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学位授与の日付	２０２５年３月３１日
論　文　名	Comparative analysis of three distinct cytolethal distending toxin (*cdt*) genes and biological activities of their gene products in *Campylobacter fetus* （カンピロバクター・フィータスの３種類の細胞膨化致死毒素（*cdt*）遺伝子とその産物の生物活性の比較解析）
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論文要旨

Introduction

Campylobacter is one of the major causative agents of diarrhea worldwide. Among the 32 currently recognized species in the genus *Campylobacter*, *C. jejuni* and *C. coli* are predominantly isolated from diarrheal patients and domestic animals such as poultry and cattle. *C. fetus* is known as a zoonotic pathogen, causing not only diarrhea but also septicemia and meningitis in humans, and abortions in cattle and sheep. However, the precise virulence mechanisms of *C. fetus* remain largely unknown. Cytolethal distending toxin (CDT) is one of the most well-characterized virulence factors in *Campylobacter* spp. CDT consists of three subunits (CdtA, CdtB and CdtC) encoded by three adjacent genes, *cdtA*, *cdtB*, and *cdtC*, respectively. CdtB functions as the active subunit with DNase activity, while CdtA and CdtC are responsible for the receptor binding and internalization of CdtB into cytosol. This toxin induces cell distention and DNA damage on target mammalian cells, resulting in irreversible cell cycle arrest and eventual cell death. It has been reported that *C. jejuni*, *C. coli* and *C. fetus* ubiquitously possess a single *cdt* gene-cluster, and CDT production by *C. jejuni*, *C. coli* and *C. fetus* strains has been confirmed in several studies. However, a previous study from our lab found that the cell distention caused by *C. fetus* strains in CHO cells was not neutralized by anti-rCfCdtB serum of *C. fetus* strain Col-108, suggesting the production of variant of

CfCDT or an unknown toxin(s) in the *C. fetus*.

Our preliminary *in silico* analysis suggested the presence of three different *cdt* gene clusters, which we named *Cfcdt-I*, *Cfcdt-II* and *Cfcdt-III* in the *C. fetus* ATCC27374^{T} genome. It is known that CDT can promote the invasion of *Campylobacter* spp. into host cells and help evade the host immune system by affecting immune cells. Nevertheless, it remains unclear whether these *Cfcdt* gene clusters are ubiquitously present in *C. fetus* strains and their association with diseases in humans and animals. Therefore, we hypothesized that *C. fetus* may cause extraintestinal infections in humans and animals more frequently than *C. jejuni* and *C. coli* by utilizing these distinct CfCDTs. Our final goal is to understand the potential association between the pathogenesis and *cdt* gene products in *C. fetus*. The objective of this study was to evaluate the role of the *cdt* gene products as potential virulence factors in *C. fetus*.

Chapter 1. Distribution and genetic diversity of three *cdt* gene clusters in *C. fetus*

To investigate the distribution of three *cdt* gene clusters in *C. fetus*, 137 *C. fetus* strains isolated from human patients (n=18) and healthy cattle (n=119) were analyzed. The presence of three types of *Cfcdt* genes was assessed by colony hybridization assay using nine specific ^{32}P-labeled probes for each *cdt* subunit gene (*cdtA*, *B*, and *C*), showing that all *C. fetus* strains carry three different *Cfcdt* gene clusters. Clonal relatedness for all *C. fetus* strains was determined by pulsed-field gel electrophoresis (PFGE), further grouped *C. fetus* strains into 38 different pulsotypes. Whole-genome sequencing of 38 representative *C. fetus* strains (8: human, 30: cattle) was conducted to investigate differences among the *Cfcdt* gene sequences and sequence type (ST). Each *Cfcdt* gene cluster displayed high similarity (>99%) across the 38 strains, and interestingly, some strains shared similar sequence variations at the same positions within the *cdt* gene sequences, leading to the identification of five types of CfCDT variants among these strains. To highlight these differences, multilocus sequence typing was performed. A total of 6 sequence types (STs) were identified among 38 *C. fetus* strains. Human strains were mostly typed as ST-6 (7 out of 8), while half of the cattle strains (16 out of 30) belonged to ST-3. ST-specific variations were observed in the *Cfcdt* gene sequences, including deletions and insertions in some cases that indicate truncation in the subunit proteins. Moreover, some human and cattle strains with different pulsotypes, but belonging to ST-6, exhibited very similar PFGE patterns, suggesting that *C. fetus* ST-6 associated with cattle may serve as a source of infection to humans, and ST-6 in cattle could be more virulent than other STs to human. Notably, *C. fetus* ST-6 strains carried a mutated *Cfcdt-IIB* gene, indicating that CfCDT-II may not play an important role in causing diseases.

Altogether, these data suggest that three distinct *Cfcdt* genes (*Cfcdt-I*, *Cfcdt-II*, and *Cfcdt-III*) are highly conserved within the *C. fetus* strains examined, and that strains belonging to ST-6 may be more related with human illness.

Chapter 2. Functional characterization of three *Cfcdt* gene products in recombinant *E. coli* strain

To understand the role of each *Cfcdt* gene-product, the three *Cfcdt* gene products

from *C. fetus* ATCC27374^T were analyzed for the CDT activities, such as cell distention and DNase activity. Each of three full length *Cfcdt* gene clusters were cloned into pET28a(+) vector and expressed separately in *E. coli* BL21(DE3), generating rCfCDT-I, rCfCDT-II, and rCfCDT-III, and used as each toxin sample. CDT toxicities including cell distention, cell cycle arrest and γH2AX were observed in HeLa cells cultured with rCfCDT-I and rCfCDT-III, but not rCfCDT-II. Since rCfCDT-II did not show any CDT-like activities against HeLa cells, cytotoxicity assays were extended to five additional cells lines, which are Caco-2, HepG2, Jurkat, Vero and CHO, to further explore the biological activity of each rCfCDT. Consistent with previous results, rCfCDT-I and rCfCDT-III induced cell distention against Caco-2, HepG2, Vero and CHO cells, and caused cell damage in Jurkat cells. Interestingly, cytotoxic cell tropism of these two CfCDTs were not identical. rCfCDT-I showed stronger cytotoxicity against HeLa, Caco-2, HepG2 and Vero cells, while rCfCDT-III exhibited higher activity on CHO and Jurkat cells. In contrast, rCfCDT-II showed no detectable cytotoxicity against any of the tested cell lines even though the expression of CfCdt-IIB could be detected by using antiserum against rCfCdt-IIB. To investigate whether the receptor-binding subunits (CdtA and CdtC) contribute to the observed differences in cell tropism between CfCDT-I and CfCDT-III, six chimeric products (CfCdtIA-IB-IIIC, CfCdtIA-IIIB-IIIC, CfCdtIA-IIIB-IC, CfCdtIIIA-IIIB-IC, CfCdtIIIA-IB-IC, CfCdtIIIA-IB-IIIC) were constructed and expressed in *E. coli* BL21(DE3). The CDT activity of filter sterilized chimeric cell lysates was tested against HeLa and CHO cells. Unexpectedly, none of the six chimeric CDTs induced cell distention against either cell lines, suggesting that the receptor-binding subunits between CfCDT-I and CfCDT-III are likely incompatible in forming a functional structure. Additionally, to determine whether the CfCdt-IIB subunit is active, receptor-binding subunits from CfCDT-II and CfCDT-III, which have higher homology in the *CfcdtB* gene than CfCdt-I, were swapped to create two chimeric toxins (CfCdtIIA-IIIB-IIC and CfCdtIIIA-IIB-IIIC). However, no chimeric toxins induced cell distention against HeLa or CHO cells.

Taken together, these results suggest that *C. fetus* produces biologically active CfCDT-I and CfCDT-III, both of which could serve as potential virulence factors. Moreover, CfCDT-I and CfCDT-III show different cell tropisms and may utilize different toxin receptors, suggesting that they might have different potential roles *in vivo*.

Chapter 3. Characterization of CfCDTs in *C. fetus* wild-type strains

To understand if the truncation of CfCDTs happened in *C. fetus* wild-type strains could affect the toxicity, cytotoxicity assays were performed with HeLa, CHO, Vero, Caco-2, and Jurkat cells using lysates from *C. fetus* strains with different STs. All tested strains showed CDT ability on cell lines tested, indicating that *C. fetus* strains could produce biologically active CfCDTs. *C. fetus* ST-3, -5, -6, and -20 strains showed similar CDT titers, regardless of whether a truncated CfCDT subunit was present. Notably, *C. fetus* strains belonging to ST-2 and ST-81, which have a truncated CfCdt-IIIB subunit, showed reduced CDT titers in all tested cell lines. In contrast, ST-6 strains with a truncated CfCdt-IIB subunit exhibited no reduction in CDT titer, suggesting that CfCDT-II may not be essential for *C. fetus* cytotoxicity as described in chapter 2. To determine whether *Cfcdt-II* genes are transcribed in *C. fetus*, the transcriptional levels of nine *Cfcdt* subunit genes [*Cfcdt-I* (*A*, *B*, *C*), *Cfcdt-II* (*A*, *B*, *C*), and *Cfcdt-III* (*A*, *B*, *C*)] were analyzed by RT-qPCR. The relative transcription of each *Cfcdt* gene was

normalized by that of the housekeeping gene *rpoA*. Results showed that *Cfcdt-II* genes are consistently transcribed at the same levels as *Cfcdt-I* and *Cfcdt-III* genes. To ascertain which CfCDT predominantly contributes to the cytotoxicity of *C. fetus* wild-type strains, neutralization assays were carried out using antiserum against each rCfCdtB. Expectedly, the cytotoxicity caused by rCfCDT-I and rCfCDT-III could be neutralized only by antiserum against rCfCdt-IB and rCfCdt-IIIB, respectively. Under treatment with antiserum against rCfCdt-IB or rCfCdt-IIIB, cytotoxicity caused by *C. fetus* wild-type strains was not fully neutralized. However, when treated with a combination of antisera against rCfCdt-IB and rCfCdt-IIIB, cytotoxicity caused by *C. fetus* wild-type strains could be fully neutralized. To further study the contribution of each CfCDT to the cytotoxic activities of *C. fetus*, isogenic *cdt* mutants (Δ*cdt-I*, Δ*cdt-III*, and Δ*cdt-I*/Δ*cdt-III*) were constructed in *C. fetus* ATCC27374^{T} strain. Cytotoxicity assays with the lysates of *C. fetus* mutant Δ*cdt-I* or Δ*cdt-III* revealed that each mutant strain could still show cytotoxicity against HeLa, CHO and Jurkat cells, cause G_2/M cell cycle arrest and damage DNA in HeLa cells. In contrast, double mutant strain (Δ*cdt-I*/Δ*cdt-III*) exhibited no detectable CDT activity in any of the tested cell lines even though the expression of CfCdt-IIB could be detected using anti-rCfCdtIIB serum, further supporting the finding that CfCDT-II is not biologically active. In summary, both CfCDT-I and CfCDT-III act as active toxins in *C. fetus*, while CfCDT-II does not appear to be an active toxin to the pathogenicity of *C. fetus* strains, at least toxic assays used in this study.

Conclusions

1. *C. fetus* ubiquitously possesses three distinct *Cfcdt* gene clusters, and the variations of *Cfcdt* genes sequences were conserved in each *C. fetus* ST at both nucleotide and deduced amino acid sequence levels.
2. *C. fetus* strains belonging to ST-6 may be more pathogenic to human.
3. *C. fetus* strains produced biologically active CfCDT-I and CfCDT-III which could be the potential virulence factors of this organism.
4. CfCDT-I and CfCDT-III showed different cell tropisms and immunogenicity, suggesting that these toxins might have different target cell/tissue and/or different virulent role *in vivo*.
5. *Cfcdt-II* genes were transcribed in *C. fetus* cells but may not form active holotoxin or be toxic against cell lines which were used in this study.

審査結果の要旨

Campylobacter は我が国のみならず世界的に問題となっている食中毒細菌である。現在 32 菌種知られている *Campylobacter* 属菌の中で *C. jejuni* と *C. coli* が最も高頻度に胃腸炎患者から分離されている。一方、*C. fetus* はヒトに胃腸炎のみならず敗血症や髄膜炎の原因となり、家畜には流産の原因

となる。しかしながら、人や家畜に対する病態発症に関わる詳細なメカニズムは明らかとなっていない。細胞膨化致死毒素（CDT）は *Campylobacter* 属菌の中で最もよく解析されている病原因子の 1 つである。CDT は CdtA、CdtB、CdtC の 3 つのサブユニットから構成されるホロ毒素で、CdtA と CdtC が感受性細胞の受容体への結合に関与し、CdtB が DNase 活性を有する毒素活性本態である。CDT は感受性細胞を膨化させた後、致死させる毒素として知られている。以前の研究で *C. fetus* の細胞毒性が CfCdtB の抗体で中和できなかったことから *C. fetus* は複数の CDT を産生している可能性が考えられた。*C. fetus* 標準株の登録された全塩基配列を解析したところ 3 種類の *cdt* 遺伝子を見出し、それぞれ *CfcdtI*、*CfcdtII*、*CfcdtIII* と命名した。しかしながら、これら 3 種類の *cdt* 遺伝子が *C. fetus* に普遍的に存在しているのか、また、遺伝子産物は細胞毒性を発揮できるのか、*C. fetus* の病原性にどのように関わっているかは明らかとなっていない。そこで、本研究では、*C. fetus* における *Cfcdt* 遺伝子の分布と塩基配列の多様性と 3 種類の CfCDT の細胞毒性について解析した。

第一章では 3 種類の *cdt* 遺伝子の *C. fetus* での分布と遺伝子配列の多様性について調べた。患者由来 18 株と健康な牛由来の 119 株の合計 137 株についてそれぞれのサブユニット遺伝子、合計 9 遺伝子の分布について調べたところ全ての遺伝子が 137 株に保存されていた。137 株を PFGE による遺伝子型別で選別した 38 株について全ゲノム解析を行い、3 種類の *cdt* 遺伝子の塩基配列と MLST 解析により ST を決定した。3 種類の *Cfcdt* 遺伝子間の類似性は 50-60%であった。38 株の 3 種類の *Cfcdt* 遺伝子内の類似性はそれぞれ 99%以上で 6 つの ST に分類された。ST-6 は家畜由来株と患者由来株で検出され、人への病原性に関係する ST と考えられた。いくつかの *cdt* 遺伝子に変異も見つかり、完全長とならない Cdt サブユニットも存在した。ST 型と *Cfcdt* 遺伝子の変異に相関性が認められた。

第二章では、3 種類の組換え *cdt* 遺伝子を大腸菌で発現させ、3 種類の rCfCDT の細胞毒性について種々の培養細胞を用いて調べた。rCfCDT-I の rCfCDT-III は細胞膨化、G_2/M での細胞周期の停止、ヒストンリン酸化が確認されたが、rCfCDT-II ではこれらの活性は見られなかった。また、rCfCDT-I と rCfCDT-III の細胞指向性は異なっていた。それぞれの A、B、C サブユニットの 8 種類のキメラ毒素を作製したが全てのキメラ毒素で細胞毒性は観察されなかった。以上より、*C. fetus* は少なくとも 2 種類の細胞毒性のある CDT を産生していることが明らかとなった。

第三章では不完全長な *Cfcdt* 遺伝子を含め野生株を用いてそれぞれの CDT の細胞毒性を種々の細胞を用いて解析した。rCfCDT-II はどの細胞に対しても毒性

を示さなかったが、*CfcdtI* と *CfcdtIII* と同様、*CfcdtII* も含めそれぞれのサブユニット遺伝子の mRNA は同レベルで転写されていた。また、rCfCDT-I と rCfCDT-III の活性はそれぞれの抗 CdtB 血清で中和された。野生株の細胞毒性は抗 CdtIB と抗 CdtIIIB の両血清で完全に中和された。野生株の *CfcdtI* 欠損株、*CfcdtIII* 欠損株および *CfcdtI* と *CfcdtIII* の両欠損株を作製し細胞周期とヒストンリン酸化に与える影響を調べたところ単独欠損株では残りの CfCDT のそれぞれの活性が認められたが、両欠損株では CfCDT の活性は全く認められなかった。一方、両欠損株において Western blotting で CfCdt-IIB の発現がわずかながら確認された。以上の結果より、CfCDT-I と CfCDT-III は活性のある毒素として産生され、CfCDT-II は活性がないか、発現量が少なく細胞毒性等が認められていない可能性が考えられた。

以上の結果は、*C. fetus* には 3 種類の *cdt* 遺伝子が普遍的に存在すること、3 種類の CDT のうち I 型と III 型のみが細胞毒性を示すことなど *C. fetus* がヒトに胃腸炎のみならず腸管外感染症を、また家畜に流産を引き起こす *C. fetus* の病原性を考える上で有益な基礎的情報を提供するものである。本研究成果は獣医学のみならず医学の分野において多大な貢献をすると考えられる。従って、本論文の審査ならびに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。