称号及び氏名	博士（獣医学）　OBI, OKECHUKWU JOHN
学位授与の日付	２０２５年3月３１日
論　文　名	Identification of potential reservoirs of virulent *Providencia* strains in Japan, and their zoonotic relevance （日本における病原性プロビデンシア属細菌の潜在的自然宿主の同定と人獣共通感染症との関連性）
論文審査委員	主査　山﨑　伸二 副査　三宅　眞実 副査　堀江　真行 副査　畑中　律敏

論文要旨

Introduction

Providencia species are members of the family *Morganellaceae* formerly classified as *Enterobacteriaceae*. They are widely distributed in the environment including water, soil, and plants. In addition, they are considered as part of human and animal intestinal flora. However, some strains are known to cause gastroenteritis in humans and animals including human food borne outbreaks and were recently designated as enteropathogenic.

The enteropathogenic mechanisms of *Providencia* remain largely speculative. However, the possession of invasins or cytolethal distending toxin (CDT) that is limited to a few strains among two species (*P. alcalifaciens* and *P. rustigianii*) isolated from diarrheic patients, are considered as mediators of virulence. Consistently, species-specific CDTs are established virulence-mediators in several enteropathogens including *Campylobacter* spp., *Escherichia coli*, etc. CDT is a tripartite-holotoxin consisting of three subunits CdtA, CdtB and CdtC acting in tandem to produce a functional toxin. CdtA and CdtC are considered the receptor binding subunits, whereas CdtB is the effector domain with DNase I-like activity that is translocated into the cell nucleus resulting in cell cycle arrest, irreversible cell distension and subsequent cell death.

Despite the isolation of *cdt* gene-positive *Providencia* in diarrhea cases in Japan and elsewhere, the reservoir and transmission route of such strains remain unknown. Initial probe of wild raccoons captured as alien and invasive species in Osaka wild reserves found by

serendipity that the animals harbor *Providencia* strains bearing *cdt* genes. This finding suggests that wild raccoons might serve as the reservoir of this pathogen, ultimately posing a risk to humans through contamination of the environment, agricultural products and the food animals.

This study is aimed at identifying the potential reservoir of the *cdt* gene-positive *Providencia* strains and elucidating their zoonotic relevance. In chapter 1, wild raccoons in Osaka were extensively surveyed for the presence of the virulent *Providencia*. The retail meats (chickens, pork and beef) and environmental water were also analyzed to obtain a wider view of possible transmission dynamics. In chapter 2, the genotypic and phenotypic characterization of CDTs identified in the *Providencia* strains were ascertained. In chapter 3, the diarrheagenic potential of the *cdt* gene-positive *Providencia* was evaluated.

Chapter 1. Distribution and genomic diversity of *cdt* gene-positive *Providencia* in raccoon, retail meats, and environmental water

To identify the potential reservoir and transmission route of *cdt* gene-positive *Providencia*, 384 raccoon rectal swabs, 143 meats (chicken 72, pork 46, beef 25) and 58 environmental water samples collected in Osaka were analyzed for the distribution of *cdt* gene-positive *Providencia* species, using a duplex PCR specifically developed for *Providencia* spp. by targeting *Providencia*-genus 16S rRNA and *cdt* genes. *Providencia* spp. were detected in 60% (232/384), 61% (87/143) and 84% (49/58) of raccoon, meat and water samples, respectively. However, *cdt* genes were only found in 16% (38/232) of raccoon and 18% (9/49) of water samples. *Providencia* species were isolated from the samples using a *Providencia* specific medium (PMXMP), and determined to be various species including *P. alcalifaciens, P. heimbachae, P. huaxiensis, P. rettgeri, P. rustigianii, P. stuartii,* and *P. vermicola* by conventional biochemical tests and sequencing of *rpoB* gene. Of the seven identified species, *cdt* genes were distributed in two, *P. alcalifaciens* (raccoon and water) and *P. rustigianii* (only in raccoon). The possible presence of other CDT variants which cannot be detected by the duplex PCR assay was assessed in the *cdt* gene-negative *Providencia* strains using a PCR-RFLP assay capable of distinguishing *cdtB* genes of *E. coli*, *E. albertii*, and *Providencia* species, coupled with sequencing of the PCR products. Interestingly, *P. rettgeri* strains isolated from eight raccoon samples yielded amplicons corresponding to the *cdtB* gene in size, but with different restriction patterns. Sequencing of the amplicons yielded nucleotides with > 90% similarity with *Providencia cdtB* genes. Altogether, the *cdt* gene-positive *Providencia* were most frequently detected in *P. alcalifaciens* (63%), *P. rettgeri* (21%), and *P. rustigianii* (16%) in raccoons and *P. alcalifaciens* (100%) in environmental water.

To ascertain how diverse the isolated *cdt* gene-positive strains are, pulsed-field gel electrophoresis was carried out with one to two strains selected at random from *cdt* gene-positive samples and divergent pulsotypes consisting of 29, 4 and 3 distinct clones among *P. alcalifaciens, P. rettgeri*, and *P. rustigianii*, respectively, were observed.

The abundance of divergent *cdt* gene-positive *Providencia* pulsotypes in raccoons, coupled with similarities to those of the environmental water samples, suggests that raccoons could be the probable reservoir of *cdt* gene-positive *Providencia* strains in Japan.

Chapter 2. Genotypic and phenotypic characterization of CDTs identified in the *Providencia* strains

Previous studies have shown the presence of functional CDTs in *P. alcalifaciens* and *P. rustigianii*. However, this information is limited to a few strains of human clinical origin. To ascertain whether *cdt* gene-positive *Providencia* strains isolated in the present study produce biologically active CDT, the *Providencia* strains cell lysates toxicities on eukaryotic

cells were determined. Sonicated filter-sterilized cell lysates of the *P. alcalifaciens, P. rustigianii,* but not *P. rettgeri* caused distension of CHO cells. Unexpectedly, the current study strains were found to be more toxic to the eukaryotic cells compared to the clinical strains previously isolated. Intriguingly, cell lysates from the *P. rustigianii* had higher toxic effects compared to the other *Providencia* species. Neutralization of cell distension activities by the addition of anti-rPaCdtB rabbit serum strongly suggests that the toxicities observed in this study were due to CDT. To ascertain the expression of *cdt* genes in *P. rettgeri*, and to further validate the CDT activities in *P. alcalifaciens* and *P. rustigianii* strains, an anti-rPaCdtB serum was used to detect the presence of CdtB in the strains cell lysates by Western blotting. CdtB production was detected in all the strains, excluding the *P. rettgeri* strains.

Based on CDT titer differences observed, and to understand why *P. rettgeri* CDT had no biological activity, the entire nucleotide sequences of the *cdt* gene clusters were determined by genome walking based on the *cdt* amplicons obtained. Nucleotide sequences of *cdt* genes in all the strains were determined, however, analysis of the *cdt* gene sequences revealed a conserved CdtB sequence of about 269 amino acids in the *P. rettgeri*, but with truncations in the *cdtA* and *cdtC*. In contrast, the nucleotide sequences of the *cdt* gene clusters in the *P. alcalifaciens* and *P. rustigianii* strains were devoid of any truncations, and their predicted amino acid sequences were not titer specific.

Since *cdt* genes were not evenly distributed in all the *Providencia* strains, and to understand their possible acquisition routes, the location of the *cdt* genes in the *cdt* gene-positive *Providencia* was determined by S1-PFGE with subsequent Southern hybridization assay using a ^{32}P-labeled *cdtB* gene-probe from a *P. alcalifaciens* clinical strain. Large plasmids of about 140-210 kb in size were hybridized with the *cdtB* gene-probe in all the tested strains. Intriguingly, the *cdtB* gene-probe also hybridized with a smaller plasmid of about 50 kb in size in *P. rustigianii*, commonly present in the *cdt* gene-positive *P. rustigianii* strains.

Having identified *cdt* genes on two different plasmids in the *cdt* gene-positive *P. rustigianii*, high-throughput sequencing with PacBio long-read Revio platform was used to elucidate the *cdt* gene clusters on the plasmids. The *cdt* genes designated as *Pruscdt-I* on the large plasmid were found to be identical (100% nucleotide sequence similarities) to previously reported *Pruscdt* genes in a *P. rustigianii* strain JH-1 isolated from a diarrheic child in Japan. However, *cdt* genes designated as *Pruscdt-II* on the small plasmid differed with *cdt-I* in nucleotide sequences (95% similarity). Nevertheless, the newly identified *Pruscdt*-II considered novel, clustered among the *Providencia cdt* genes clade based on maximum likelihood comparison. To ascertain the functionality of *Pruscdt-I* and *Pruscdt-II* gene-products, the *cdt* gene clusters were separately cloned into pET-28a(+) vector and expressed independently in *E. coli* BL21(DE3), generating rPrusCdt-I and rPrusCdt-II. The two recombinant CDTs were found to be biologically active based on cytotoxicity assay in CHO cells. However, rPrusCdt-II seems to have different tropisms due to its distension of HeLa cells, unlike rPrusCdt-I. To determine the horizontal transferability of the small plasmid bearing the newly identified *cdt-II*, bi-parental mating was carried out using a donor *P. rustigianii* strain bearing a genetically marked plasmid derivative (chloramphenicol resistant gene insert). The *Pruscdt-II* bearing plasmid was found readily mobilizable to all the recipient strains including *P. alcalifaciens* GTC2020, *P. rustigianii* GTC1504, *P. rettgeri* GTC1263, *P. stuartii* GTC1444, and *E. coli* KC95, suggesting that the donor strain (*cdt* gene-positive *P. rustigianii*) could transform related commensal bacteria strains into pathogenic strains by transfer of the *Pruscdt-II* bearing plasmid.

These findings suggest that *cdt* gene-positive *Providencia* produced functional CDTs and were more toxic compared to the clinical strains in eukaryotic cell cytotoxicity assay.

Chapter 3: Diarrheagenic potential of *cdt* gene-positive *Providencia*

It has recently been reported that the acquisition of a plasmid-mediated T3SS distinct from the chromosomally encoded T3SS is associated with invasion of HeLa cells and fluid accumulation in rabbit illeal loop by a clinical *cdt* gene-positive *P. rustigianii*. Additionally, the acquired T3SS was found to be co-localized with *cdt* genes on the same plasmid in *cdt* gene-positive clinical *Providencia* strains designated as enteropathogenic.

In view of this observation, the *cdt* gene-positive *Providencia* strains isolated from raccoons and environmental water in this study were assumed to also have the plasmid-mediated T3SS. To test this assumption, the presence of one of the plasmid-T3SS related genes (*invF,* a transcriptional regulator of the AraC/XylS family) was ascertained by PCR amplifications. Concordantly, the plasmid-T3SS was detected in all the *cdt* gene-positive strains (100%, 46/46), unlike in 11% (39/356) of the *cdt* gene-negative *Providencia* strains used to ascertain the general distribution of the plasmid-mediated T3SS in the *Providencia*.

To evaluate the potential invasiveness of the present study strains bearing the plasmid-T3SS, gentamicin protection assay was conducted using HeLa cells. The *cdt* gene-positive strains (40%), but not the *cdt* gene-negative *Providencia* strains were found to have invasive capabilities comparable to the clinical *P. rustigianii* JH-1 control strain.

Moreover, preliminary fluid induction assay in an animal model (rabbit ileal loop) revealed that the current study strains could induce diarrhea like their human clinical counterparts. These findings suggest that the current study *cdt* gene-positive *Providencia* strains might cause diseases in humans, thus highlighting their zoonotic relevance.

Conclusions

1. *Providencia* species distributions in a wild animal (raccoon), and environmental water were ascertained, paving the way for future epidemiological studies.
2. Raccoons were identified as the probable reservoir of the *cdt* gene-positive *Providencia* strains in Japan.
3. Novel *cdt* gene clusters were identified in *P rettgeri* and *P. rustigianii* isolated from raccoons, respectively.
4. Dual functional *cdt* genes on different plasmids were identified in *P. rustigianii*.
5. The *cdt* gene-positive *Providencia* isolated from raccoons and environmental water have the potential to invade intestinal epithelial cells and cause diarrhea.

審査結果の要旨

Providencia 属菌は腸内細菌目モルガネラ科に属し、水、土壌や植物など環境中に広く分布している。一部の *Providencia* 属菌はヒトや動物に胃腸炎を引き起こすが、その病原発現機構については明らかとなっていない。少なくとも、細胞侵入性や細胞膨化致死毒素（CDT）の産生性が *P. alcalifaciens* と *P. rustigianii* で報告されているが、これらがどのように病態と関係しているかは不明である。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、細胞を致死させる毒素である。CDT を産生する *P. alcalifaciens* と *P. rustigianii* が小児下痢症患者から分離されているが、自然宿主は明らかとなっていない。アライグマは様々な病原体の宿主となっていることが報告されている。本研究では、大阪府内で捕獲されたアライグマ、市販の食肉および環境水を対象とし、*Providencia* 属菌の分布、分離した *Providencia* 属菌の菌種同定、*cdt* 遺伝子の分布と病原因子を含む細菌学的性状を解析した。

第一章ではアライグマ、市販食肉および環境水における *Providencia* 属菌の分布と *cdt* 遺伝子の分布を duplex-PCR で解析した。アライグマ、市販食肉および環境水からそれぞれ 60% (232/384)、61% (87/143)、 84% (49/58)で *Providencia* 属特異的遺伝子が PCR で検出され、*Providencia* 属特異的遺伝子 PCR 陽性検体のみから 16% (38/232)、0% (0/87)、 18% (9/49)で *cdt* 遺伝子が検出された。*Providencia* 属特異的 PCR で陽性となった検体から *Providencia* 属菌を分離した結果、*P. alcalifaciens* 226 株、*P. heimbachae* 8 株、*P. huaxiensis* 12 株、*P. rettgeri* 82 株、*P. rustigianii* 40 株、*P. stuartii* 23 株、*P. vermicola* 11 株と同定された。*cdt* 遺伝子陽性の *Providencia* 属菌はアライグマ由来 *P. alcalifaciens* 24 株と *P. rustigianii* 6 株、環境水由来 *P. alcalifaciens* 8 株であった。*Pcdt* 遺伝子が陰性となった *Providencia* 属菌の *cdt* 遺伝子を異なる PCR 法で調べたところ *P. rettgeri* 8 株で陽性となった。

第二章では分離菌の *cdt* 遺伝子と CDT の性状について解析した。3 菌種の *cdt* 遺伝子の塩基配列を解析した。既報の *P. alcalifaciens* と *P. rustigianii* の *cdt* (*Pacdt, Pruscdt*) 遺伝子の類似性はそれぞれ 97%<と 100%であった。一方 *P. rettgeri* の *cdt* (*Pretcdt*) 遺伝子は *cdtA* と *cdtC* の変異により完全長の ORF を形成できなかった。それぞれの CDT の細胞毒性は *P. alcalifaciens* と *P. rustigianii* では認められたが、*P. rettgeri* では予想通り認められなかった。S1-PFGE で *cdt* 遺伝子の分布を調べたところ全てがプラスミド上に存在し、*P. rustigianii* では大きさの異なる 2 つのプラスミド上に検出された。全ゲノム解析を行ったところ新規の *Prucdt*(*Pruscdt-II*) 遺伝子は 50 kb、既存の *Prucdt* (*Pruscdt-I*) 遺伝子は 20.9 kb のプラスミド上に検出された。これらのプラスミドは同種を含め他種の *Providencia* 属にも接合伝達性を示した。PrusCDT-II は PrusCDT-I と比べ CHO 細胞に対して 8 倍強い細胞毒性を示した。

第三章では *cdt* 遺伝子陽性の *Providencia* 属菌の病原性について調べた。*cdt* 遺伝子陽性の *Providencia* 属菌はプラスミド上に 3 型分泌装置（T3SS）関連遺伝子を保持していることが報告されている。今回分離した *Providencia* 属菌における 46 株全ての *cdt* 遺伝子陽性菌と *P. alcalifaciens cdt* 遺伝子陰性の 39 株で T3SS 関連遺伝子が検出された。

細胞侵入性は *P. rettgeri* を除く 18 株で陽性となった。ウサギ腸管ループ試験で *cdt* 遺伝子 2 コピー持つ *P. rustigianii* 1 株と細胞侵入性 *P. alcalifaciens* 3 株について調べた。*P. alcalifaciens* 2 株で液体貯留が認められたが、残り 2 株では認められなかった。*cdt* 遺伝子を 2 コピー持つ *P. rustigianii* は T3SS 関連遺伝子中の *sipA* 遺伝子に変異があり、この変異が下痢原性に影響を与えている可能性が考えられた。

以上の結果は、アライグマが病原性 *Providencia* の自然宿主の１つであること、新規の *cdt* 遺伝子を *P. rettgeri* が保菌することや *P. rusutiganii* が 2 コピーの *cdt* 遺伝子を大小異なるプラスミド上に保持していることなど *Providencia* 属菌の自然界での分布や病原因子の一端を明らかとした。本研究成果は獣医学のみならず公衆衛生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。