acid residues in CdtA of strains ATCC 43954 (Cu-I) and 99-1 (Cu-III) were conserved but differ from those in CdtA of strain 40-I (Cu-II). The difference in toxin titer could result from the difference in production level of CDT. However, the production of CdtC of strain 99-1 (Cu-III) was lowest, whereas that of ATCC 43954 was highest among these 3 strains by western blotting. It's also possible that antiserum may have different sensitivities to CdtC from prototype strains. Western blotting with purified rCdtC protein generated from each respective strain demonstrated that the sensitivity of antiserum was identical. Interestingly, western blotting with non-denaturing protein showed that mobility of CdtC of strain 99-1, showing the highest titer, was different from that of ATCC 43954 and 40-1 even though predicted isoelectric points of holotoxin and individual toxin subunit protein did not show much difference.

In summary, *C. helveticus* strains produced biologically active CDT which could be the potential virulence factor of this organism, suggesting their possible emergence as important zoonotic pathogen for human. CDT activity titer produced by *C. upsaliensis* is much higher than other *Campylobacters* suggesting that CuCDT might contribute more to human diseases in comparison to other CDTs. Difference in CDT activity among *C. upsaliensis* strains could be due to amino acid substitutions in CdtA and CdtC subunits as well as altered natural conformation of holotoxin, but not due to variation at the production level.

Chapter 3: Comparative analysis of CDTs produced by pet-predominant *Campylobacters* with other CDTs in *Campylobacters*

High sequence homology was observed at amino acid level between CheCDT and CuCDT, but less to those of CjCDT and *C. hyointestinalis* CDT-II (ChCDT-II). Therefore, CheCDT and CuCDT may produce characteristics different from CjCDT and ChCDT-II. Importantly, in case of *Escherichia coli* CDT-I (EcCDT-I), transmembrane protein 181 (TMEM181) have been reported to be essential for intoxication. Therefore, the characteristics of CheCDT and CuCDT were analyzed and compared with other CDTs, in terms of cell tropism and immunogenicity. CheCDT, CuCDT, CjCDT and EcCDT-I displayed similar cell tropism. This might be due their utilization of identical toxin receptor on host cell surface. Thus, competition assay was carried out by using purified

rCdtA and rCdtC, considered to be responsible for the binding of the receptors. Interestingly, rCheCdtA or rCheCdtC could competitive inhibit the activity of CheCDT and CuCDT but not CjCDT and EcCDT-I. Additionally, ChCDT-II displayed different cell tropism. On the other hand, rCuCdtC could inhibit cytotoxicity of CheCDT Neutralization assay with anti-rCheCdtA and anti-rCheCdtC sera showed inactivation of the activity of only CuCDT and vice versa. Anti-rChCdt-IIC, anti-rCjCdtB, anti-rCfCdtB and anti-rEcCdt-IB sera evaluated in this study could not neutralize CDT activity of other CDTs except their own CDT.

In summary, CheCDT has biological characteristics related to CuCDT such as cell tropism, toxin receptor utilization and immunogenicity which are different from other CDTs in *Campylobacters*. These data indicate that CheCDT and CuCDT which are associated with pet predominant *Campylocaters* have similar biological characteristics.

Conclusions

1. *C. helveticus* strains ubiquitously conserved *cdt* gene cluster whereas *C. upsaliensis* strains analyzed in this study harbored identical *cdt* gene variant, although exhibiting different RFLP patterns.
2. *C. helveticus* strains produced biologically active CDT which could be the potential virulence factor of this organism, suggesting their possible emergence as important zoonotic pathogen for human.
3. *C. upsaliensis* strains produced biologically active CDT and yielded much higher toxin titer than other *Campylobacters* suggesting that CuCDT might contribute more to human diseases in comparison to other CDTs. The observed difference in toxin titer among the *C. upsaliensis* strains might not be related to the production level, but due to amino acid substitutions in CdtA and CdtC, or altered natural conformation of holotoxin.
4. CheCDT and CuCDT which are produced by the *Campylobaters* species predominant among the pet animals have similar biological characteristics such as cell tropism, toxin receptor utilization and immunogenicity, which in turn were different from CDTs produced by other *Campylobacters.*

審査結果の要旨

Campylobacter 属菌は通常、動物の腸管内に不顕性に存在しており、動物との過度の接触や畜産食品を介してヒトに感染する。多くの *Campylobacter* 属菌はヒトの胃腸炎の原因となるが、ある種の *Campylobacter* 属菌は敗血症など腸管外感染症の原因ともなる。現在少なくとも 26 菌種知られている *Campylobacter* 属菌の中で、*C. jejuni* と *C. coli* が食中毒の原因菌として最も高頻度に分離されている。しかし、近年、非選択培地を用いることや PCR 法による遺伝子検査法の導入で、*C. jejuni*

や *C. coli* 以外の *Campylobacter* 属菌の分離、検出例が増加してきている。

申請者の研究グループは、ある種の *Campylobacter* 属菌は細胞膨化致死毒素（*cdt*）遺伝子を、菌種特異的かつ普遍的に保有することを見出し、*cdt* 遺伝子を標的とした PCR 法や PCR-RFLP 法を開発し、*Campylobacter* 属菌の簡便・迅速な菌種同定法としての有用性を報告してきた。その過程で、*C. helveticus* も *cdt*(Che*cdt*)遺伝子を保有すること、*C. upsaliensis* が保有する *cdt*(Cu*cdt*)遺伝子には多様性があることを見出した。

そこで本研究では、*C. helveticus* が生物活性を示す CDT を産生しているのか？産生している場合どのような生物学的、免疫学的性状を有するのか？また、*C. upsaliensis* が PCR-RFLP 解析で示した多様性は CuCDT にどのような違いをもたらしているのか？を生物学的、免疫学的に解析することによって明らかにすることを目的とした。

第１章では、*C. helveticus* が保有する *cdt* 遺伝子の全塩基配列と PCR-RFLP 法で３つに型別された *C. upsaliensis* の *cdt* 遺伝子（Cu-I、Cu-II、Cu-III）の全塩基配列を解析した。その結果、*C. helveticus* に CheCdtA、CheCdtB、CheCdtC をコードした完全な Ch*cdt* 遺伝子が存在すること、また３種類の Cu*cdt* 遺伝子はそれぞれの Cu*cdtA*、Cu*cdtB*、Cu*cdtC* 遺伝子間で 96%、97%、96%以上の相同性を有することを明らかとした。CheCDT と CuCDT はアミノ酸レベルで CdtA、CdtB、CdtC それぞれで 91%、92%、86%と他の CDT と比べて最も高い相同性を有することを明らかとした。

第２章では、CheCDT と３種類の CuCDT の生物活性について解析した。その結果、大腸菌で発現した CheCdtA、CheCdtB、CheCdtC を再構成した rCheCDT は細胞膨化活性と核内の DNA 傷害に基づく細胞周期の G_2/M 期の停止とそれに基づく細胞致死活性を有することを確認した。さらに、抗 CheCdtA、抗 CheCdtB、抗 CheCdtC 血清により *C. helveticus* の CDT 活性が中和されたことより野生型の *C. helveticus* も生物活性を有する CDT を産生していることを明らかとした。一方、*C. upsaliensis* も同様の解析で野生型の株が生物活性のある CDT を産生していることを明らかとし、さらに、Cu-III の生物活性が３種類の *C. upsaliensis* の中で最も強い活性を示したのみならず他の *Campylobacter* 属菌が産生する CDT よりも 500 倍活性が強いことを明らかとした。３種類の *C. upsaliensis* の中で最も強い CDT 活性を呈した理由が、*cdt* 遺伝子のコピー数や発現量の違いではなく、産生された CDT の性状の違いに基づく可能性が示された。

第３章では、CheCDT と CuCDT の細胞指向性、それぞれのサブユニットのレセプターの認識能や免疫学的性状を含めたそれぞれの CDT の性状を比較解析した。その結果、用いた６種類の細胞に対して両 CDT は同じ細胞指向性を示し、それぞれの CdtA、CdtC 単独あるいは抗 CheCdtC または抗 CuCdtC 血清でそれぞれの CDT 活性が中和された。*C. helveticus* と *C. upsaliensis* が産生する CheCDT と CuCDT は、免疫学的、

細胞指向性やレセプターを含め性状が酷似していること示した。

以上の結果は、新興人獣共通感染症菌の可能性が考えらている２菌種の CDT について以下に記す新知見をもたらした。すなわち、*C. helveticus* が生物活性のある CDT を産生することを初めて明らかとし、特定の *C. upsaliensis* 株がタイター 65,536 という強い CDT 活性を示した理由が *cdt* 遺伝子のコピー数や産生量の増加によるものでなく産生された CDT の性状の違い基づく可能性があることを示し、ペットが感染源となる *C. helveticus* と *C. upsaliensis* が産生する CDT は生物学的、免疫学的に酷似した性状を有することを明らかとした。本研究成果は、獣医学の分野のみならず医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。