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論　　文　　名	「Phenotypic and genotypic characterization of *Campylobacter* strains isolated in Thailand（タイで分離されたキャンピロバクター属細菌の表現型および遺伝型による性状解析）」
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論文要旨

Introduction:

Campylobacter is a leading cause of gastroenteritis worldwide. Among 17 species currently known in the genus *Campylobacter*, *C. jejuni, C. coli, C. fetus, C. lari, C. upsaliensis,* and *C. hyointestinalis* are implicated in human diseases. Many animals, in particular poultry, pigs and cattle, are reservoirs of these organisms which are transmitted to human by direct contact or consumption of contaminated food or water. *Campylobacte*r infections are self-limiting and do not require antimicrobial treatment except complicated infections which macrolides and fluoroquinolones are often drugs of choice. However, resistance of *Campylobacter* to antimicrobials has substantially increased in many countries including Thailand and has become a matter of concern.

Campylobacters are gram negative, highly motile, spiral-shaped microaerophilic bacteria. Conventional methods for the identification of *Campylobacter* species are costly, laborious, time-consuming and considered biochemical inert which is

resulted in misidentification or unidentification. A rapid, sensitive, and specific method for identification of these organisms is required to provide important information for tracing sources of transmission and selecting an appropriate treatment. Our group recently developed a multiplex PCR assay for the species-specific detection of *C. jejuni*, *C. coli,* and *C. fetus* based on cytolethal distending toxin (*cdt*) genes.

In the present study, firstly *Campylobacter* strains isolated from human patients and healthy poultry in Thailand were identified by the phenotypic and genotypic methods and the results were compared with those obtained by the *cdt* gene-based species-specific multiplex PCR. Secondly the *cdt* genes detected in *C. hyointestinalis* were analyzed and biological activity of their gene products were investigated. Finally antimicrobial resistance profile and mutations in *gyrase A* (*gyrA*) gene of *C. jejuni* and *C. coli* strains were analyzed.

Chapter I: Identification of *Campylobacter* strains isolated in Thailand

A total of 112 *Campylobacter* strains (78 from human patients and 34 from poultry) obtained from Thailand between 2001 and 2002 were identified as species level by API Campy, detection of hippuricase (*hipO*) gene, and 16S rRNA gene sequence. The collective results of these three methods were compared with those identified by *cdt* genes-based species-specific multiplex PCR.

Among 34 poultry strains, 20, 10, and 1 were identified as *C. jejuni*, *C. coli*, and *Arcobacter cryaerophilus*, respectively, whereas 3 strains were unidentified by API Campy. By 16S rRNA gene sequence, all 34 strains were identified as *C. jejuni/C. coli*. Thus, the *hipO* gene, which is known to be specifically present in *C. jejuni* was examined. The *hipO* gene was detected in only 20 strains, which were initially identified as *C. jejuni* by API Campy. The collective results of these three methods could conclude 20 strains as *C. jejuni* and 14 strains as *C. coli*, respectively. By contrast,

the *cdtA*, and *cdtC* gene-based multiplex PCR could identify as *C. jejuni* 19 strains, and *C. coli* 13 strains whereas *cdtB* gene-based multiplex PCR could identify 20 strains as *C. jejuni* and 14 strains as *C. coli* which is well correlated with the result of the collective results of the three methods.

On the other hand, among 78 human strains, the *cdtA*, *cdtB*, and *cdtC* gene-based multiplex PCR could identify 40, 45, and 41 strains as *C. jejuni*, 29, 31, and 30 strains as *C. coli*, and 0, 1, and 1 strain as *C. fetus*, respectively. One strain was unidentified by *cdt* genes-based multiplex PCR though *cdtB* common primer yielded a specific PCR product. In contrast, API Campy identified 45, 23, 2, and 1 strains as *C. jejuni, C. coli, C. fetus,* and *A. cryaerophilus,* respectively and 7 strains were unable to be identified. By 16S rRNA gene sequence, however, 77 and 1 were identified as *C. jejuni /C. coli,* and *C. fetus,* respectively. One strain which was unable to be identified by the *cdt* genes-based multiplex PCR was identified as *C. hyointestinalis* by 16S rRNA gene analysis. This is the first report of isolation of *C. hyointestinalis* from a human patient in Thailand. The *hipO* gene was detected in all 45 strains identified as *C. jejuni* by API Campy but not in any other strains. The collective results obtained by three different methods are well correlated with those by the *cdtB* gene-based multiplex PCR.

Campylobacter strains were further characterized by Pulse-field gel electrophoresis (PFGE). Although PFGE patterns revealed extensive genetic diversity which is mostly specific to either human or animal strains, identical and similar PFGE patterns were also identified in poultry and human strains, suggesting that the transmission of *Campylobacter* from poultry to human might occur in Thailand.

Chapter II: Cytolethal distending toxin genes in *C. hyointestinalis*

There exists the evidence to support an important role for *C. hyointestinalis* in human and animal disease. However, the mechanism of the disease has been yet unclear.

To examine the importance of CDT produced by *C. hyointestinalis* in its pathogenesis, the *cdt* genes detected in *C. hyointestinalis* isolated from a diarrheal patient in Thailand were sequenced by a genome walking method. The *cdt* genes of *C. hyointestinalis* consist of three closely linked genes termed *cdtA, cdtB,* and *cdtC* whose sizes are 798 bp (266 aa), 804 bp (268 aa), and 537 bp (178 aa), respectively. Each Cdt subunit in *C. hyointestinalis* has highest homology to those in *C. coli* (40.9% for CdtA, 61.6% for CdtB, and 29.8% for CdtC). The *cdtB* gene of *C. hyointestinalis* was amplified by PCR and cloned into an expression vector, pET-28a (+). The recombinant plasmid was transformed into *E. coli* BL21 (DE3). The expression of recombinant CdtB protein with His_6-tagged (HisCdtB) was induced in the presence of IPTG. The HisCdtB protein was purified by an affinity column chromatography followed by gel filtration of FPLC and immunized against the rabbit for producing antiserum. Anti-HisCdtB serum was specifically reacted to both purified HisCdtB and ca. 30 kDa protein corresponding to CdtB in sonic lysate of *C. hyointestinalis* culture. Subsequently, CDT activities in sonic lysate of *C. hyointestinalis* were investigated by HeLa cell assay and FACS with or without antiserum, respectively. Sonic lysate of *C. hyointestinalis* exhibited progressive cell distension and death in HeLa cells resulted in G_2/M arrest. Both CDT activities were neutralized by anti-HisCdtB. This is the first report demonstrating the entire *cdt* genes sequence and biological activity of *cdt* genes product in *C. hyointestinalis*.

Chapter III: Analysis of antimicrobial resistance patterns

Since reports of *Campylobacter* strains resistant to antimicrobial agents, particularly macrolides and fluoroquinolones, have been appearing with increasing frequency worldwide, analysis of antimicrobial resistance profile was conducted with 110 *Campylobacter* strains isolated from Thailand including 65 *C. jejuni* (45 humans and 20 poultry) and 45 *C. coli* (31 humans and 14 poultry) by E-test. Eight

antimicrobial agents such as erythromycin (ERY), azithromycin (AZM), clindamycin (CLI), nalidixic acid (NAL), ciprofloxacin (CIP), tetracycline (TET), chloramphenicol (CHL), and gentamicin (GEN) were included in this study.

High degree of resistances to NAL, CIP, and TET were observed in *C. jejuni* (93-95%, 89-93%, and 60-85%, respectively) and *C. coli* (90-93%, 84-93%, and 68-86%, respectively) whereas the resistances to ERY, AZM, CLI were observed only in *C. coli* strains which resistance rates of poultry strains (57%, 57%, and 57%, respectively) were higher than those of human strains (13%, 16%, and 13%, respectively). There were no resistant strains of *C. jejuni* and *C. coli* to CHL and GEN. In *C. jejuni*, most strains isolated from human and poultry showed resistances to NAL and CIP in the high MIC values of >256 and >32 µg/ml, respectively whereas resistance to TET in the high MIC values from 64 to >256µg/ml was observed in most strains isolated from poultry. In *C. coli*, most strains isolated from human and poultry showed resistances in the high MIC values of >256µg/ml to ERY, AZM, CLI, NAL, 64 - >256µg/ml to TET and >32 µg/ml to CIP.

Furthermore, the *gyrA* gene, a target of quinolone antimicrobial agents, in quinolone resistant strains was sequenced to investigate its mutation. A Thr86Ile mutation in the *gyrA* gene product was identified in all NAL-resistant isolates in addition to other various different mutations in the *gyrA* gene in many *C. jejuni* strains, indicating that A Thr86Ile mutation in GyrA could be involved in quinolone resistance as reported previously.

Conclusion

1. *CdtB* gene-based multiplex PCR is a simple, rapid and reliable method for identification of *C. jejuni*, *C. coli*, and *C. fetus* isolated from both healthy animals and human patients.

2. PFGE analysis indicated that *C. jejuni* and *C. coli* analyzed in this study were genetically diverse. Some PFGE patterns between humans and poultry strains showed identical or similar, suggesting that transmission of *Campylobacter* might occur between poultry and human in Thailand.
3. Analysis of entire *cdt* genes sequence of *C. hyointestinalis* revealed the presence of *cdtA, cdtB,* and *cdtC* genes with 798 bp (266 aa), 804 bp (268 aa), and 537 bp (178 aa), respectively. CDT was demonstrated to be a potential virulence factor in *C. hyointestinalis*.
4. Most *C. jejuni* and *C. coli* strains isolated in Thailand are highly resistant to fluoroquinolone and tetracycline. Some *C. coli* strains are highly resistant to macrolides and clindamycin. The Thr86Ile mutation in GyrA could be involved in quinolone resistance in *Campylobacter* strains isolated in Thailand.

審査結果の要旨

Campylobacter 属細菌による食中毒は我が国の食中毒の発生件数で、近年、常にトップの座を占め、世界的にも増加傾向にある。*Campylobacter* 属細菌は、家畜、特に家禽の腸管内に常在し、*Campylobacter* 属細菌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。現在 *Campylobacter* 属細菌は、17 菌種が知られているがヒトに病気を引き起こすものとして、*C. jejuni*、*C. coli*、*C. fetus*、*C. lari*、*C. upsaliensis* と *C. hyointestinalis* の 6 菌種がある。*Campylobacter* 属細菌は 1)微好気性細菌であり、培養には特殊な装置が必要である、2)増殖が遅い、3)生化学的性状が菌種間で類似しており菌種同定が容易でない、4) 菌種によって温度感受性が異なるなどの理由から、本菌の分離・同定に時間を要するだけでなく、誤同定や同定できないなどの問題も生じている。一方、家畜や医療現場における抗菌薬の不適切な使用がキノロン薬やマクロライド薬を含め多剤耐性 *Campylobacter* 属細菌の出現に繋がり医療現場で大きな問題となっている。よって、家禽や患者から分離される *Campylobacter* 属細菌の薬剤感受性を知ることは重要である。

本研究では、タイの家禽および下痢症患者から分離された*Campylobacter*属様細菌について菌種同定、病原因子および薬剤感受性について解析した。以下はそれらの成績の概要である。

第1章では、タイにおいて*Campylobacter*属様細菌として分離・同定された112菌株（患者由来78株、家禽由来34株）を従来法、すなわち、生化学的性状試験、16S rRNA遺伝子の塩基配列の解析および馬尿酸水解酵素遺伝子の有無を総合的に判定し、65株を*C. jejuni*、45株を*C. coli*、そして1株を*C. fetus*と同定した。また得られた結果は、cytolethal distending toxin（*cdt*）遺伝子に基づく種特異的なMultiplex PCR（*cdtB* gene-based Multiplex PCR）法の結果と完全に一致し、*cdtB* gene-based Multiplex PCR法が簡便、迅速でより正確な菌種同定法として有用であることを示した。また、*cdtB* gene-based Multiplex PCRで増幅産物が得られず、複数菌種の*cdtB*遺伝子を増幅出来る共通プライマーで増幅バンドが得られた1株は、16S rRNA遺伝子の解析から*C. hyointestinalis*と同定した。

第2章では、タイの下痢症患者から分離された*C. hyointestinalis*が保有する*cdt*遺伝子の全塩基配列とその遺伝子産物が細胞膨化致死活性を有するかどうかについて解析した。その結果、*C. hyointestinalis*の*cdt*遺伝子は他の*Campylobacter*属細菌の*cdt*遺伝子と同様*cdtA*, *cdtB*および*cdtC*の3つの遺伝子から構成され、アミノ酸レベルでは*C. coli*のCdtと最も相同性が高かった。また、組換え*C. hyointestinalis* CdtB（ChCdtB）を精製し、ChCdtBと特異的に反応する抗体を作成し、*C. hyointestinalis*が示す細胞膨化致死活性および細胞周期のG_2/M期阻害が抗体により中和されてことから、これらの活性はCdtに基づく特異的なものであることを明らかとした。

第3章では、タイで分離された65株の*C. jejuni*（患者由来45株、家禽由来20株）及び45株の*C. coli*（患者由来31株、家禽由来14株）、合計110株について薬剤感受性を調べた。その結果、*C. jejuni*及び*C. coli*は家禽及び患者由来株でキノロン薬及びテトラサイクリンに対して高い耐性率と高いMIC値を示した。一方、マクロライド薬に対しては、*C. jejuni*は家禽及び患者由来株の両方に感受性であったが、*C. coli*では患者由来株に比べて家禽由来株で高い耐性率を示した。

以上の結果は、*cdtB* gene-based Multiplex PCR法が*Campylobacter*属細菌の菌種を簡便、迅速、正確に同定できること、*C. hyointestinalis*で見いだしたCdtが新たな病原因子となる可能性があること及びタイで分離された*C. jejuni*や*C. coli*が高い割合でかつキノロン薬とテトラサイクリンに高度耐性化していることを示した。これらの成果は、*Campylobacter*属細菌による感染症の制御に寄与するとともに、感染症学および防疫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。