称号及び氏名	博士（獣医学）　S. M. Lutful Kabir
学位授与の日付	平成２３年３月３１日
論　文　名	Comparison of molecular methods for the species identification of clinical Campylobacter strains and their antimicrobial resistance （カンピロバクター属菌の遺伝学的菌種同定法の比較と臨床分離菌の薬剤耐性）
論文審査委員	主査　山崎 伸二 副査　小崎 俊司 副査　笹井 和美

論文要旨

Introduction

Campylobacters are microaerophilic spiral Gram-negative bacteria. They are motile and possessing a single flagellum at one or both poles and they are non-sporeformer. Among more than 17 species currently recognized in the genus *Campylobacter*, *C. jejuni* and *C. coli* can infect poultry, livestock and pets and these animals are source of human infection. Most infections are believed to result from the ingestion of contaminated food such as undercooked meats, contaminated water, or raw milk. Furthermore, *C. jejuni* and *C. coli* infections are considered to be the leading causes of human gastroenteritis in the developed countries particularly in Japan.

The accurate identification of *C. jejuni* and *C. coli* is needed for the identification of their diseases spectrum, tracing their source of infection as well as their route of transmission. However, conventional identification methods for *Campylobacter* species are tedious and time-consuming, because these organisms are slow growing, biochemically less reactive and require microaerobic conditions. In particular, it is often difficult to differentiate *C. jejuni* from *C. coli* by biochemical methods and 16S rRNA gene sequence because of the extensive similarity between these two

species. Hippuricase activity is the only key marker for the differentiation of *C. jejuni*; however, this activity is sometimes very weak in *C. jejuni* and hippuricase-negative strains of this species are also well recognized. Therefore, detection of hippuricase (*hipO*) gene by PCR is more reliable than biochemical test for the differentiation of *C. jejuni* from *C. coli*. However, deletion and mutation of the primer binding sites in *hipO* gene resulting in no amplification by PCR has been also noticed. In addition, several PCR-based assays targeting 16S or 23S rRNA gene or species-specific genes have been developed to facilitate the differentiation of *C. jejuni* from *C. coli*, however, the ability of these assays for exact identification of *Campylobacter* species has not yet been established.

Cytolethal distending toxin (CDT), which is one of the well-characterized virulence factors in *Campylobacter* spp., is encoded by three linked genes termed *cdtA*, *cdtB* and *cdtC*. The *cdt* gene clusters in *C. jejuni*, *C. coli* and *C. fetus* were demonstrated to be ubiquitously distributed in each species in a species-specific manner. Recently, a cytolethal distending toxin (*cdt*) gene-based species specific multiplex PCR assay has been developed for the detection and identification of *C. jejuni*, *C. coli* and *C. fetus*. However, among the *cdtA/B/C* gene-based multiplex PCR, which one is the best for the monitoring of clinical samples is also a matter of great concern.

Campylobacter infections pose a serious public health problem for which many countries are monitoring their infection and antimicrobial resistance patterns. There are some reports about increasing trend in antimicrobials resistance particularly to quinolones in *Campylobacter* in many parts of the world. The emergence of quinolone resistance is mediated by several mechanisms. Resistance to fluoroquinolones typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV) and its decreased accumulation inside the bacteria due to an over expression of efflux pump systems. Both of these mechanisms are chromosomally mediated.

Considering the potential threat of *Campylobacter* infections associated with their risks of antimicrobial resistance in Japan, this study was designed for the *Campylobacter* strains isolated from diarrheal patients in Japan to achieve the following objectives: (1) evaluation of a *cdt* gene-based species-specific multiplex PCR assay, (2) comparison of other molecular methods and (3) genotypic characterization of *C. jejuni* and *C. coli* strains resistant to fluoroquinolone.

Chapter 1: Evaluation of a *cdt* gene-based species-specific multiplex PCR assay for the identification of *Campylobacter* strains isolated from diarrheal patients in Japan

A total of 325 *Campylobacter* strains isolated from stool specimens of patients with diarrhea who visited the Tokyo Women's Medical University Hospital, Japan during the period between 1997 and 2005, were used in this study. These strains were identified as *Campylobacter* spp., *C. jejuni* or *C. coli* by culture methods, microscopy and biochemical tests (catalase, oxidase and hippuricase activity) at the Tokyo Women's Medical University Hospital. In this study, bacteria were grown on blood base agar at 37°C for 48 h under microaerobic conditions and DNA templates were prepared by boiling method. DNA templates were used for *hipO* gene PCR, 16S rRNA gene sequence and *cdtA/B/C* gene-based multiplex PCR. A total of 314 strains were identified as *C. jejuni* by *hipO* gene PCR and 11 strains were negative by the same PCR. These 11

strains were subjected to 16S rRNA gene sequencing (1,335 bp) followed by homology search using BLAST program and all were identified as *C. jejuni*/*C. coli*. Later, all the (n=325) strains were subjected to *cdtA/B/C* gene- based multiplex PCR for further identification and all the strains were identified as it were by combined methods of *hipO* and 16S rRNA gene-based identification except 5, 4, and 1 strains that were negative by *cdtA, cdtB* and *cdtC* gene-based multiplex PCR, respectively. In order to elucidate the reasons for getting these negative results, amplification using common primers, sequencing and sequence data analyses of the *cdt* gene clusters of these strains were carried out. Clustal V alignment of the sequences from test strains with the published *cdt genes* sequence of *C. jejuni* 81-176 (Acc. No. U51121) revealed 667-bp deletion between *cdtA* and *cdtB* plus additional 51-bp deletion within the *cdtB* genes in 4 *C. jejuni* strains, consistent with the shorter PCR amplicon size than expected size. Furthermore, two strains comprising *C. jejuni* and *C. coli* that did not yield specific PCR products contained mutations in the primer binding sites of *cdt*A and *cdtC* genes, respectively. These data indicate that *cdtC* gene-based multiplex PCR is more accurate and reliable than *cdtA* or *cdtB* gene-based multiplex PCR to identify the species of *Campylobacter* strains such as *C. jejuni* and *C. coli.*

In order to investigate the genetic diversity of randomly selected 50 *C. jejuni* and 11 *C. coli* strains, pulsed-field gel electrophoresis (PFGE) was employed. *C. jejuni* (n=50) and *C. coli* (n=11) strains were classified into 42 pulsotypes and 4 subtypes, and 8 pulsotypes, respectively. These data indicate that the *C. jejuni* and *C. coli* strains used in this study were genetically diverse. Interestingly, the 4 *C. jejuni* strains that carried deletion between *cdtA* and *cdtB* as well as in *cdtB* gene, 2 of them were clonal even isolated at different time point. These data demonstrate that such type of *C. jejuni* strains having deletions in *cdt* gene clusters might be spread from the common or different sources.

Chapter 2: Comparison of other molecular methods for the species identification of *Campylobacter* strains

To compare the sensitivity and specificity of the *cdt* gene-based multiplex PCR with those of other PCR-based species identification methods, the applicability of different published PCR assays targeting different genes such as 23S rRNA, *ceuE*, *hipO*, *ask*, *lpxA* and *mapA* were further tested with 58 *C. jejuni* of clinical origin, 31 *C. coli* of different origins (clinical and environment) and 14 reference strains (3 *C. jejuni*, 2 *C. coli*, 2 *C. fetus* and 5 other *Campylobacters* and 2 *Arcobacter* spp.). When *C. jejuni* specific 23S rRNA gene-based PCR was used out of 58 *C. jejuni*, 31 *C. coli* and 14 reference strains, 56 *C. jejuni*, 1 *C. coli* and 3 *C. jejuni* reference strains gave specific amplification, respectively, although size of the amplified fragments was variable (590 bp, 640 bp and 740 bp) and different from the previously described. On the other hand, when *C. coli* specific 23S rRNA gene-based PCR was used out of 58 *C. jejuni*, 31 *C. coli* and 14 reference strains, 2 *C. jejuni*, 31 *C. coli* and 3 reference strains (2 *C. coli* and 1 *C. lari*) gave specific amplification, respectively. When *C. jejuni* specific *ceuE* gene-based PCR was applied to the same set of the strains, 51 *C. jejuni* and 1 *C. jejuni* reference strains gave specific amplification, respectively. On the other hand, when *C. coli* specific *ceuE* gene-based PCR was applied to the same set of the

strains, 30 *C. coli* and 2 *C. coli* reference strains gave specific amplification, respectively. When *C. jejuni* specific *lpxA* gene-based PCR was applied, 58 *C. jejuni*, 3 *C. coli* and 3 *C. jejuni* reference strains gave specific amplification, respectively. On the other hand, when *C. coli* specific *lpxA* gene-based PCR was applied, 4 *C. jejuni*, 31 *C. coli* and 2 *C. coli* reference strains gave specific amplification, respectively. When *mapA* gene-based PCR was used, 55 *C. jejuni*, 3 *C. coli* and 4 reference strains (3 *C. jejuni* and 1 *C. coli*) gave specific amplification, respectively. However, all *C. jejuni* and *C. coli* strains used in this study were accurately identified by combination of *hipO* and *ask* gene-based PCR.

In order to verify the failure of the species identification by 23S rRNA, *ceuE*, *lpxA* and *mapA* gene-based PCR, new primers covering the flanking regions of these genes were designed for checking the nucleotide sequences of primer binding sites of PCR negative strains. A few *C. jejuni* strains negative by *C. jejuni* specific *ceuE* gene-based PCR and 1 *C. coli* strains negative by *C. coli* specific *ceuE* gene-based PCR were amplified and sequenced by using newly designed primers. These strains were found to have mutations in both primers binding sites. On the other hand, 3 *C. coli* strains positive (faint band) by *C. jejuni* specific *lpxA* gene-based PCR and 4 *C. jejuni* strains positive (faint band) by *C. coli* specific *lpxA* gene-based PCR were amplified and sequenced by using newly designed primers. These strains were found to have a few mutations in the forward primer-binding site but there were no mutations in the reverse primer-binding site. However, a few *C. jejuni* strains negative by 23S rRNA gene-based PCR didn't give any specific amplification by newly designed primers as well. In addition, a few *C. jejuni* strains negative by *mapA* gene-based PCR, 3 *C. coli* and 1 *C. coli* reference strains positive by *mapA* gene-based PCR also didn't give any specific amplification by newly designed primers. As several investigators already reported the less sensitivity of *hipO* gene-based PCR and there is no evidence yet for the simultaneous occurrence of deletions or mutations in the *cdtA*, *cdtB* and *cdtC* genes of the same *Campylobacter* strain, combination of *cdtC* and *cdtB* or *cdtA* gene-based multiplex PCR might be one of the best option for the accurate identification of *C. jejuni*, *C. coli* and *C. fetus.*

Chapter 3: Antimicrobial resistance profiles and genotypic characterization of fluoroquinolone resistance in *C. jejuni* and *C. coli* strains isolated from diarrheal patients in Japan

Since the antimicrobial resistant *Campylobacter* strains have been increasing worldwide, analysis of antimicrobial resistance profile was carried out with the 325 *Campylobacter* strains including 314 *C. jejuni* and 11 *C. coli* by disk diffusion method. Although fluoroquinolones and macrolides are the antimicrobial agents of choice when therapeutic intervention is warranted and tetracyclines have been suggested as an alternative choice in the treatment of clinical campylobacteriosis, a series of antimicrobial agents such as ampicillin, tetracycline, azithromycin, erythromycin, chloramphenicol, gentamicin, nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin, fosfomycin and sulphamethoxazole-trimethoprim were used in this study. Tetracycline and fluoroquinolone resistances were observed in both *C. jejuni* (29 and 24%, respectively) and *C. coli* (36 and 36%, respectively) strains analyzed in this study. On the other hand, most of the *C. jejuni*

and *C. coli* strains analyzed in this study were susceptible to macrolides, gentamicin and chloramphenicol. These data suggest that macrolides could be used as first choice of the drug for the treatment of campylobacteriosis in Japan. However, multidrug resistance (when considered resistance to 3 or more agents) was found in 28 and 46% of *C. jejuni* and *C. coli* strains, respectively. These data suggest that the prevalence of multidrug resistance may be higher in *C. coli* in comparison to *C. jejuni* strains in Japan.

In order to analyze genetic background of fluoroquinolone resistant *C. jejuni* and *C. coli* strains, PCR amplifications of *gyrA* and *parC* genes were performed. However, there was no strain positive for *parC* gene in this study. On the other hand, the *gyrA* genes in 75 *C. jejuni* and 4 *C. coli* strains were sequenced to investigate whether there was any mutation. A total of 73 out of 75 *C. jejuni* strains which were resistant to CIP (MIC, 8-64 μg/ml) had the T to I substitution at codon 86 in GyrA that is considered to be the main contributing factor for fluoroquinolone resistance in *Campylobacter* spp. The additional mutations were observed at codons 203 (N203S), 203 (N203S) and 206 (A206V), 149 (V149I) and 203 (N203S), and 90 (D90N) and 203 (N203S) in 23, 11, 5, and 1 strains, respectively. On the other hand, no point and silent mutations were found in 1 strain (K278) although this strain was phenotypically resistant and only silent mutations were noticed in 1 strain (K170). Furthermore, in case of *C. coli*, a total of 4 strains, which were resistant to CIP (MIC, 16-64 μg/ml), had the T to I substitution at codon 86 in GyrA. These data suggest that the amino acid substitution at codon 86 (T86I) in the quinolone resistance determining region (QRDR) of GyrA may play a major role in the acquisition of resistance to fluoroquinolone in the *C. jejuni* and *C. coli* strains tested.

To elucidate the role of efflux pump in the acquisition of resistance to fluoroquinolone in the *C. jejuni* and *C. coli* strains, PCR amplifications of *cmeR* genes in 75 *C. jejuni* and 4 *C. coli* strains were performed. One strain (K170; MIC:16 μg/ml) having silent mutations in the *gyrA* sequence displayed mutations in the HTH DNA binding motif of CmeR as well as in the promoter region of CmeABC efflux pump. Another strain (K278; MIC: 8 μg/ml) having no mutations in the *gyrA* sequence displayed mutations in the CmeR as well as in the inverted repeat of the promoter region of CmeABC efflux pump. Overall, there were no mutations in the CmeR and promoter region of CmeABC efflux pump in the 17 fluoroquinolone resistant *C. jejuni* strains and in the remaining 60 fluoroquinolone resistant *Campylobacter* strains, there were mutations either in CmeR or in promoter region of CmeABC efflux pump or in both regions. These observations suggest that generally low level of fluoroquinolone resistance occurred due to point mutations of the *gyrA* gene but high level of fluoroquinolone resistance might be occurred due to point mutations in the *gyrA* gene as well as mutations in the CmeR and promoter region of CmeABC efflux pump. Furthermore, PFGE analysis of 45 fluoroquinolone resistant *C. jejuni* strains revealed 32 pulsotypes and 3 subtypes, indicating that these fluoroquinolone resistant *C. jejuni* strains were genetically diverse. However, a few fluoroquinolone resistant *C. jejuni* strains were clonal and this was the indication of spreading of a few fluoroquinolone resistant *C. jejuni* strains from the common sources.

Conclusions

1. *cdtC* gene-based multiplex PCR appears to be the most useful for the simple and rapid identification of *C. jejuni* and *C. coli*.
2. Among several PCR-based species identification methods of *C. jejuni* and *C. coli*, *hipO* and *ask* gene-based method appeared to be the best.
3. But combination of *cdtC* and *cdtB* or *cdtA* gene-based multiplex PCR may be more accurate and might be best for the accurate identification of *C. jejuni* and *C. coli*.
4. Emergence of resistance of *C. jejuni* and *C. coli* to a variety of antimicrobials especially tetracycline and fluoroquinolones among the diarrheal patients in Japan.
5. Generally low level of fluoroquinolone resistance occurs due to point and silent mutations of the *gyrA* gene only but high level of fluoroquinolone resistance may occur due to point mutations in the *gyrA* gene as well as mutations in the CmeR and promoter region of CmeABC.

審査結果の要旨

Campylobacter 属細菌は、我が国においても最も問題となっている食中毒原因菌の一つである。現在 *Campylobacter* 属細菌は、少なくとも 17 菌種知られているが、食中毒原因菌に指定され、特に重要なのが *C. jejuni* と *C. coli* の2菌種である。特に家禽の腸管内に常在する *C. jejuni* や *C. coli* で汚染した鶏肉を加熱不十分な状態で食することにより、食中毒を引き起こすと考えられている。*C. jejuni* は末梢神経障害であるギランバレー症候群の原因となることから *C. jejuni* と *C. coli* を鑑別して検査することは重要である。C. *jejuni* や *C. coli* は(1)微好気性細菌であり、培養には特殊な装置が必要で、(2)増殖が遅く、(3)生化学的性状が酷似しており菌種同定が容易でないなどの理由から、本菌の分離・同定には時間を要するだけでなく、誤同定や同定できないなどの重要な問題がある。これらを克服するため、*C. jejuni* や *C. coli* の特異的な遺伝子を PCR で検出することにより、この2菌種を鑑別する方法が多数開発されている。しかしながら、どの方法が最適かについては充分検討されていない。一方、家畜や医療現場における抗菌薬の不適切な使用の結果、ニューキノロンやマクロライドを含む多剤耐性菌の出現が医療現場で大きな問題となっている。それゆえ、患者から分離される *C. jejuni* や *C. coli* の薬剤感受性を把握することは重要である。

本研究では、我々の研究グループが既に開発し報告した細胞膨化致死毒素(*cdt*)遺伝子を検出する PCR 法について 325 株の臨床分離株を用いて、その有用性の評価を行い、さらにその他の菌種特異的遺伝子を検出する PCR 法と比較した。さらに、わが国の下痢症患者から分離された *C. jejuni* や *C. coli* について薬剤感受性について解析した。以下はそれらの結果の概要である。

第1章では、我が国の小児下痢症患者から分離され、*Campylobacter* 属様細菌として同定された株について、16S rRNA 遺伝子の解析と馬尿酸水解酵素(*hipO*)遺伝子の有無を調べ、得られた結果を総合的に判定し 314 株を *C. jejuni*、11 株を *C. coli* と同定した。また得られた結果は、*cdtA*、*cdtB* 及び *cdtC*

gene-based Multiplex PCR 法で得られた結果と一致した。しなしながら、*cdt* gene-based Multiplex PCR 法で PCR 産物を得られなかった株が少数認められ、その株の *cdt* 遺伝子の解析から、プライマー結合部位の変異と *cdt* 遺伝子の一部が欠失していることが原因であることがわかった。以上の結果より、*cdtC* 遺伝子と *cdtA* 或いは *cdtB* 遺伝子を標的とした PCR 法を組み合わせることが最良であることを示した。

第2章では、既に報告されている *C. jejuni* と *C. coli* の菌種を鑑別できる PCR 法即ち、*C. jejuni* を特異的に検出できる 23S rRNA、*ceuE*、*lpxA*、*hipO*、*mapA* PCR あるいは、*C. coli* を特異的に検出できる 23S rRNA、*ceuE*、*lpxA*、*ask* PCR と *cdt* gene-based multiplex PCR 法について 61 株の *C. jejuni*、33 株の *C. coli*、それ以外の *Campylobacter* 属菌 7 株と *Arocbacter* 属菌 2 株を含む 103 株を用いて評価した。その結果、*hipO* と *ask* は、*C. jejuni* と *C. coli* を 100%の特異性及び感度で検出できたが、その他の PCR 法は特異性と感度に問題があった。*hipO* と *ask* を標的とした PCR 法は個別に行う必要があり、*cdt* 遺伝子を標的とした PCR 法は一度の PCR で *C. jejuni* と *C. coli* 鑑別することができ、*cdt* 遺伝子を標的とした PCR 法の方がより実用的であることを示した。

第3章では、わが国の下痢症患者から分離された 314 株の *C. jejuni* と 14 株の *C. coli* の合計 325 株について薬剤感受性を調べた。その結果、*C. jejuni* 及び *C. coli* はニューキノロン及びテトラサイクリンに対して約 30%前後の耐性率と一部の菌で高い MIC 値を示した。一方、マクロライドに対しては *C. jejuni*、*C. coli* 共にほとんどが感受性であり、マクロライドが治療に用いる第一選択薬となることを明らかにした。また、ニューキノロンに対する耐性について遺伝子レベルで解析した結果、*gyrA* 遺伝子の変異或いは薬剤排出ポンプをコードした遺伝子のプロモーター領域或いはその転写を調節している遺伝子の変異が関与している可能性を示した。

以上の結果は、*cdt* gene-based Multiplex PCR 法、特に *cdtC* 遺伝子を標的とした PCR 法が他の菌種特異的な PCR 法と比べ、*C. jejuni* や *C. coli* の菌種を簡便、迅速、正確に同定・鑑別できること、及びわが国で分離された *C. jejuni* や *C. coli* がニューキノロンとテトラサイクリンにある程度の割合で耐性化しているが、マクロライドに関してはほとんどの株が感受性であることを示した。これらの成果は、*C. jejuni* や *C. coli* による食中毒の制御に寄与するとともに、細菌学および感染制御学に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。