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論文名 Studies on prevalence, serotype, antimicrobial resistance and clonal relationship of *Salmonella* in foods, waters and humans in Ho Chi Minh City, Vietnam
(ベトナムホーチミンシティにおける食品、水およびヒトでのサルモネラの汚染状況、分離菌の血清型、薬剤感受性とクローナリティーに関する研究)

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Introduction

Foodborne diseases caused by non-typhoidal *Salmonella* are a very important public health problem all over the world with 85% of those cases being linked to contaminated foods and waters. Non-typhoidal *Salmonella* gastroenteritis accounts for 80.3 million global cases of foodborne illness each year. Particularly, *Salmonella* was the fourth most frequent causative agent of infant diarrhea in southern Vietnam and invasive non-typhoidal *Salmonella* disease was leading to a high mortality rate (26%) in HIV patients in Vietnam. Notwithstanding, there was no nationwide surveillance program for monitoring *Salmonella* food contamination. Emergence and spread of multidrug resistant (MDR) bacteria, particularly MDR-*Salmonella*, have become a major public health concern. This phenomenon occurred by selective pressure due to inappropriate usage of antimicrobials in humans and animals. Along with these reasons, spread of antimicrobial resistant bacteria has contributed to the acquisition of antimicrobial resistance (AMR) determinant through horizontal gene transfer. Currently, extended-spectrum β lactamase (ESBL) and AmpC- β lactamase (AmpC)-producing *Salmonella*, which can hydrolyze third-generation cephalosporins, are also prevalent in many countries. ESBL and *ampC* genes are often located on large plasmids, which also carry other AMR genes including fluoroquinolones. Nevertheless, cephalosporin and fluoroquinolone are used to treat the *Salmonella* infections in humans. Therefore, this is a serious threat to global public health because of limited treatment options. Because of emergence of MDR bacteria, colistin, a reused antimicrobial, is a crucial last-resort for the treatment of MDR infections. However, the occurrence of plasmid-mediated colistin resistance (Mcr) has been increasing in many countries. In addition, there is paucity of information about the presence of plasmid-mediated colistin resistant *Salmonella* in Vietnam. Currently, presence of *Salmonella* in different foods (meat, fish, vegetable and fruit) and waters as well as in humans (symptomatic and asymptomatic), their antimicrobial resistance profile, and the genetic relatedness of *Salmonella* serotype, which were concurrently detected in humans and foods/waters, have been lacking in Vietnam.

In this study, we investigated the prevalence of *Salmonella* and their serotype in symptomatic and asymptomatic humans, raw meats, fishery products, vegetables, fruits and waters in Ho Chi Minh City (HCMC), Vietnam. Furthermore, phenotypic and genotypic characteristics of the isolates were examined particularly focusing on ESBL and Mcr.

Chapter 1. Prevalence, serotype and antimicrobial resistance of non-typhoidal *Salmonella* isolates in raw meat, fish and shrimp samples in Ho Chi Minh City, Vietnam

Prevalence, serotype and AMR of *Salmonella* isolates in a total of 409 samples (255 raw meats and 154 seafoods) collected between October 2012 and March 2015 from slaughterhouses, wholesale fish market and retail markets in HCMC, Vietnam were examined. A high rate of *Salmonella* contamination was detected in pork (69/99, 70%), poultry (47/72, 65%), beef (49/84, 58%), shrimp (26/53, 49%), and farmed freshwater fish samples (37/100, 37%). A total of 53 *Salmonella* serotypes were found, of which *S. Rissen* (38) was most prevalent, followed by *S. Weltevreden* (24), *S. London* (23), *S. Anatum* (22), *S. Typhimurium* (19) and *S. Corvallis* (18). In addition, 4 monophasic *S. Typhimurium* strains were identified using a PCR for the detection of a specific IS200 fragment within the *fliB-fliA* intergenic region. A total of 336 *Salmonella* isolates were examined for antimicrobial susceptibility and had a high prevalence (62%) of resistance to antimicrobial agents, particularly tetracycline (TET) (53%), ampicillin (AMP) (44%), chloramphenicol (CHL) (38%), and trimethoprim/sulfamethoxazole (SXT) (31%). *Salmonella* with MDR being resistance to three or more classes of antimicrobials were commonly found (40%) and, *S. Saintpaul* and *S. Bovismorbificans* exhibited resistance to 6 classes of antimicrobials (3.3%). All 7 *S. Indiana* isolates were resistant

against 4 to 6 classes of antimicrobials including ciprofloxacin (CIP), which is used for the treatment of human *Salmonella* infections. Moreover, plasmid-located *bla*_{CMY-2} gene was identified in 4 *S. Braenderup* and 1 *S. Typhimurium* isolated from poultry and plasmid-located *bla*_{CTX-M-55} and *bla*_{TEM-1} genes were detected in 1 *S. Bovismorbificans* and 1 *S. Newport* isolated from fish. None of *Salmonella* isolates were positive for *mcr-1~8* genes. This study indicated that MDR *Salmonella* is widely disseminated not only in meats but also in seafood in the food distribution system of HCMC, Vietnam.

Chapter 2. Prevalence, serotype and antimicrobial resistance of non-typhoidal *Salmonella* in vegetable, fruit, and water samples in Ho Chi Minh City, Vietnam

Next, prevalence, serotype and AMR of *Salmonella* in vegetable, fruit and water samples in HCMC, Vietnam were examined. *Salmonella* was isolated from 75% (30/40), 57% (12/21), 18% (28/160), and 2.5% (1/40) of river water, irrigation water, vegetable and ice water samples, respectively. However, no *Salmonella* was isolated from 160 fruit and 40 tap water samples examined. A total of 102 isolates, obtained from the 71 samples, belonging to 33 different serotypes, of which *S. Rissen* (10 isolates) was most prevalent, followed by *S. London* (9), *S. Hvitittingfoss* (8), *S. Newport* (7) and *S. Weltevreden* (7). It should be emphasized that there was the marked similarity in serotypes of *Salmonella* isolates between vegetables and river or irrigation water. AMR was most prevalent against TET (35%), followed by CHL (34%), AMP (31%), SXT (24%) and nalidixic acid (NAL) (11%). In water samples, *S. Agona* harboring plasmid containing *bla*_{CMY-2} and *bla*_{TEM-1} genes was identified. However, *mcr-1~8* genes were not detected in all the 102 *Salmonella* isolates. Of the 102 isolates, 52 (51%) showed resistance to at least 1 antimicrobial class while 28 (28%) showed MDR phenotype. Taken together, these data indicated that environmental water and vegetable but not fruit in HCMC were contaminated with *Salmonella* including MDR strains, and environmental water might be the source of *Salmonella* contamination in vegetables.

Chapter 3. Prevalence, serotype and antimicrobial resistance of non-typhoidal *Salmonella* in fecal samples of diarrheal and healthy humans in Ho Chi Minh City, Vietnam

Fecal specimens were collected from 306 diarrheal and 380 healthy humans, and 30 *Salmonella* were isolated from 14 patients (15 isolates) and 12 healthy humans (15) by conventional methods, corresponding to 4.6% and 3.2% of positive rate of *Salmonella*, respectively. Fifteen *Salmonella* strains of each matrix were grouped into 10 *Salmonella* serotypes. A total of 18 *Salmonella* serotypes were identified, of which *S. Kentucky* (4) was most prevalent, followed by *S. Enteritidis* (3) and *S. Indiana* (3). AMR was most prevalent against AMP (73%), followed by TET (70%), CHL (60%), SXT (50%) and NAL (43%). Of the 30 isolates, 24 (80%) showed resistance to at least 1 antimicrobial class while 20 (67%) showed MDR phenotype. Plasmid-mediated *bla*_{CTX-M-55} and *bla*_{TEM-1} genes were detected in *S. Indiana* (3) and monophasic *S. Typhimurium* (1) isolated from healthy and diarrheal humans, respectively. The chromosomally located *bla*_{CTX-M-15} and plasmid-located *bla*_{CTX-M-65} harboring *S. Kentucky* (2) and *S. Infantis* (1) were also isolated from diarrheal patients, respectively. Notably, all ESBL-producing *Salmonella* were resistant to 6 or 7 antimicrobial classes. Especially, ESBL-producing *S. Kentucky* (2) and *S. Indiana* (3) isolates were co-resistant to CIP. Noteworthy, a monophasic *S. Typhimurium* co-harboring *mcr-3* and *bla*_{CTX-M-65} genes was identified in a patient sample and *mcr-3* gene was located on plasmid. These data indicate that prevalence of *Salmonella* in diarrheal patient and healthy human was relatively low but *Salmonella* exhibited MDR, especially against CIP and third generation cephalosporin, are a serious risk to public health.

Chapter 4. Transferability of *mcr-3*, *bla*_{CTX-M-1}, *bla*_{CTX-M-9} and *bla*_{CMY-2} genes, and genetic relatedness between *Salmonella* serotypes concurrent in human and food/ water samples

As described in Chapter 3, *bla*_{CTX-M-1}, *bla*_{CTX-M-9}, *bla*_{TEM-1}, *bla*_{CMY-2} and *mcr-3* genes were located on plasmid. Transferability of the R-plasmid was examined by a conjugation assay. All *bla*_{CMY-2}, *bla*_{CTX-M-65} and *mcr-3* gene-harboring plasmids were transferred from *Salmonella* donor strains to a recipient *E. coli*. *bla*_{CTX-M-55} and *bla*_{TEM-1} gene-harboring plasmids were transferred to a recipient *E. coli* from 3 *Salmonella* donor strains but not 6. Moreover, transconjugants harboring ESBLs were co-resistant to TET, CHL, kanamycin and SXT and reduced the susceptibility to gentamicin, CIP and NAL. These data indicated that ESBL-, *ampC*- and *mcr*-gene harboring *Salmonella* in food, water and human could be a potential reservoir and source of dissemination of plasmid-mediated ESBL, *ampC*, *mcr* and other AMR genes, contribute to spread MDR, and pose a serious concern for public health.

Representational *Salmonella* serotypes that were concurrently detected in humans and foods/waters were analyzed for plasmid replicon profile and the genetic relatedness by MLST and PFGE methods. Among 18 plasmid replicons belonging to Enterobacteriaceae, the most popular one was IncHI1 (41%), followed by IncFIAs (12%), IncN (5.8%), IncI1 (5.8%), IncA/C (2.9%) and untypable (41%). Moreover, *Xba*I-digested PFGE profile and MLST of *Salmonella* generated 17 major clusters and 10 STs, respectively. *S. Typhimurium* and monophasic *S. Typhimurium* were divided into 3 STs: ST19, ST34 and ST36 and corresponding to 3 PFGE patterns. Six *S. Enteritidis* strains belonging to ST11 and IncFIAs were comprised of 3 clusters based on the AMR profile and their sources. Twelve *S. Kentucky* strains were divided into 3 STs: ST198, ST314 and ST2410, and corresponding to 3 clusters, in which ST314 isolate was susceptible to all the tested antimicrobials, while ST198 isolates were MDR including CIP. Ten *S. Indiana* strains fell the same sequence type, ST17, but divided into 2 PFGE patterns. Taken together, there were strong correlations of STs, PFGE patterns and serotypes and a good association among STs, PFGE patterns and antimicrobial resistance phenotype in *S. Kentucky*. These data suggest that the genetic characterization of *Salmonella* strains belonging to the same serotype that was derived from different sources might be closely related, except for isolates from irrigation samples. Impressively, MDR *S. Indiana* ST17, MDR *S. Kentucky* ST198, and MDR monophasic *S. Typhimurium* ST34, a trend of the world, were also isolated in this study. Meats, fishery products, vegetables, river waters might be the source and reservoir of *Salmonella* as well as antimicrobial resistance genes for humans in HCMC, Vietnam. It is recommended that the Vietnamese government needs to develop the national food safety monitoring and surveillance system, as well as the wastewater treatment system in HCMC.

Conclusions

In this study, *Salmonella* were detected in various foods, waters, healthy individuals and diarrheal patients in HCMC, Vietnam. In addition, *Salmonella* in foods, water and humans could be the potential sources of AMR genes including ESBLs, *ampC*, and *mcr* genes. Besides, some AMR determinants were successfully transferred among bacteria through plasmid, indicating a challenge of public health. Moreover, the genetic relatedness of representative *Salmonella* serotypes from human and food/water were closely related. Therefore, meat, fishery product, vegetable and river water might be the sources of *Salmonella* contamination as well as AMR determinants for humans in HCMC, Vietnam. These data suggest that Vietnamese government needs to develop and improve the national foodborne disease surveillance and AMR surveillance system for restriction of foodborne disease as well as the spread of AMR bacteria.

審査結果の要旨

サルモネラ食中毒は世界的な問題であり、ベトナムでは 4 番目に多い小児下痢症の起因菌でもある。しかしながら、ベトナムでは各種食品におけるサルモネラ汚染に関する情報はほとんどない。多剤耐性 (MDR) サルモネラの出現・拡散も大きな問題となっている。特に、基質特異性拡張型 β ラクタマーゼ (ESBL) AmpC β ラクタマーゼや伝達性コリスチン耐性 (*mcr*) 遺伝子を保持するプラスミドは多剤耐性を示し、菌種内のみならず菌種間を越えて伝達されることがある。MDR サルモネラの出現は抗菌薬治療を困難にし、医療上大きな問題となっている。これらの耐性菌出現の背景として、ヒトや動物への不適切な抗菌薬の使用が挙げられる。

サルモネラは通常、家畜の腸管内に不顕性に感染し、と畜解体時に食肉が便で汚染されヒトへの感染源となる。さらに、家畜の便が環境水、野菜や果物等を汚染しヒトへの感染源となる可能性もある。それゆえ、サルモネラの食中毒対策を講じるためには、食品や水のサルモネラの汚染実態を把握することが重要である。そこで本研究では、ホーチミンシティ (HCMC) を対象地域とし食肉、魚介類、野菜、果物や水、そしてヒトにおいて、どのようなサルモネラがどの程度分布しているか、分離菌の血清型や遺伝子型、薬剤耐性と耐性に関わる遺伝子を調べるとともに、それらの性状の伝達性を明らかとすることを目的とした。

第 1 章ではと畜場、魚介類の卸売市場や市中市場で入手した牛肉、豚肉、鶏肉、魚やエビのサルモネラの汚染状況を解析した。その結果、それぞれの検体の 59%、70%、65%、37%、49% からサルモネラを分離した。分離したサルモネラ 336 株は 53 の血清型に分類され、うち Rissen が最も多く、次いで Weltevreden、London であった。分離株の 62% が何らかの抗菌薬に耐性を示し、テトラサイクリン (TET) 耐性が最も多く 53%、次いでアンピシリン (AMP) の 44%、クロラムフェニコール (CHL) の 38% であった。約 2% の菌が第 3 世代セフェムに耐性を示した。分離株の 41% が多剤耐性菌 (MDRO) で、血清型 Bovismorbificans や Saintpaul では 6 剤に耐性を示し、Indiana では 7 株全てが 4-6 剤に耐性を示した。以上より、MDR サルモネラが HCMC の食肉や魚介類に広く分布していることが明らかとなった。

第 2 章では卸売市場や市中市場で入手した野菜、果物、水におけるサルモネラ汚染を解析した。その結果、河川水、灌漑用水、野菜、氷のそれぞれの 75%、57%、18%、2.5% からサルモネラが分離されたが、果物と水道水からは分離されなかった。分離したサルモネラ 106 株は 34 の血清型に分類され、うち Rissen が最も多く、次いで London、Hvittingfoss であった。また、河川水と野菜からは同じ血清型のサルモネラが分離された。最も耐性率が高かったのが TET 耐性の 35%、次いで CHL の 34%、AMP の 31% であった。分離株の 51% が何らかの抗菌薬に耐性を示し、22% が MDRO であった。環境水が野菜への汚染源となっている可能性が示された。

第 3 章では下痢症患者 306 人と健常者 380 人からサルモネラの分離を試みた。その結果、それぞれ 14 人から 15 株、12 人から 15 株分離され、18 種類の血清型に分

類され、うち Kentucky が最も多く、次いで Enteritidis、Indiana であった。分離株の 80% が何らかの抗菌薬に耐性を示し、67% が MDRO であった。ESBL 産生菌 7 株全てが多剤耐性菌で、うち 3 株は健常者から分離された *bla*_{CTX-M} 遺伝子陽性の Indiana、下痢症患者からは *mcr-3* と *bla*_{CTX-M} 遺伝子を持つ非定型 Typhimurium1 株、*bla*_{CTX-M} 遺伝子陽性の Kentucky 2 株と Infantis 1 株が分離された。食品と比べヒトでのサルモネラの陽性率は低かったが、ヒト分離株は食品分離株より高い多剤耐性率を示した。

第 4 章ではヒトや食品・水由来のサルモネラが保有する主要な薬剤耐性遺伝子の水平伝播や分離株のクローナリティーを調べた。その結果、*bla*_{CTX-M-1}、*bla*_{CTX-M-9}、*bla*_{CMY-2} や *mcr-3* 遺伝子を保持した MDR プラスミドがサルモネラから大腸菌に接合伝達され、MDR プラスミドがサルモネラが多剤耐性化に寄与している可能性を示した。食品や患者由来のサルモネラが同じ血清型で類似の遺伝子型を示し、食品がヒトへの感染源、またヒトも食品への汚染源となりうる可能性が示唆された。

以上の結果は、HCMC の食肉、野菜や環境水が高率にサルモネラで汚染されていること、健常者も含めヒト由来サルモネラではより多剤耐性率が高いこと、ESBL 遺伝子を保有する MDR プラスミドがサルモネラが多剤耐性化に寄与している可能性、食品や水由来のサルモネラがヒトへの感染源となっている可能性等、今後のサルモネラ対策に有益な情報を提供するものであり、本研究成果は獣医学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、本論文の審査及び学力確認の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。