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論　　文　　名	Development of a multiplex PCR targeting *eae*, *stx* and *cdt* genes, and characterization of *cdt* gene-positive *Providencia rustigianii* isolated from a diarrheic child in Japan （*eae*, *stx* および *cdt* 遺伝子を検出できるマルチプレックス PCR の構築と日本の小児下痢症患者から分離された *cdt* 遺伝子陽性プロビデンシア・ラスティゲニーの性状解析）
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論文要旨

Introduction

Food borne zoonoses is a major concern worldwide. Zoonotic transmission is considered to be a key driver in the current emergence and re-emergence of novel diseases. It has been estimated that 60% of all communicable and 75% of emerging infectious diseases of people were originated from animals. Recently, zoonoses due to *Salmonella* spp. *Campylobacter* spp. and pathogenic *E. coli* seems to be increased significantly. *E. coli* in general are normal flora of human and animal intestine, however, some of them are pathogenic to human and animals. Pathogenic *E. coli* are grouped into two groups, diarrheagenic *E. coli* (DEC) and extra-intestinal pathogenic *E. coli* (ExPEC), which are associated with intestinal and extra-intestinal infections, respectively. Some of the DEC such as enterohemorrhagic *E. coli* (EHEC) or Shiga toxin (Stx)-producing *E. coli* (STEC) carrying *eae* (attaching and effacing) and/or *stx* genes and enteropathogenic *E. coli* (EPEC) carrying *bfp* (bundle forming pili) and/or *eae* genes are zoonotic pathogens. EPEC can be further divided into two groups such as typical EPEC (tEPEC) having both *bfp* and *eae* genes and atypical EPEC (aEPEC) having *eae* gene only. aEPEC has been considered to be originated from

animals while tEPEC is originated from human. Stx is divided into two types such as Stx1 and Stx2. Stx2-producing *E. coli* are associated with more severe clinical conditions such as hemolytic-uremic syndrome (HUS) and neurological disorder than Stx1-producing *E. coli*. Furthermore, EHEC (*eae* gene-positive STEC) is generally more virulent than STEC (*eae* gene-negative). It should be noted that aEPEC would be automatically converted to EHEC if Stx2-phage lysogenize aEPEC. Therefore, it is considered that aEPEC is more virulent than tEPEC. Along with these *E. coli* pathotypes, cytolethal distending toxin (CDT)-producing *E. coli* (CTEC) has been also isolated from children with diarrhea. CDT consists of 3 subunits such as CdtA, CDtB and CdtC. Among 5 types of CDT (I to V)-producing *E. coli* so far known, CDT-II-producing *E. coli* has been demonstrated to be *Escherichia albertii*, which is also known to be an emerging zoonotic pathogen. Because several *E. albertii* strains have been isolated from wild finch, pigeons and raccoons, and have been associated with various outbreaks of food-poisoning.

Recently, aEPEC, CTEC, STEC and *E. albertii* are increasing reported from clinical cases but the association of those bacterium with diarrhea is not epidemiologically established. To understand the epidemiological significance of these pathogens in diarrheal diseases, detection and isolation of causative bacteria from stool samples is necessary. Several simplex and multiplex PCR have been described for the detection of those bacteria based on their important virulence determinants (*eae*, *stx* and *cdt*). However, none of the existing PCR systems are suitable to detect *eae*, *stx*, particularly *stx2*, and *cdt* genes in a single PCR. Thus, to facilitate rapid detection of these bacteria in diarrheic stool samples, this study was designed to develop a multiplex PCR (m-PCR) which can amplify *eae*, *stx* and *cdt* in a single reaction mixture. By using the m-PCR, untypable *cdtB* gene was detected from stool of diarrheic child during routine surveillance, and untypable *cdtB* gene-positive bacterium was isolated and identified as *Providencia rustigianii*. *cdt* gene-positive *P. rustigianii* was further analyzed genetically and phenotypically.

Chapter 1. Development of a multiplex PCR for the detection of *eae*, *stx* and *cdt* genes, and its application to clinical samples

There are different variants of *eae*, *stx* and *cdt* genes with distinct biological features. Specific PCR primers for each gene were designed and a multiplex PCR (m-PCR) for the detection of these 3 genes was developed. For this purpose, primers for *eae* and *stx* were designed aligning sequences of all the reported variants of the target genes. In case of *cdt* gene amplification, primers were taken from Hinenoya *et al*. (2009), which can detect all the 5 subtypes of *cdtB* genes in *E. coli*. This m-PCR could amplify *eae* genes and all the reported subtypes of *stx* and *cdtB* genes except *stx2f* with 100% sensitivity (58/ 58 isolates) and specificity (51/ 51 isolates). Detection limit of the m-PCR for *eae*, *stx* and *cdtB* were 100, 10-100, and 1-10 CFU/assay, respectively. This m-PCR could also detect *eae*, *stx* and *cdtB* genes in spiked stool samples with considerable detection limits. Thus, to determine the prevalence of *eae*, *stx* and *cdt* genes in

diarrheic children in Japan, this m-PCR was employed. A total of 555 rectal swab samples were examined upon enrichment in TSB where 4.9 and 5.8% of the samples were positive for *eae* and *cdt* genes, respectively. Further, restriction fragment length polymorphism (RFLP) assay (Hinenoya *et al*., 2009) was used to subtype the *cdt* genes in positive samples. From 3 samples *cdt-II* positive bacteria which were also positive for *eae* genes were isolated and identified as *E. albertii*. In addition, one untypable *cdtB* (UT-*cdtB*) gene having similar RFLP pattern to that of *P. alcalifaciens* was detected. The results indicated that this m-PCR can be used for surveillance of diarrheic stool samples not only for *eae, stx* and *cdt* gene-positive aEPEC, STEC, CTEC, *E. albertii* but also *Providencia* spp. The UT-*cdtB* was detected in a stool sample collected from a 15 months old infant where no other pathogenic virus (*Adenovirus*, *Norovirus*, *Rotavirus*) and bacterial pathogens (*Aeromonas* spp., *Campylobacter* spp. *Klebsiella* spp., *Salmonella* spp., and *Yersinia* spp.) has been detected indicating that the UT-*cdtB* carrying bacteria might be associated with diarrhea in that infant. The UT-*cdtB* gene sequence had 95% homology with *cdtB* gene of *P. alcalifaciens*. CDT production has been reported in very limited number of *P. alcalifaciens* strains and the association of CDT producing *P. alcalifaciens* with diarrhea has not been elucidated. Thus, this study was further extended to isolate and characterize the UT-*cdtB* bearing bacterium and determine its association with diarrhea by in-depth genotypic and phenotypic analysis.

Chapter 2. Identification the untypable *cdtB* positive bacterium as *P. rustigianii* and its genotypic analysis

P. alcalifaciens like UT-*cdtB* positive bacteria was isolated using *Providencia* specific PMXMP media. Interestingly, this isolate when further characterized by biochemical tests, 16S rRNA and *rpoB* gene sequencing was identified as *P. rustigianii* (strain JH-1) but not *P. alcalifaciens.* This is the first report regarding the detection of *cdtB* gene in *P. rustigianii*. *P. rustigianii* is rarely isolated from human diseases and strain JH-1 is the only known strain in *P. rustigianii* having *cdtB* gene. Thus, the whole *cdt* gene cluster of strain JH-1 was sequenced by primer walking which revealed a 2,223 bp sequences including 2,143 bp long *cdtABC* gene cluster. The deduced amino acid sequences of CdtA, CdtB and CdtC were 94, 97 and 94% homologous to CDT of previously reported *P. alcalifaciens* respectively. As *cdt* is not frequently associated with *Providencia*, we have speculated that *Providencia* spp. might have acquired *cdt* genes through horizontal transfer events. Interestingly, S1-PFGE and Southern hybridization revealed the presence of *cdtB* gene on a large plasmids of *P. rustigianii* strain JH-1 and other 4 *cdt* gene-positive *P. alcalifaciens* strains tested. Furthermore, *cdt* bearing plasmid in strain JH-1 (pJH-1) could be transferred to other enterobacteria including *cdt* negative *P. rustigianii* strain GTC1504. This *in vitro* transferability suggests that this plasmid is horizontally transferable. To further characterize the plasmid, *P. rustigianii* strain JH-1 was sequenced by whole genome sequencing (WGS) using MiSeq and PacBio RSII platform. WGS revealed a 3,992,833 bp chromosome and 168,819 bp plasmid with 41.2 and 38.7% GC contents, respectively. Annotation

of the plasmid sequences confirmed the presence of *cdt* genes on the plasmid along with type three secretion system (T3SS) and conjugation related genes. Further analysis revealed another T3SS related genes on the chromosome. The T3SS on the chromosome (cT3SS) belonged to Esc family whereas plasmid T3SS (pT3SS) belonged to the Inv/Mxi-Spa family. *In-silico* analysis revealed that cT3SS of strain JH-1 was ≥64% homologous to the T3SS's of other *Providencia* spp. but pT3SS was found to be unique. Furthermore, cT3SS-*spaL* (c*spaL*) like gene sequences could be detected on other strains of *P. rustigianii* and *P. alcalifaciens* by S1-PFGE, but pT3SS was present only on the plasmids of *cdt* positive *Providencia* tested. This result indicated that cT3SS might be conserved among *Providencia* strains but pT3SS might be associated with the *cdt* bearing plasmid. Presence of *cdt* and pT3SS genes on the same plasmid indicated that the *cdt* genes positive *Providencia* spp. might be sharing similar type of plasmids. From the findings of genetic analysis it could be said that *P. rustigianii* strain JH-1 carried all the machineries to be a potential virulent bacteria and its virulence might be regulated by the transferable *cdt* bearing plasmid (pJH-1). However, *in-vitro* and *in-vivo* experiments are necessary to establish the virulence and role of *cdt* and T3SS genes on the virulence of *P. rustigianii* strain JH-1.

Chapter 3. Phenotypic characterization of *P. rustigianii* strain JH-1

To understand the virulence of strain JH-1 biological activity of *cdt* and T3SS gene-products were examined. Western blotting and cytotoxicity assay with CHO cells revealed production of biologically active CDT by strain JH-1. T3SS is reported to be associated with invasion in *Salmonella* Typhimurim. As strain JH-1 possesses two T3SSs similar to *S.* Typhimurium, we have attempted to determine the association of the T3SSs with the invasiveness of this strain. HeLa cell invasion assay under gentamicin protection revealed strain JH-1 as invasive. In addition to T3SS, CDT production is also associated with the invasion in Campylobacter spp. To determine the association of CDT and T3SS's with the invasiveness, *cdtB* and *spaL* (ATPase) gene mutants of cT3SS (c*spaL*) and pT3SS (p*spaL*) were used. Invasion assay with the mutants revealed that, *cdtB* and *cspaL* mutation did not but p*spaL* gene mutants (Δp*spaL*) lost their invasiveness. Interestingly, invasiveness was increased in Δc*spaL*. To understand the underlying mechanisms of invasion loss or increase, relative expression of pT3SS genes were determined by qRT-PCR. Relative expression of 10 tested pT3SS genes were significantly downregulated in Δp*spaL*, whereas in Δc*spaL* relative expression of genes downstream of p*spaL* were significantly up-regulated, indicating that p*spaL* might be essential for the expression of pT3SS gene but cT3SS possibly suppress pT3SS through interfering the regulation of pT3SS genes, however, the actual mechanism of interaction between the T3SSs could not be uncovered. In *P. alcalifaciens* invasion was considered as the major mechanism of diarrhea induction. As strain JH-1 could invade HeLa cells, further rabbit ileal loop test was performed to determine its diarrheagenicity. Strain JH-1 could cause fluid accumulation in the ileal loop. In addition, Δc*spaL* could but Δp*spaL* could not induce fluid accumulation, indicating that pT3SS was associated with diarrheagenicity of strain

JH-1. Furthermore, CDT and T3SS activity could be determined in transconjugants carrying pJH-1.

Conclusions

Taken together, it can be concluded that, m-PCR developed in this study would be useful in detecting the target genes in diarrheic stool samples. *P. rustigianii* strain JH-1 could produce functional CDT and diarrheagenic. The diarrheagenicity of strain JH-1 was regulated by a T3SS located on a plasmid which was horizontally transferable to other enterobacteria with its properties. As *cdt* producing *P. alcalifaciens* strains also carries similar plasmid and pT3SS like sequences we assume that *P. alcalifaciens* also use similar T3SS to invade and induce diarrhea, however, further descriptive studies are necessary to establish this assumption.

審査結果の要旨

動物および動物性由来食品による食中毒が多発し、特に先進国において大きな問題となっている。腸管出血性大腸菌（EHEC）はその代表格であるが、近年、腸管病原性大腸菌（EPEC）や *Escherichia albertii*（Ea）も問題となっている。腸管病原性大腸菌（EPEC）は、*eae* と *bfp* の両病原遺伝子を有する定型腸管病原性大腸菌（tEPEC）と *eae* 遺伝子のみを保有し、動物が感染源となる非定型腸管病原性大腸菌（aEPEC）が存在する。また、斃死した野鳥や健康な野生動物から分離され，新興人獣共通感染症菌として注目を集めている Ea による集団食中毒事例が過去 7 年間、我が国で多発している。しかしながら、Ea は細菌学的性状が EPEC や EHEC と酷似していることから、過去の事例において EPEC や EHEC と誤同定されていた。EHEC、EPEC 及び Ea の共通な病原因子として *eae* 遺伝子を有すること、また EHEC の主要な病原因子である *stx* 遺伝子を保有する Ea も一部存在することもその一因である。一方、ある種の大腸菌は細胞膨化致死毒素を産生し、病気の家畜、健康な家畜及び下痢症患者から分離されている。近年、II 型の細胞膨化致死毒素（CDT）を産生する大腸菌（CTEC）は、大腸菌でなく Ea であることが明らかとなった。Ea が共通に保有する病原因子として *eae* と *cdt* 遺伝子がある。このように、誤同定が起こる背景として Ea 、EHEC と EPEC を鑑別し特異的に検出できる系が無いことが原因である。

第一章では、EHEC、EPEC、Ea と CTEC の疫学的調査を目的にこれら 4 種類の細菌の病原因子である *eae*、*stx* 及び *cdt* 遺伝子を検出できるマルチプレックス PCR（M-PCR）の構築を試みた。構築した M-PCR の感度、特異性を当該遺伝子陽性及び陰性の 58 及び 55 株を用いて調べた結果、それぞれ 100%であった。検出下限は、純培養菌では *eae*、*stx* 及び *cdt* 遺伝子、PCR チューブあたりそれぞれ 100、10-100、1-10 CFU であった。糞便を用

いたスパイクサンプルでは、それぞれ 10^5、10^5 と 10^4 CFU/mL、増菌培養後では、それぞれ 10^3、10^2 と 10^2 CFU/mL であった。さらに、小児下痢症患者便 555 検体を用いて当該遺伝子を検出したところ、4.9%、5.5%の検体で *eae* と *cdt* 遺伝子が検出されたが、*stx* 遺伝子は全く検出されなかった。興味深いことに型別できない *cdt* (UT-*cdt*)遺伝子が検出され、その塩基配列は *Providencia alcalifaciens*（Pa）の *cdt* 遺伝子と高い相同性を有していた。

第 2 章では、UT-*cdt* 遺伝子が Pa の *cdt* 遺伝子と高い相同性を示したことから、UT-*cdt* 遺伝子陽性菌を *Providencia* 属菌の選択培地を用いて分離し、菌種同定を試みた。その結果、UT-*cdt* 遺伝子陽性菌は *P. rustigianii*（Prus）であることが明らかとなった。さらに、*cdt* 遺伝子の全塩基配列を明らかとし、*cdt* 遺伝子は染色体上でなく、接合伝達性プラスミド上に見つかった。*Cdt* 遺伝子以外の病原遺伝子が存在しているかどうかを調べるため、本菌の全ゲノム配列を解析した。その結果、染色体は 3,992,833 bp から、プラスミドは 168,819 bp からなり、プラスミド上には *cdt* 遺伝子に加え 3 型分泌装置（pT3SS）に関わる遺伝子と接合伝達に関わる遺伝子も存在することを明らかとした。さらに染色体上にもプラスミド性とは異なる T3SS（cT3SS）をコードした遺伝子を見出した。これらは、それぞれサルモネラで見出された T3SS と類似したものであった。以上の結果は、Prus の病原因子として *cdt* 遺伝子及び T3SS 関連遺伝子の存在を初めて示す結果であった。

第 3 章では、第 2 章で見出した Prus の病原遺伝子が実際に病原性の発現に関与しているかについて調べた。CDT 活性は CHO 細胞を用いて証明され、T3SS の細胞侵入性は HeLa 細胞によって確認された。しかし、pT3SS の変異体は細胞侵入性を失ったが、cT3SS の変異体は細胞侵入性を失わせるどころかむしろ細胞侵入性を促進した。この変異体は、pT3SS の遺伝子発現を促進していた。さらに、Prus の下痢原性をウサギの腸管ループ試験で調べた結果、液体貯留活性が認められ、下痢原性が確認された。しかも、pT3SS の変異体はウサギの腸管ループ試験による液体貯留活性が消失したのに対し、cT3SS の変異体は野生型の株を投与した時と同様、液体貯留活性を示した。ウサギ腸管組織の病理組織学的解析を行なった結果、腸管組織の傷害が観察された。また、Prus の病原性プラスミドは、非病原性の *P. ruttgeri*（Pret）にも接合伝達され CDT の産生及び細胞侵入性を示すことを明らかとした。以上の結果より、Prus の病原遺伝子は *in vitro* および *in vivo* において実際に Prus の病原性に関わっていることが示された。

以上の結果は、誤同定が問題となっていた EHEC、EPEC 及び Ea のみならず、人獣共通感染症菌の可能性のある病原性 *Providencia* 属菌の分離にも有用な検出系を構築したことに加え、*Providencia* 属菌の病原性に CDT と T3SS が関わっていることを示唆するものである。特に T3SS については *Providencia* 属菌に 2 コピー存在し、pT3SS が病原性発現に直接関与する一方、cT3SS は pT3SS の発現調節を介して病原性に関与していることを明らかとした。これらの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、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。