Studies on the virulence of Shiga toxin-producing *Escherichia coli* O157 and on the epidemiologic relationship between human and cattle isolates

(志賀毒素産生性大腸菌 0157 の病原性およびヒト・牛分離株間の疫学的関連に関する研究)

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1.1.1 Prevalence of Shiga toxin-producing *Escherichia coli* in human

Shiga toxin-producing *Escherichia coli* (STEC) is defined by its ability to produce Shiga toxin (Stx), which is also called Vero toxin (VT), that is cytotoxic to Vero cells. This pathogen was first described by Konowalchuk *et al.* in Canada in 1977 (84). The first food poisoning outbreak caused by STEC was reported in 1982 in the U.S.A. In this outbreak, Riley *et al.* isolated an *E. coli* O157:H7 strain as a new kind of bacteria to cause diarrhea (136). Symptoms associated with this organism were quite severe with abdominal cramps, bloody diarrhea, and hemolytic uremic syndrome (HUS). O'Brien *et al.* (119) found that *E. coli* O157:H7 reported by Riley neutralized by an antitoxin to Shiga toxin produced by *Shigella dysenteriae* type 1. This finding was quite unique at that time as the toxins produced by two different bacterial species were immunologically related each other. Subsequently, successive mass outbreaks occurred in Canada, the U.S.A., Europe, and Australia (119,133). The first mass outbreak in Japan resulted from STEC O145:H- contamination, which developed in an elementary school in Tokyo in 1984 . However, STEC was widely recognized throughout Japan when two children died in a mass outbreak that occurred in a kindergarten in Urawa in 1990 (1). In 1996, successive mass outbreaks occurred, involving over 11,000 human cases and 12 deaths in different

prefectures in Japan. One of these outbreaks caused by contaminated school lunches in 47 primary schools in Sakai City resulted in an outbreak on an unprecedented scale in the world, involving more than 6,000 children (105,182). Subsequently, a law that all cases of STEC infection must be reported was established in 1999 in Japan, and since that time, 3,000-4,500 persons infected with STEC have been officially reported annually.

1.1.2 Clinical manifestations in human infection with STEC

STEC can cause clinical manifestations of a broad spectrum ranging from asymptomatic to death in humans. Most cases present watery diarrhea without further complications. However, some patients progress to bloody diarrhea or hemorrhagic colitis. 5-10 % patients with hemorrhagic colitis may progress to life-threatening, HUS or thrombocytopenic purpura (TTP) (1, 77, 113, 136,182, 178), and may be accompanied by long-term sequelae (56). Although the kidneys are frequent targets, a wide range of organs, including the central nervous system, the lungs, pancreas, and heart, may be affected. Children less than 5 years old and the elderly are most susceptible to severe complications (58,175).

Most aspects of bloody diarrhea and HUS appear to be attributable to the action of Stx on vascular endothelial cells, and thrombotic microangiopathy is a central feature of the disease (134). This is evident in the lesions and symptomatology of the STEC associated diseases in animals and humans. Bloody diarrhea is associated with lesions in small blood vessels in the colon. The HUS is associated with renal glomerular lesions

that are due to damage to endothelial cells, which become swollen and detach from the basement membrane. Fibrin thrombi develop, and there is narrowing or occlusion of the capillary lumen. The compromise in the blood supply to the glomeruli is the major contributor to loss of kidney function, but damage to glomerular epithelial and proximal tubular epithelium may also contribute to kidney damage.

1.1.3 Classification of STEC by serotyping

STEC have been characterized by serotyping which is used extensively to categorize *E.coli* strains (143). The serotype of an *E.coli* isolate is based on the O (Ohne) antigen determined by the polysaccharide portion of cell wall lipopolysaccharide (LPS) and H (Hauch) antigen determined by flagella protein. Many serotypes of STEC have been isolated from humans with disease (16,143), however, less than 10 O groups, such as O157, O26, O103, O111, O121, O145, are responsible for the majority of cases (58). Association of serotypes with diseases of varying severity in humans and with sporadic diseases or outbreaks has led to the proposal that STEC is classified into 5 seropathotypes, A to E (79). Seropathotype A consists of O157:H7 and O157:HNM, and these serotypes were considered to be most virulent. Seropathotype B consists of O26:H11, O103:H2, O111:HNM, O121:H19, and O145:HNM, and they are similar to the STEC O157 in causing severe diseases and outbreaks but occur at lower frequency. Seropathotype C consists of serotypes that are infrequently implicated in sporadic HUS but are not associated with outbreaks and include O91:H21 and O113:H21.

Seropathotype D is composed of numerous serotypes that have been implicated in sporadic case of diarrhea, and Seropathotype E is composed of many STEC serotypes that have not been implicated in disease in humans. Thus, O157:H7/HNM are the most important serotypes because of its high frequency and severe virulence (58).

1.2 Overview of the process of STEC infection in humans

1.2.1 Intestinal colonization by STEC

Colonization of the intestine and damage due to toxins is considered to be the disease process. Colonization is the process by which STEC overcome host defense mechanisms and establishes itself in the intestine. Gastric acidity is an important host defense mechanism in the gastrointestinal tract, but acid resistance is a general feature of *E. coli* (52,87) and has been demonstrated for O157 (110) and other STEC serotypes (87,176). STEC O157:H7 has been shown to survive in acidic foods such as apple juice and salami and to be capable of causing disease in humans who have ingested low numbers of the bacteria (7,163). However, there is considerable strain-to-strain variation within this serotype. Exposure to weakly acidic environments induces an acid tolerance response, which enhances resistance to more acidic pH (39).

Adherence to intestinal epithelial cells is an early feature of STEC infection. The patterns of attachment and interaction between STEC and epithelial cells are markedly different between *eae*-positive and *eae*-negative STEC.

1.2.2 Adherence to intestinal epithelial cells of *eae*-positive STEC

The *eae*-positive STEC colonizes the intestinal mucosa and induces a characteristic histopathological lesion referred to as attaching and effacing (A/E) lesions (74,113). The A/E lesion is characterized by effacement of microvilli and bacterial adherence to the epithelial cell membrane. Attached bacteria stimulate host cell actin polymerization accumulation, resulting in a raised attachment pedestal (28).

Although the AE lesion is not essential for bloody diarrhea and HUS in humans, the vast majority of strains implicated in these syndromes are *eae*-positive (52). Thus, most STEC are *eae*-positive, and the combined presence of *eae* and *stx2* genes is an important predictor of HUS (47). The *eae*-positive STEC possess a pathogenicity island called the locus of enterocyte effacement (LEE), which encodes the bacterial proteins necessary for formation of the AE lesion. The LEE of STEC is conserved in enteropathogenic *E. coli* (EPEC) as well, and it is well known that the presence of the LEE is strongly associated with disease (6,56,153). The LEE of STEC O157:H7 is 43 kb in size and contains an additional 7.5 kb prophage sequence compared with EPEC strains. The role of this additional sequence is not clearly defined. The LEE is composed of at least 41 different genes organized into three major regions; (i) a type III secretion system (TTSS) that exports effecter molecules; (ii) an adhesion called intimin and its translocated receptor, Tir, which is translocated into the host cell membrane by the TTSS; and (iii) several secreted proteins (Esp) as a part of TTSS, which are important in modification of host cell signal transduction during the formation of A/E lesions (95,127).

Furthermore, the LEE is organized into 5 major polycistronic operons called LEE1 to LEE5. The products of the LEE are a type III secretion apparatus (LEE1 to LEE3); a protein translocation system (LEE4); an adherence system consisting of an outer membrane protein called intimin or Eae (*E. coli* attaching and effacing protein) and its receptor, translocated intimin receptor (Tir), both encoded by LEE5; and effector proteins that are translocated by the secretion system. The secretion apparatus is a molecular syringe structure, and it begins inside the bacterial cytoplasm, extends through the inner and outer membranes, and passes through the host cell membrane. Secreted proteins are transferred from the bacterial cytoplasm to the host cell through this structure. The secreted proteins encoded by the LEE include Tir, mitochondrion-associated protein, EspF (*E. coli* secreted protein F), EspG, EspH, and EspZ. A number of nonLEE-encoded proteins are also translocated by the LEE secretion apparatus (6). The TIR protein is inserted into the host cell membrane and it acts as the receptor for intimin on the bacterial surface, but certain host cell compounds also bind intimin. The TIR and other secreted proteins activate a number of signaling cascades that result in rearrangement of the intestinal epithelial cell architecture and in changes in the cell physiology.

1.2.3 Adherence to intestinal epithelial cells of *eae*-negative STEC

Much less is known about adherence of *eae*-negative STEC. Dytoc *et al.* investigated attachment of an *eae*-negative STEC of serotype O113:H21 to cultured epithelial cells (HEp-2) and to rabbit intestine *in vivo* (43). They showed that there were

areas of microvillus effacement beneath the organism but that the cytoskeletal rearrangement characteristic of the AE lesion did not develop. Vidal *et al.* showed that the STEC strain 472-1 adhered to epithelial cells in an *eae*-independent matter and that *saa* and *psu-int* participated in this adhesion process (171). The *E. coli* O104:H4 strain causing a large outbreak of HUS and bloody diarrhea in Germany in May and June, 2011, possessed an unusual combination of pathogenic features typical of enteroaggregative *E. coli* (EAggEC) together with the capacity to produce Shiga toxin (144).

1.3 Shiga toxin

1.3.1 Shiga toxin－types

Shiga toxin is the critical virulence factor in STEC diseases. Two types of Stx, called Stx1 and Stx2, were originally recognized. Stx1 and Stx2 are different immunologically, whereas they have the same toxin activity, specifically inhibition of protein synthesis (143,157). The Stx1 molecule is a highly conserved structure that is identical to that of Shiga toxin of *Shigella dysenteriae* type 1 (68,69,118,159). Recently, variants of Stx1, called Stx1c and Stx1d, were reported (185,24). In contrast, there are many variants of Stx2, named Stx2c, Stx2d, Stx2d-activatable, Stx2e, and Stx2f, Stx2g that differ in their biological activity and association with disease (24,50,146).

The *stx2c* gene was found at similarly low frequencies (about 5%) in isolates from

patients with HUS and from patients with diarrhea examined the association of *stx2* gene variants with disease in 626 STEC isolates from humans (50). Friedrich *et al.* determined that *stx2d* and *stx2e* were associated with mild disease or asymptomatic carriage, were produced by *eae*-negative strains, and were never present in 268 STEC isolates from patients with HUS (50).

1.3.2 Shiga toxin—structure and role

All Stx molecules have an A1B5 structure, a heterohexamer in which 1 A subunit (~ 32 kDa) is noncovalently associated with a pentamer of B subunits (~7.7 kDa each). Although they possess the same mechanism of action, there is only 55% identity in amino acid sequence between the A subunits of Stx1 and Stx2. The A subunit possesses enzymatic activity that enables the toxin to cleave a specific adenine base from the 28 S rRNA and thereby prevent protein synthesis. Apoptosis may follow the inhibition of protein synthesis as a result of ribocytotoxic stress response (152) or it may develop rapidly due to signaling by Stx (32,50).

The B subunits bind to specific glycolipid receptors on the surface of cells, permitting internalization of the toxin molecule. The Stx toxins bind to globotriaosylceramide (Gb3). The Stx2e implicated in edema disease of pigs, uses globotetraosylceramide (Gb4) as its preferred receptor, but it can also bind Gb3.

Following binding of toxin to its receptor, Stx is taken up into the cell by receptor-mediated endocytosis (140,141). Binding of Stx induces signaling that resulted

in Syk activation and an increase in Stx entry into the cell. The toxin thus appears to regulate its entry into cells. There is evidence of an association of Stx2 with a higher risk of developing HUS and the presence of both *eae* and *stx2* in an STEC isolate is considered to be a predictor of HUS (18,47). It is not known whether the association of Stx2 with HUS is the result of the action of Stx2 or whether Stx2 is simply a marker for increased disease severity. However, Stx2 is about 1,000 times more toxic for human renal microvascular endothelial cells than Stx1 (99,150).

1.3.3 Shiga toxin—function

It has been known that Shiga toxin inhibits protein synthesis in eukaryotic cells by inhibiting elongation factor 1 (EF-1)-dependent aminoacyl-tRNA binding to 60S ribosomal subunits (21). Igarashi *et al.* demonstrated that not only Stx1 but also Stx2 inactivated 60S ribosomal subunits and inhibited EF-1-dependent aminoacyl-tRNA binding in eukaryotic cells (66,121). Further, they also demonstrated that Stx1 and Stx2 exhibited an RNA N-glycosidase activity and cleaved the N-glycosidic bond of the adenosine residue at specific position of the 28S ribosomal RNA of 60S ribosomal subunit.

1.3.4 Shiga toxin – expression

The overview of the expression of the shiga toxin has been summarized by Wagner *et al.* and Shimizu *et al.* We introduce it here (172,150).

The *stx1* and *stx2* genes are located on the genomes of resident prophages (called Stx-encoding phage or Stx-converting phage) of the λ family immediately downstream of the phage late promoters ($p_{R'}$). The production of Stx1 and Stx2 is closely related to induction of the Stx-encoding phage. Stx-encoding phages are related to the λ phage, which is well characterized in both its genome arrangement and transcription patterns. The patterns of transcription initiating at, p_L and p_R, is shown below.

In the lysogenic state of the λ phage, the repressor (*cI*) silences the transcription of most phage genes. Removal of repression, which can occur when DNA damage activates the bacterial SOS response causing RecA-mediated cleavage of the repressor, leads to a cascade of regulatory events beginning with expression of the N transcription antitermination protein. Terminator read-through mediated by the N protein results in expression of delayed early genes that encode products involved in replication and prophage excision, as well as the Q antitermination protein. Q acts at a site, qut, within the phage late promoter, $p_{R'}$, modifying RNA polymerase to a highly processive form that reads through downstream terminators. Thus, late gene expression by lambdoid prophages is a consequence of prophage induction. *stx1* and *stx2* genes are found within the late operons of the Stx-encoding phages, suggesting that they transcribe from the late phage promoter along with lysis genes and morphogenesis genes following prophage induction (130).

Then, Stx-encoding phage genome is excised in the form of cyclic DNA from STEC genome by the recombinant gene products (108). The cyclic phage genome is amplified

dramatically by the reproduction gene products, and consequently, Stxs are produced in large quantities, as well as the head protein and tailpiece protein of the phage. A large number of phage particles are formed of a large quantity of head, tailpiece protein and phage genomic DNA, and the phage particles are released into the extracellular. Then, at the same time, Stxs are also released. However, most Stx2 toxins are released into the extracellular by this mechanism, but most Stx1 toxins are kept in the bacterial cells without being released. This depends on the difference of the promoters for Stx1 and Stx2 expression in Stx-encoding phages.

For Stx1, there are two promoters located on the upper stream of *stx1* gene; one is the late phage promoter, and the other is Stx1 promoter. However, both promoters act at the same time in only about 5% of STEC, and in about 95% of STEC, only Stx1 promoter acts. The transcription starting from Stx1 promoter stops at the terminator located on the upper stream of the phage lysis gene. Therefore, this cascade finishes without transcription of the phage lysis gene, after the *stx1* gene is transcribed. Consequently, in most STEC, Stx1 production and phage lysis are not expressed at the same time, and Stx1 does not release into the extracellular.

On the other hand, Stx2-encoding phage has no specific promoter for transcription of *stx2* gene, therefore the transcription during prophage induction of the Stx-encoding phage was important for Stx2 expression (172). The transcription for Stx2 expression is started from the phage late promoter. Subsequently, *stx2* genes, phage lysis gene, and phage structural gene located in the downstream, are transcribed during phage

induction. Consequently, *stx2* gene and a phage lysis gene are included in the same transcription unit, and Stx production and phage lysis occur at the same time, and Stx2 is released into the extracellular.

Thus, these differences in the mechanisms of gene expression between *stx1* and *stx2* in STEC also may contribute to the difference in localization between Stx1 and Stx2. Furthermore, in Stx2 release from STEC, Shimizu *et al.* determined that another specific Stx2 release system, in addition with the specific induction of the Stx2-encoding phage, are involved in Stx2 release from STEC (150). This Stx2 release system strictly recognized the serine 31 residue of the B subunit of Stx2 in STEC, but the equipment involved in the system is not yet clear.

The gene arrangements around *stx1* and *stx2* genes of Stx-encoding phages in STEC are similar, but their high-expression conditions reportedly are somewhat different (25,26,38). Stx2 production is activated by phage-inducing agents, such as mitomycin C, while Stx1 production is increased under low-iron conditions (25,26).

1.4 Other virulence factors of STEC

Typical virulence factors of STEC are the Stxs and the LEE-encoded effectors, in addition, the products of several genes on the virulence plasmid and on the chromosome have been suggested to play a role in virulence. These products include adhesins, toxins, proteases, iron acquisition systems, LPS, and flagellin. The nonfimbrial adhesins include EfaI, chromosomal Iha (iron-regulated gene A homologue), outer membrane protein A

(OmpA)(Torres and Kaper, 2003), STEC autoagglutinating adhesion (Saa), and plasmid-encoded ToxB (160,125,162). The Efa1 (EHEC factor for adherence) has homology with ToxB, and is found in EPEC as well as EHEC. This adhesin was detected in a clinical STEC O111:H- isolate. The EfaI adhesin is known to contribute to colonization of the bovine intestine (154). The Iha is an outer membrane protein with homology to iron-regulated gene A of *Vibrio cholera* that is widely distributed among STEC serotypes (161,164). The outer membrane protein A is an adhesin for O157:H7 cells. The Saa (125) is produced by strains of certain serotypes of LEE-negative STEC (e.g., O113:H21 and O91:H2), including some strains that have been isolated from patients with HUS. This adhesion may be important in colonization of cattle. Jenkins *et al.* showed that the gene for Saa was significantly more associated with STEC from cattle than with those from humans (70). The ToxB protein, which has homology with *Clostridium difficile* toxins A and B, was shown to contribute to adherence of EHEC to epithelial cells. The gene for ToxB is present in a wide range of STEC serotypes (161,164).

The fimbrial adhesins include long polar fimbriae (Lpf) (41,165,166,167), SfaA (153), StpA (49), and StcA (153). Long polar fimbriae are encoded by 2 regions of the O157:H7 chromosome and are highly related to the long polar fimbriae of *Salmonella* Typhimurium. *Escherichia coli* O113:H21 has only a single copy of the lpf operon. However, recent studies cast doubt on the likelihood that Lpf are virulence factors for STEC.

Although the critical importance of Shiga toxin in the pathogenesis of STEC diseases

is well established, there are other toxins produced by STEC that may play roles in disease. Cytolethal distending toxin (CDT) was detected in a small proportion of *eae*-negative STEC and was associated with disease (12). More recently the toxin was reported in O157:H- STEC (11). The *astA* gene for enteroaggregative *E. coli* heat-stable enterotoxin (EAST1) is found in O157:H7 STEC (142). This gene is present in a wide range of diarrheagenic *E. coli* of human and animal origins. The contribution of EAST1 to STEC disease is not known but it has the potential to contribute to watery diarrhea. The plasmid-encoded hemolysin (Ehly) is produced by both *eae*-positive and *eae*-negative STEC. Among strains from humans in Finland, 92% of the *eae*-positive STEC were positive for enterohemolysin compared with 35% of the *eae*-negative strains (46). Subtilase cytotoxin is a recently discovered toxin that has homology with subtilase of *Bacillus anthracis* (126). It was produced by an *eae*-negative O113:H21 STEC strain, and the gene was detected in a high percentage of other STEC strains. The toxin was lethal for mice and induced microvascular thrombosis and necrosis in several organs including the kidneys in mice. The toxin has an A1:B5 structure, is a serine protease, and may contribute to pathogenesis of disease.

Several other proteases are produced by STEC, and their activities suggest that they could contribute to disease (22,23,57). These activities include mucinolysis, cleavage of coagulation factor V, degradation of apolipoproteins, reducing activity, and adherence. The StcE product is suggested to play a major role of pathogenesis. The plasmid encoded type 2 secretion system may contribute to virulence through its secretion of StcE (145).

The role of H7 flagellin is to induce an inflammatory response. A H21 flagellin also play a role in virulence, albeit through different mechanisms. Rogers *et al.* showed that a *fliC* deletion mutant of an *eae*-negative O113:H21 STEC was less virulent in a mouse model of infection (137). Some serotypes of STEC such as O5:NM, have a urease-positive phenotype (62,51). Urease could be involved in acid resistance or cytotoxicity *in vivo*.

1.5 Source of STEC infection in human and transmission route of STEC

1.5.1 Various transmission routes

In U.S.A., the most frequent route of STEC O157:H7 infections is via consumption of contaminated food and water (133). Contaminated beef product, especially undercooked ground beef, has been the most common vehicle in sporadic cases and outbreaks of STEC O157 infection. Beef products may become contaminated during slaughter, where fecal contamination of the carcasses may occur. Subsequent process of beef may transfer pathogens from the surface of the meat to the interior. Therefore, if ground beef is incompletely cooked, the bacteria can survive.

There are a variety of contaminated food vehicles other than ground beef that have been linked to STEC O157 incidences, including unpasteurized milk, drinking water, salami, beef jerky, and fresh produce such as lettuce, radish sprouts, fresh spinach, and apple cider. The largest outbreak of STEC O157 infection in 47 primary schools in Sakai city, Osaka, Japan, involving more than 6,000 children, was suspected to have come from

radish sprout contamination (1996) (37,105,182).

However, it can also be spread directly from person to person, particularly in child day-care facilities, from animal to person, and from environment to person. Infections have been documented from people visiting petting zoos, dairy farms, or camp grounds where cattle have previously grazed (95,158, 64,173)

Of the 350 outbreaks reported to the CDC from 1982 to 2002, the determined transmission routes were foodborne (52%), unknown (21%), person-to-person(14%), waterborne (9%), and animal contact (3%) (95). These various transmission routes can be explained by the very low infectious dose (~50 CFU) of STEC O157:H7.

STEC infection can be very serious, or fatal, and consequently such illness must be prevented. Reduction of STEC O157 contamination of beef products will reduce the risk of infection to consumers. Such reductions of the potential risks of consuming contaminated beef products has to be achieved by good hygine practices by both producers (farm level ,slaughterhouse level, and treatment of end-products) and consumers (education and providing adequate information on proper handling of foods) (37).

1.5.2 Cattle as a reservoir of STEC

STEC can be found from numerous animal species (10), but ruminants have been identified as a major reservoir of STEC that are highly virulent to humans, in particular STEC O157. Cattle are considered to be the most important source of human infections

with STEC O157, being asymptomatic excretors of the organism, which is a transient member of their normal gut micro flora. Studies conducted with experimental infections have confirmed that STEC O157 shedding is longer and more intense in calves than in adult cattle (74) and that it largely increases after weaning (136).

Studies on the presence of STEC in cattle have been performed worldwide (28,57,95). In North America, cattle are of most significance as a reservoir of STEC, whereas, in countries such as Australia, sheep are of greater significance. In North America, STEC have been isolated both from beef cattle and dairy cattle (33,42). Hancock *et al.* reported the isolation of STEC O157 from 0.28% of dairy cattle (8.3% of the tested herds) and from 0.71% of beef cattle (16% of the herds)(59). In Australia, *stx* genes were present in 16.7% of fecal samples from dairy cattle (34) and STEC O157 and STEC O26:H11 were isolated from 1.9 and 1.7% of the samples respectively. In Japan, *stx* genes were detected in the feces of 46% of tested calves, 66% of heifers and 69% of cows (83); STEC O26, O111 and O157 were isolated in 9 of the 78 herds tested (11.5%). In Europe, studies on STEC fecal shedding have been performed in many countries, ranging from 2.8% of the tested animals in the UK (189) to 75% in Norway (169). The isolation rate of STEC O157 from animals ranging from 0.2% in France(129) to 16.6% in Italy (20). In the studies by Blanco *et al.* in Spain, the main STEC serogroups isolated were O8, O20, O22, O77, O113, O126 and O162 (14). Most of the isolates were negative for the *eae* gene (14,15). STEC belonging to serogroups other than O157 can frequently be isolated from young calves with diarrhea (88).

However, the results of the investigations on the prevalence of STEC and/or STEC O157 in cattle are clearly influenced by the sampling and detection methods adopted. It is difficult to determine whether the results reported reflect true differences in colonization rates or are the consequence of the different methodologies adopted. Therefore, to evaluate these results, it is necessary to pay attention to the sampling and detection methods adopted.

1.5.3 Other ruminant species as a reservoir of STEC

STEC exists also in the intestine of other ruminants such as, sheep, goat, and deer. STEC, including O157 and other serotypes associated with human infections such as O91, O128 and O146, have been frequently isolated from the intestinal content of sheep (63,169). STEC O157 has also been found in both meat and milk (31) and sheep are now considered as an important reservoir for human infection in some countries such as Australia (17). STEC O157 has also been isolated from goats (131), and goat milk has been associated with an outbreak (13). Small ruminant flocks may also have a relevant role in spreading STEC contamination in the environment (65,122). STEC O157 has been repeatedly isolated from deer (135) and the consumption of deer venison has been associated with human infections (82,132).

1.5.4 Non ruminant animals as a reservoir of STEC

STEC have also been recovered from pigs, horses, dogs, and birds. In pigs, the STEC serotypes that are usually detected are associated with edema disease and are specific for pigs. Although pigs are not considered to be a major source of STEC O157 infection and other STEC associated with human infections, STEC O157 have been isolated from pigs.

STEC have also been isolated from birds. The isolation of STEC O157 (174) and non-O157 STEC (101) from seagull droppings has been reported. The seagulls were not considered as a true reservoir of STEC but rather as potential vectors for their dissemination. STEC strains producing a particular variant of Stx2, designated Stx2f, have been frequently isolated from feral pigeons (107,146). It is difficult to establish whether Sxt2f-producing strains may represent a cause of avian disease or even a potential health hazard for humans. Pigeons seem to be a natural reservoir for these particular STEC strains, which could be host adapted.

1.5.5 Shedding and persistence

STEC O157 naturally colonizes the gastrointestinal tracts of cattle, and the lymphoid follicle-dense mucosa at the terminal rectum, called the rectoanal junction (RAJ) mucosa, is known as a principal site of colonization in cattle (96,114).

Three distinct patterns of *E. coli* O157:H7 carriage in cattle have been described previously (95). First, animals can be transiently culture positive for short durations of a few days and are considered passive shedders and are likely not colonized at the RAJ mucosa. Second, cattle can be colonized and shed the bacteria for an average of 1 month

and typically not longer than 2 months. Third, a few rare animals are colonized for a long duration and shed the bacteria from 3 to 12 months or longer. This unique situation, in which a few animals develop long-duration colonization (>2 months) with *E. coli* O157:H7, is likely due to bacterial association at the RAJ mucosa; however, it may be due to unique colonization by the bacteria at a site(s) in addition to the RAJ mucosa.

In a recent study conducted on a breeding farm in Japan (179), fecal shedding of EHEC O157 and EHEC O26 persisted up to ten weeks and three weeks, respectively. The magnitude of fecal shedding was around 10^4 cfu/gram for STEC O157 and 10^2 cfu/gram for STEC O26. McDowell *et al.* have shown that STEC appear to be well adapted to survive in animal feces, where they can remain viable for periods ranging from several weeks to many months (104). Some strains of STEC can persist many years in a same cattle herd, and that could explain the role of cattle as a reservoir. Moreover, introductions of new STEC strains via feeds and drinking water are always possible (61). The importance of the farm environment as a potential source or reservoir of STEC O157 has been extensively addressed. In the USA, a same well-identified STEC O157 strain persisted in a farm environment for more than two years (147). The strain was isolated from cattle but also from other animals such as birds or flies, and from feeds and drinking water. The persistence of STEC in calves kept in a cowshed might result from continuous contact between the animals and/or with their environment, that allow regular re-infection.

When cattle feces contaminated experimentally with10^8 cfu/gram of STEC O157

were kept on the surface of grazing and, the O157 numbers decreased by 4.0–5.0 log10 cfu/gram within 50 days, but the organism was still detectable in the surrounding soil for up to 99 days (19). Persistence of STEC in the soil (54,55) favors the infection of cattle and makes environmental exposure a risk factor for human infection (35,65,122,155).

Age, diet, and immunity of individual cattle could also potentially affect bacterial colonization. Cray and Moon reported that calves shed *E. coli* O157:H7 longer than adult cattle given the same level of *E. coli* O157:H7 inoculums (36).

Reducing the level of carriage of *E. coli* O157:H7 in cattle, as a major source of *E. coli* O157:H7 infection, would play a key role in decreasing the risk of human infection. Animal wastes and effluents from farming operations, including manure and slurries, are frequently applied as a fertiliser to land used for crop or silage production and cattle grazing. In such cases, appropriate handling of these products is necessary to control the spread of potentially present STEC and limit the risks of human infection (60,67). Cattle manure composting before its spread onto land may reduce the risk of transmission of STEC through contaminated vegetables (72,73,100). Cattle husbandry is likely the major source of environmental contamination with STEC. However, it should also be considered that the presence of STEC may result from throwing out contaminated wastewaters of human origin (86) or spreading contaminated sewage- sludge onto the land (170).

1.5.6 Environmental survival

STEC O157:H7 can survive and persist in numerous environments such as soil, water, and food as well as in animal reservoirs. STEC O157:H7 has been shown to survive for a year in manure-treated soil and for 21 months in raw manure that had not been composted (71). Composting manure is effective in destroying STEC O157:H7, if the temperature is maintained above 50℃ for 6 days. STEC O157:H7 can survive for a long time in water, especially at cold temperatures. Water trough sediments contaminated with bovine feces can serve as a long-term (>8 months) reservoir of *E. coli* O157:H7, and the surviving bacteria in contaminated troughs is a source of infection (90). Barker *et al.* showed that *E. coli* O157:H7 survives and replicates in *Acanthamoeba polyphaga* (5). *A. polyphaga* is a common environmental protozoan that is widely distributed in soil, water, and fecal slurry. Thus, it can be an efficient transmission vehicle of STEC O157:H7 in these environments.

To survive in varied environments, STEC O157:H7 requires the ability to adapt to variations or extreme changes in temperature, pH, and osmolarity conditions commonly encountered in nature.

These environmental adaptations of STEC O157:H7 play an important role in the persistence and dissemination of this microorganism on farms and the increasing transfer from cattle to cattle. In addition, the ability to survive outside the host reservoir increases the risk that the pathogen may contaminate crops and produce via bovine manure contamination, irrigation with contaminated water, or direct contact with infected animals (103).

1.6 Overview of my thesis

1.6.1 Background

In Miyazaki Prefecture in Japan where this study was carried out, many human STEC infections have occurred recently. During 10 years from 2000 to 2009, STEC patients have been reported officially between 39 and 132 every year. The dominant serotype of human-origin STEC in Miyazaki is O157, and clinical manifestations vary from no symptoms to serious systemic complications such as HUS.

On the other hand, Miyazaki is an area where the production of livestock for meat is very prolific and many cattle are bred. It is well known that cattle are reservoirs for STEC O157 which is an important pathogen of STEC infection in human. Misawa *et al.* detected *stx* genes by PCR in 76.3% of calves in Miyazaki (106).

1.6.2 Overview of my thesis

The goal of this study is to better understand STEC infections and to apply my results to preventive measure against STEC outbreaks. In this study, the following two researches were carried out.

The aim of this first research is to clarify the factors affecting pathogenicity of STEC O157 strains for a better understanding of diversity of symptoms including severe symptoms such as HUS and encephalitis. For this aim, STEC O157 strains isolated in Miyazaki Prefecture were examined for *stx* genotypes, the amount of Stxs, the

stx specific mRNA expression, DNA fingerprinting patterns of STEC O157 strains, and analysis in correlation with clinical manifestations of STEC O157 infections. Based on the results, the relationship between *stx* genotype and pathogenicity for humans in STEC O157 strains was studied. This research is summarized in chapter 2 in this thesis.

The aim of the second research is to investigate distribution pattern of STEC O157 epidemiologically in the cattle population which has been prolific, and to clarify the epidemiological relationship between STEC O157 in the cattle population and in human population. For this aim, the *stx* genotype distribution of cattle origin O157 strains in the Miyazaki region was surveyed and compared with that of strains from humans who are living in the same area. Furthermore, the relationship between O157 strains originating in human and cattle was investigated by two molecular techniques, pulsed-field gel electrophoresis analysis (PFGE) (102) and insertion sequence (IS) printing analysis (124). This research is summarized in chapter 3 in this thesis.

2. The role of Stx2 variants in STEC O157 infections in human

Relationship between *stx* genotype and pathogenicity for humans in STEC O157 strains

2.1 Introduction

Shiga toxin (Stx)-producing *Escherchia coli* (STEC) is an emerging pathogen of significant clinical and public health concern that causes various symptoms such as diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans (26). Recently, many human STEC infections have occurred in Japan, and this has become an important public health issue.

Various factors, such as type of toxin, dose of bacteria, and age of humans infected, influence the diversity (severity) of symptoms caused by STEC infection. Shiga toxins (Stx1 and Stx2) have a prominent role in the pathogenesis of STEC and are classified with their restrictive variants according to the nomenclature proposed by Calderwood *et al.* (27). Stx1-related toxins (or Stx variants) include Stx1, Stx1c, and Stx1d, whereas Stx2-related toxins (or Stx2 variants) include Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g (117,143,156). These toxins are encoded by genes designated *stx1*, *stx1c*, *stx1d*, *stx2*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2g*, respectively. Some reports indicate that Stx type (or *stx* genetype) is associated with the virulence level of STEC (45,50,53).

In Miyazaki Prefecture in Japan, many human STEC infections have occurred recently, and stockbreeding is prolific in this area. Misawa *et al.* detected *stx* genes by PCR in 76.3% of calves in Miyazaki (106). The dominant serotype of human-origin STEC in Miyazaki is the O157 which is one most associated with epidemic cases.

The aim of this study is to examine why infected people with STEC O157 develop

various clinical manifestations including severe symptoms such as HUS and encephalitis. For this aim, STEC O157 strains isolated in Miyazaki Prefecture were examined for *stx* genotypes, the amount of Stxs, the *stx*-specific mRNA expression, DNA fingerprinting patterns of STEC O157 strains, and the results were analysed in correlation with clinical manifestations of STEC O157 infections.

2.2. Materials and methods

2.2.1 Bacterial strains and subjects

STEC O157 strains comprising 211 isolates from 210 human fecal samples collected in 2000-2008 and 8 isolates from 8 cattle fecal samples collected in 2008-2009 in Miyazaki prefecture, were used in this study. Human samples were derived from 145 occurrences; these occurrences comprised 8 group outbreaks (n=39 isolates), 26 family outbreaks (n=60), 52 sporadic diarrhea cases (n=53), and 59 healthy carriers (n=59) (Table1). The strains were used for *stx* genotype analysis, quantitative analysis of toxin using reversed passive latex agglutination (RPLA) and Vero cell assay, mesurement of *stx2*-related mRNA expression, genomic DNA analysis using pulsed-field gel electrophoresis (PFGE), and analysis in correlation with clinical manifestations.

2.2.2 Isolation of STEC serotype O157 strains

Escherichia coli (*E. coli*) strains were isolated from fecal samples with selective media of CT-SMAC (Oxoid Ltd., Hampshire, England), CHROMagar O157 TAM (CHROMagar, Paris, France), and DHL (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The detection of Stxs was performed by detecting *stx* genes using PCR.

STEC isolates were serotyped with commercial *E.coli* antisera (Denka-seiken Co., Ltd., Tokyo, Japan). Possession of *eae* gene was also confirmed in all O157 strains using PCR (9).

2.2.3 PCR

Detection and typing of *stx* gene was performed by PCR, as follows: A boiled bacterial supernatant (DNA solution) was added to PCR reagent including 10×PCR buffer, dNTPs, appropriate primers, TaKaRa Ex Taq (TaKaRa Bio Inc., Shiga, Japan), and ultrapure water in total volume of 25 μl, followed by amplification for 25 cycles using a thermal cycler (PE Applied Biosystems 9700, Foster City, CA, USA)(75,40,112,168,181). The amplification products were electrophoresed on a 2% agarose gel (NuSieve 3:1 Agarose; Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) and visualized using a UV transilluminator after ethidium bromide staining. Primers, Probes, and restriction enzymes used in this study were shown in Table 2.

2.2.4 Typing of *stx* genes

Detection of *stx* genes was performed by PCR screening as described by

Karch and Meyer (75). This PCR strategy can detect *stx1*, *stx2*, *stx2c*, *stx2d*, *stx2e*, and *stx2g*, but not *stx2f* (146). Inability to detect *stx2f* does not seem to be a serious epidemiologic problem, since detection of STEC O157 strain carrying the *stx2f* gene has never been reported.

Typing of the *stx* genes was performed by PCR as described by Nakao *et al.* (112), and each strain was classified into *stx1*, *stx2*, and three *stx2* variant groups (*stx2c*, *stx2d*, or *stx2e*). To distinguish between *stx2 and stx2* variants (*stx2v-a* and *stx2v-b*), PCR and subsequent restriction fragment length polymorphism (RFLP) analysis using three restriction enzymes, *Hae*III, *Nci*I, and *Rsa*I (TaKaRa Bio Inc., Shiga, Japan), was performed according to the method reported previously (168). Furthermore, Sablet *et al.* reported that *stx2v-a* could be further divided into two groups, *stx2vha* and *stx2c* (40). For this distinction, we performed PCR-RFLP analysis using the restriction enzyme *Eco*RV (TaKaRa Bio).

2.2.5 Bacterial preparation for RPLA, Vero cell assay, and *stx2*-related mRNA measurement

For RPLA and the Vero cell assay, bacteria were grown with starting concentrations at around 10^7 cfu/ml in CAYE broth (Denka Seiken Co. Ltd., Tokyo, Japan) at 37°C with shaking, and were grown to 2.4×10^8—6.6×10^8 cfu/ml up to 24 hours. The culture was further incubated for 30 min in the presence or absence of polymyxin B (final

concentration of 5000U/ml). Culture supernatants were then obtained by centrifugation (13,000*g*, 5 min) and filtered through 0.22-μm pore size membrane filter units (Millex-GV; Millipore, Bedford, MA, USA). The filtrate was used as the Stx extract for RPLA and the Vero cell assay.

For *stx2*-related mRNA measurement and RPLA carried out at the same time, bacterial cultures were grown in CAYE broth with starting concentrations at 9×10^4 to 1.1×10^5 cfu/ml at 37°C with shaking for 2, 4, 5, 6, 7, 8, 9, or 24 h. Total RNA was extracted and purified from 0.5 ml of each bacterial culture using the RNA protect Bacteria Reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA was removed from total RNA samples via DNase I (RNase-free, TaKaRa Bio Inc., Shiga, Japan) digestion, and 2 μl of each total RNA sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Aliquots (2 μl) of the resulting cDNA samples were used as templates for real-time PCR.

2.2.6 RPLA

Stx1 and Stx2 were quantitated using a Commercial RPLA test kit (VTEC-RPLA, Denka Seiken Co. Ltd., Tokyo, Japan), according to the manufacturer's instructions (76). Briefly, serial two-fold dilutions of Stx extract from 1 to 1024 or 2048 times (25 μl) were mixed with equal volumes of latex particles sensitized with rabbit polyclonal anti-Stx1 IgG or anti-Stx2 IgG in 96-well V-bottom microtiter plates. The plates were incubated at room temperature and examined for latex agglutination for 20–24 hours. The titers of

Stx1 and Stx2 were expressed as the reciprocal of the highest dilution, where "x" was used as a unit of dilution, that caused agglutination by the respective latex reagent.

2.2.7 Vero cell assay

Stx1 and Stx2 were quantitated using the Vero cell assay method reported by Karmali *et al.* (78). Briefly, 1.5×10^4 Vero cells in Eagle MEM medium (100 µl) (Nissui Pharmaceutical Co. Ltd.) containing 10% fetal bovine serum (ICN Biomedical Inc., Aurora, OH, USA) were inoculated into each well of 96-well microtiter tissue culture plates and cultured at 35°C in a 5% CO_2 atmosphere. After 24 hours of incubation, two-fold dilutions of the Stx extract (100 µl) were added to the Vero cell monolayers in each well and the plates were further incubated under the same conditions. After three days of incubation, viability of cells was examined after staining with crystal violet. The cytotoxic titer was expressed as the reciprocal of the highest dilution, where "x" was used as a unit of dilution, that killed 50% of the cells in the monolayer (CD_{50}).

2.2.8 Real-time PCR

Real-time PCR was performed using the 7500 Fast Real-time PCR System (Applied Biosystems) according to the Taqman PCR method described by Nielsen *et al.* to measure *stx2* related mRNA levels (115)(Table 2). Briefly, Real-time PCR assays were carried out in 20 µl volumes containing 2 µl of template DNA, 600nM of each primer, 200nM of each probe, and the TaqMan Universal Master Mix (Applied Biosystems)

using the 7500 Real Time PCR System (Applied Biosystems). Thermal cycling consisted of two initial steps, 50°C for 2min and 95°C for 10min, which were followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The amounts of *stx2*-related mRNAs were calculated based on corresponding standard curves that were generated from PCR amplification of genomic DNA from one STEC isolate (absolute quantification) (30,44,115). Nielsen *et al.* reported that these primers and probes could identify, but not differentiate between, all *stx2* variants except *stx2e*.

To accurately assess the differences in *stx2*-related mRNA expression among STEC strains, we made relative quantifications; this method normalizes expression values of the target gene using expression of a housekeeping mRNA (an internal standard) in RNA samples (40). The *tufA* gene, which encodes the EF-Tu elongation factor of protein synthesis—an abundant cellular housekeeping protein in *E. coli*—was used as an internal standard (148,149,183). Primers and a probe for *tufA* real-time PCR were prepared using the method reported by Sablet *et al.* with the following changes; the forward primer was *mtufAq*F (CATGGTTGATGACGAAGAGCTG), the reverse primer was *mtufAq*R (CACGCTCTGGTTCCGGAAT) (40), and a new probe, *tufAq*P, was designed (FAM-CGATCGTTCGTGGTTCTGCTCTGAA-TAMURA)(Table 2). Real-time PCR for *stx2* and *tufA* was performed in parallel in separate wells of the same multi-well plate. The amounts of *stx2*-related and *tufA* mRNAs were calculated based on corresponding standard curves that were generated from each PCR amplification of genomic DNA from one STEC isolate (40,44).

2.2.9 PFGE

PFGE was performed according to the protocol used by the PFGE-based national network set up to monitor the spread of O157 in Japan (102). Briefly, bacterial cultures grown in Trypto-Soya Broth (Nissui Pharmaceutical Co., Ltd.) at 37°C for 16-18 hours were embedded in agarose plugs and were treated with Proteinase K (1mg/plug, Wako Pure Chemical Industries, Ltd., Osaka, Japan). After removing Proteinase K, genomic DNA embedded in plugs were digested with 30 U of restriction enzyme *Xba*I (Boehringer Mannheim, Mannheim, Germany) overnight at 37°C. Agarose gels (1% SeaKem Gold Agarose; Cambrex Bio Science Rockland, Inc.) were electrophoresed using a CHEF DR III apparatus (Bio-Rad Laboratories, Richmond, CA, USA) under the following conditions: 0.5×Tris-Borate-EDTA buffer at 14°C, 6 V/cm for 20 hours, with a linearly ramped switching time from 2.2 to 54.2 sec. Images of gels stained with ethidium bromide were analyzed using Fingerprinting II software (Bio-Rad Laboratories).

2.3 Results

2.3.1 *stx* genotypes in STEC O157 isolates from human

All of 211 strains were investigated, consequently, *stx1*, *stx2*, and *stx2c* were detected but *stx2d*, *stx2e*, and *stx2f* were not detected (Table 1). In practice, the combination of these three genes generated five genotypes: *stx2* alone (hereafter *stx2*), *stx2c* alone (hereafter *stx2c*), both *stx1* and *stx2* (hereafter *stx1/stx2*), both *stx1* and *stx2c*

(hereafter *stx1/stx2c*), and both *stx2* and *stx2c* (hereafter *stx2/stx2c*). The two most frequent genotypes were *stx1/stx2* (detected in 92 isolates; 43.6%) and *stx2c* (detected in 74 isolates; 35.0%). The *stx2*, *stx2/stx2c*, and *stx1/stx2c* were detected in 27 (12.8%), 9 (4.3%), and 9 isolates (4.3%), respectively (Table 1). Consequently, 128 isolates (60.7%) carried *stx2* genes, alone or in a combination with other *stx* genes, and 83 strains (39.3%) possessed *stx2c* genes, but not *stx2* genes. No strain possessing only *stx1* gene was seen in the present study.

2.3.2 Relationship between *stx* genotypes of isolates and clinical manifestations in humans

The relationship between *stx* genotypes of isolates and clinical manifestations of the infected people is shown in Fig. 1A. HUS were caused by three *stx* genotypes (7/7 cases) —*stx1/stx2*, *stx2*, *stx2/stx2c*. Most of bloody diarrhea also caused these three genotype strains (46/49cases), and the other three bloody diarrhea cases (3/49cases) were caused by the *stx2c* genotype strains. One of these three *stx2c* genotype strains was a unique strain that showed a high Stx2 toxin titer (≧1024x) by RPLA, unlike the other *stx2c* genotype strains. In contrast, the people infected with *stx2c* genotype strains (71/74 strains), with the exception of the above three cases, and all of *stx1/stx2c* genotype strains (9/9 strains) presented mild symptoms such as watery diarrhea, stomachache, or were asymptomatic. The genotype caused mass outbreaks were *stx1/stx2* (5 cases) and *stx2* (2 cases) in Miyazaki in 2000-2008 (data not shown).

The relationship between age and clinical symptoms is also shown in Fig.1B, 1C and Table 3. The frequency of developing severe symptoms such as HUS or bloody diarrhea was higher at 46.4% in 0-9 year old patients than 9.6% in 10 or more year old patients. The frequency of asymptomatic carriers and non-bloody patients was higher at 90.4% in 10 or more year old patients than 53.6% in 0-9 year old patients (X^2 =36.2).

Taken together these findings indicated that people infected with *stx2*-carrying strains (*stx2, stx1/stx2* and *stx2/stx2c* genotypes) developed HUS or bloody diarrhea with higher frequency than people with *stx2c*-carrying strains without *stx2* (*stx2c* and *stx1/stx2c* genotype) (X^2 =36.9), and that young age of patient was a risk factor for developing severe symptoms.

2.3.3 Stx titers determined by RPLA in extracts of strains representing the five *stx* genotypes

The amount of Stx was determined by RPLA, and the relationship between *stx* genotype and their toxic activities was investigated. The RPLA Stx titers for the 64 representative isolates of the cases in 2000-2003 are shown in Table 4. Using Stx1 reagent, polymyxin B (an antibiotic that releases the *stxs* from the periplasmic space of bacteria and increases specific activity of toxin extracts)-untreated cultures of isolates carrying the *stx1* gene showed low Stx1-RPLA titers (8-64x), whereas all extracts of polymyxin B -treated cultures of them showed high titers (256-1024x).

Using Stx2 reagent, RPLA titers in strains carrying *stx2* without *stx2c* were significantly different from those carrying *stx2c* without *stx2*, as follows: isolates with *stx2* only genotypes (such as *stx2* and *stx1/stx2*) showed high Stx2 titers of ≧64-1024x. By contrast, isolates with *stx2c* only genotypes (such as *stx2c* and *stx1/stx2c*) showed low Stx2 RPLA titers of 1-32x, except one isolate with a remarkably high Stx2 RPLA titer of ≧1024x. The mean titer of Stx2c was about 1/32 of the mean titer of Stx2, if the *stx2c* strain with a remarkably high Stx2 RPLA titer was excluded from the analysis. Little difference was observed between polymyxin B-treated and -non-treated cultures.

2.3.4 Stx titers determined by Vero cell assay in extracts of strains representing the five *stx* genotypes

Vero cell assay which was a standard detection method for Stxs, was also performed to evaluate the relationship between pathogenicity and *stx* genotypes. The Stx activity measured by Vero cell assay for the 54 isolates of the cases in 2000-2003 is shown in Table 4. By this assay, extracts of the 31 *stx2c* genotype isolates had an average of eight-fold lower titer than did extracts of the two *stx2* genotype isolates (2^{10}–2^{15} *vs.* $2^{14}$and 2^{17}). When polymyxin B was not treated (Stx1 titers might be low, therefore, total toxin titer seems to be less affected by Stx1), the *stx1/stx2* and *stx1/stx2c* genotype strains showed the titers of 2^{15}–2^{19} and 2^{14}–2^{16}, respectively. In addition, among the five different *stx* genotypes, *stx2c* genotype was associated with the lowest cytotoxicity,

except for one isolate with a remarkably high titer. Thus, the Vero cell cytotoxicity (CD_{50}) varied depending on the Stx type (i.e. Stx1, Stx2, Stx2c).

On the other hand, a slight difference was observed between Stx extracts of polymyxin B-treated and -non-treated isolates, in the isolates belonging to genotypes carrying *stx1* gene, such as the *stx1/stx2* (2^{15}–2^{19} →2^{16}–2^{19}) and *stx1/stx2c* genotypes (2^{14}–2^{16}→2^{16}–2^{18}).

By comparing the RPLA titer with the Vero cell cytotoxicity titer, the Stx2 RPLA titer was shown to correlated well with the Vero cell cytotoxicity, and above all, when Stx1 level was low (which was the case when STEC cultures were not treated with polymyxin B) (r=0.82) (Fig.2).

2.3.5 The kinetics of *stx2* related mRNA expression and toxin production in three STEC O157 strains.

The kinetics of *stx2* related mRNA expression in three strains —a *stx2* strain, a *stx2c* strain, and a *stx1/stx2* strain—were compared to assess the difference of toxin production between *stx2c* and *stx2* (absolute quantification) (Fig. 3). These three strains were a *stx2* strain derived from a patient with bloody diarrhea in 2007, a *stx2c* strain derived from a healthy carrier in 2008, and a *stx1/stx2* strain derived from a patient with bloody diarrhea in 2007. Increases in the number of bacteria were similar between cultures, regardless of strain genotype, during the 24 h after inoculation. In contrast, *stx2* related mRNA expression varied among the strains. In the *stx2* and *stx1/stx2*

strains, mRNA expression reached a peak value 6 h after inoculation, but in the *stx2c* strain expression peaked 5 h after inoculation; expression in each strain did not increase thereafter. The peak value in the *stx2* and *stx1/stx2* strains were 4-fold and 8-fold greater, respectively, than that in the *stx2c* strain.

The Stx2-related toxin titer in each strain began to increase 4 h after bacterial inoculation and reached a peak 9 or more hours after inoculation. The peak toxin titers of the *stx2* and *stx1/stx2* strains were 256x, but that of the *stx2c* strain was 16x. The peak value of toxin titer for *stx2* and *stx1/stx2* strains was 16-fold greater than that of the *stx2c* strain.

2.3.6 Comparison of *stx2*-related mRNA expression and toxin production among STEC O157 isolates

The STEC O157 strains used in this examination (n=33) include 24 strains isolated during 2000-2008 and one strain in 2010 (this strain, No.1 in Fig.4, was not included in "bacterial strains described in the section of Material and methods", and this was added only for this test.) from human, and 8 strains isolated from cattle during 2008-2009. Expression of *stx2*-related mRNA in the 33 isolates, which represented five different genotypes, was measured by relative quantitative real-time PCR (Fig. 4). The mean value of *stx2*-related mRNA expression in isolates with a genotypes that include the *stx2* gene (i.e., *stx2*, *stx1/stx2*, *stx2/stx2c* strains) were not significantly different from one another, and the mean values of isolates with genotypes that included the *stx2c* gene, but

did not include *stx2*, (i.e., *stx2c* strains (except No. 29) and *stx1/stx2c* strains) were not also significantly different from one another. Interestingly, the isolates with genotypes that included *stx2* had significantly higher *stx2*-related mRNA expression than isolates with genotypes that included *stx2c*, but not *stx2* (t test, $p = 6.08E\text{-}08$); only one isolate, which had the *stx2c* genotype and a remarkably high level of *stx2*-related mRNA, did not fall in this pattern. The mean value of the toxin titer of the *stx2*-inclusive isolates was about 10-fold higher than that of strains with genotypes that included *stx2c*, but not *stx2*. For each genotype, mRNA expression in the isolates varied within a relatively narrow range, with one notable exception, the *stx2c* strain described previously. Furthermore, for each genotype, no difference in mRNA expression was evident between isolates of cattle origin and those of human origin.

Stx2-related toxin production in culture of the 33 isolates was also determined using RPLA (Fig. 4). Isolates with *stx2*-inclusive genotypes (i.e., *stx2*, *stx1/stx2*, and *stx2/stx2c* strains) had high Stx2 titers ranging from 32x to 1024x. In contrast, all isolates, but one, with genotypes that included *stx2c*, but not *stx2* (i.e., *stx2c* and *stx1/stx2c* strains) had low Stx2 titers that ranged from 1x to 32x. The mean titer of the *stx2*-inclusive strains was about 40-fold higher than the mean titer of the strains that included *stx2c*, but not *stx2*, if the *stx2c* strain with remarkably high *stx*-related mRNA expression and high toxin titer was excluded from the analysis.

The one exception was the *stx2c* strain that showed a remarkably high Stx2 RPLA titer and *stx2*-related mRNA expression described in the section of 2.3.3 and 2.3.4. Both

stx2c mRNA expression and the RPLA titer in this strain were significantly higher (about 100-fold and 1000-fold, respectively) than the mean values measured in other *stx2c* strains.

Interestingly, the *stx1/stx2c* strains had slightly lower titers than the *stx2c* strains.

2.3.7 DNA finger printing patterns of STEC O157 isolates by PFGE analysis

DNA fingerprint patterns by PFGE analysis for the 65 causative strains from 64 occurrences in 2000-2003 are shown in Fig. 5. We first used PFGE to analyze epidemiological relevancy, but then we found that PFGE identified clusters which were highly correlated with pathogenic genotype. The STEC O157 strains were divided into large four clusters designated A, B, C, and D, with one exception. The clusters bore some relationship to *stx* genotypes. Clusters A and C mainly comprised strains with *stx2c* genotype and Cluster D mainly comprised strains with *stx1/stx2* genotype. Cluster B comprised strains with various genotypes including *stx2*, *stx2/stx2c*, and *stx1/stx2c*. In this way, the PFGE pattern clusters were closely associated with *stx* genotype.

2.4 Discussion

In the present study, five *stx* genotypes (*stx2*, *stx2c*, *stx1/stx2*, *stx1/stx2c*, and *stx2/stx2c*) were detected in 211 isolates from Miyazaki Prefecture and the most common were *stx1/stx2* and *stx2c* (43.6% and 35.0%, respectively). The high frequent detections of

stx1/stx2 and *stx2c* strains seem to be the characteristics in some particular countries in the world (Table 5) (92,111,117). In Japan, a similar survey had been carried out in Osaka (111,117). *stx1/stx2* strains were detected at high frequency in both areas, but *stx2c* strains were detected more dominantly in Miyazaki than in Osaka. *stx2/stx2c* was prevalent in Finland and Argentina, unlike in Miyazaki (128,45,96).

The genotypes of causative strain in group and family outbreaks, and sporadic cases were *stx1/stx2*, *stx2*, *stx2/stx2c* and *stx2c*, whereas genotypes mainly found in healthy carries were *stx2c* and *stx1/stx2c*. In addition, people infected with *stx2*-carrying strains (*stx2*, *stx1/stx2* and *stx2/stx2c* genotypes) developed HUS or bloody diarrhea with higher frequency than people with *stx2c*-carrying strains without *stx2* gene (*stx2c* and *stx1/stx2c* genotypes) (X^2=36.9) (Fig.1). Then, of 9 people infected with the strains carrying the *stx1/stx2c* genotype, 7 people were asymptomatic and 2 people presented water diarrhea. Therefore, *stx1* seems to have lower virulence for humans. This finding was consistent with the reports by Boerlin *et al.* (18) and Siegler *et al.* (151). Accordingly, we confirmed that *stx* genotype was one of the important risk factors of disease severity, and further, that pathogenicity to humans was higher for the *stx2*-including genotype isolates than the *stx2c* genotype isolates, as supported by previous reports, Nishikawa *et al.* (117) and Friedlich *et al.* (50).

Friedlich *et al.* (50) have shown that *stx2c* is the only *stx2* variant associated with HUS, but the risk of developing HUS was significantly lower after infection with *stx2c*-bearing STEC strains than after infection with *stx2*-bearing STEC strains. Eklund

et al. and Friedrich *et al.* found that *stx2c*-bearing STEC infections can progress to HUS (45,50). Taken together, these results indicate that patients infected with *stx2c* genotype strains also are at some risk of developing severe symptoms.

RPLA is commonly used as a quantitative method to estimate the amount of toxin proteins. However, this method is based on antigen-antibody reaction. Therefore cross reaction is observed between Stx2 and Stx2c. Anti-Stx2 latex reagent reacts with Stx2c but it was 30-fold less sensitive to purified Stx2c proteins than those of Stx2 (76). Thus RPLA cannot determine the amount of each variant toxin precisely.

Vero cell assays are a well-known and standard method used to quantify Stxs. This study showed that the Vero cell cytotoxicity (CD50) varied depending on the Stx types. In the five different *stx* genotype strains (*stx2, stx2c, stx1/stx2, stx1/stx2c, and stx2/stx2c*), most of *stx2c* strains showed lower cytotoxicity on Vero cells than other four genotype strains. Lindgren *et al.* showed that purified Stx2vhb (approximately 5 pg/CD50), which has B subunit sequences that are identical to those of Stx2c, is approximately 100-fold less reactive than purified Stx2 (approximately 500 pg/CD50) on Vero cells; this difference is due to lower affinity of Stx2vhb for the glycolipid receptor Gb3 that is caused by one amino acid difference in the B subunits of Stx2vhb and Stx2 (93). Other researchers showed that the B subunit of Stx determined relative cytotoxic specificity, and therefore, the difference in cytotoxicity should be due to difference in either receptor affinity or specificity (123,139,177). Hence, in this study, the factor causing difference in cytotoxicity among different Stx type strains is likely to be Stx composition, but, in

addition, the amount of toxin can be nominated as one of the factors. However, it might be difficult to compare the amount of toxin production in strains that produce different type of toxins, due to difference in either receptor affinity or specificity caused by the composition of variants.

Here, *stx2*-related mRNA was quantified to compare the amount of Stx2-related toxins produced by O157 strains of different genotypes. *stx2*- and *stx2c*-specific mRNA can be quantified together using this method because a common part of *stx2* and *stx2c* mRNAs is used as a target sequence for real-time PCR (115). Based on measurements of *stx2*-related mRNA expressed in cultures of each of 33 O157 isolates, *stx2*-related mRNA expression in *stx2*-inclusive isolates (i.e., *stx2*, *stx1/stx2*, and *stx2/stx2c* strains) was significantly higher on average than that in isolates with genotypes that included *stx2c*, but not *stx2* (i.e., *stx2c* and *stx1/stx2c* strains). This finding strongly indicated that O157 strains with a genotype that included *stx2* produced more toxin than those with a genotype that included *stx2c*, but not *stx2*.

However, the *stx2*-related mRNA expression levels did not necessarily correlate with toxin titers by RPLA in the same *stx* genotype strains. These variations are possibly due to differences in mRNA half-life, translation efficiency, or differences in experimental conditions used for each of assays. However, the variations between mRNA and toxin production levels do not affect the conclusion drawn from these data.

Our results were consistent with those of Zhang *et al.* (184,186). They divided *E. coli* O157:H7 strains into three major genetic lineages, lineage (L) I (which includes strains

with the *stx2* gene), LI/II (which includes strains with the *stx2* or *stx2/stx2c* genes), and LII (which includes strains with the *stx2c* gene), and showed that LI and LI/II strains produced significantly higher levels of *stx2* and/or *stx2c* mRNA than LII strains (P= 8.83E−03 and P = 8.74E−04, respectively). Moreover, they showed that the LI strains produce significantly higher levels of *stx2* and/or *stx2c* mRNA than LI/II strains (P = 7.11E−03); they also showed that LI and LI/II strains are more frequently associated with human diseases than are LII strains. Their classification (lineage) seems to differ from our classification (*stx* genotype); however, both systems are highly dependent on the *stx2* genotype. Our results were thought to be comparable with their results. The strains with *stx2* inclusive genotypes clearly expressed more *stx2c* related mRNA (and also toxin) than the strains with genotypes that included *stx2c*, but not *stx2*. This difference must affect pathogenicity to humans; nevertheless, it is not currently known whether the levels of toxin production observed *in vitro* correspond to the amount of toxin produced *in vivo*. Thus, it was found that the amount of toxin production might be one of the factors that cause differences in pathogenicity among different genotype O157 strains .

From the above, *stx* genotype, clinical manifestations, *stx2* specific mRNA expression, and PFGE were well correlated with each other, and were also suggested to be correlated with pathogenecity, and therefore, determining the *stx* genotype of STEC O157 might be helpful in assessing the risk of development of severe symptoms.

Another interesting finding was the high levels of toxin production in a *stx2c* STEC O157 strain. In general, the *stx2c* O157 strains expressed less *stx2c* specific mRNA and

showed lower toxin titers. In this strain, both *stx2c* mRNA expression and the RPLA titer were significantly higher (about 100-fold and 1000-fold, respectively) than the mean values measured in other *stx2c* strains. The patient infected with this strain developed bloody diarrhea; therefore, this strain was apparently more pathogenic than other *stx2c* strains; but reason for the high level of toxin production is unknown. However, only one such strain was isolated; therefore, the probability of detecting such a strain is very low.

However, the pathogenicity of STEC for humans is complex and depends on the virulence characteristics of the infecting STEC strain and host factors (such as age), although Stxs are the major virulence factors. In this study, the data from the patient's ages and symptoms indicated that the young age of patient was a risk factor for developing severe symptoms. Accordingly, the risk for developing serious symptoms should be evaluated in consideration of various factors, such as *stx* genotype, *eae* gene, enterohemolysin, subtilase cytotoxin and age of host (45).

In conclusion, STEC O157 strains are the most important pathogens in human STEC infectious diseases, with regard to both severity of clinical symptoms and disease incidence. O157 strains that produce Stx2-related toxins are reported to be more commonly associated with serious diseases than isolates producing Stx1. Based on analysis of *stx* genotype, measurements of *stx2*-specific mRNA expression, clusters identified by PFGE using restriction enzyme *Xba* I, and clinical manifestations, we found that results of these four analyses were correlated well each other, and were also suggested to be correlated with pathogenesis. Therefore, *stx* genotype was one of the

important risk factors of disease severity and determining the *stx* genotype of the bacteria might be helpful in assessing the risk of developing severe symptoms in STEC O157 infection. In addition, these results indicated that *stx2c* genotype was less virulent than other *stx2* genotypes, as supported by previous reports (45,50).

We also found that measurement of *stx*-related mRNA expression accurately might reflect the toxin production level among STEC strains; furthermore, expression levels in *stx*-related mRNA could be used to assess pathogenicity in humans.

In this study, we found one *stx2c* strain that produced a remarkably high level of toxin, and the patient infected with this strain developed bloody diarrhea. Accordingly, caution is warranted in cases where infections are caused by *stx2c* O157 strains or *stx2* O157 strains.

2.5 Tables and figures

Table 1 STEC O157 isolates from human used in this study

A. *stx* genotypes and isolated years

Year	2000		2001		2002		2003		2004		2005		2006		2007		2008		Total	
stx genotype	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
stx2	0	(0)	0	(0)	0	(0)	1	(1)	1*	(1)	5	(1)	3	(3)	15	(4)	2	(1)	27*	(11)
stx1/stx2	3	(3)	5	(4)	1	(1)	17	(9)	1	(1)	12	(7)	26	(13)	17	(13)	10	(8)	92	(59)
stx2/stx2c	0	(0)	1	(1)	0	(0)	0	(0)	1*	(1)	2	(2)	2	(2)	2	(2)	1	(1)	9*	(9)
stx2c	20	(17)	13	(11)	6	(5)	5	(5)	1	(1)	5	(3)	9	(7)	6	(6)	9	(4)	74	(59)
stx1/stx2c	2	(2)	0	(0)	1	(1)	1	(1)	0	(0)	2	(1)	2	(2)	1	(1)	0	(0)	9	(8)
total	25	(22)	19	(16)	8	(7)	24	(16)	4	(4)	26	(14)	42	(27)	41	(26)	22	(14)	211	(146)

A: No.of STEC O157 isolates from persons.

B. No. of representative strains of STEC O157 from cases

*: One strain with *stx2* genotype and one strain with *stx2/stx2c* genotype were isolated from the same individual.

B. Origins

Human origin strains in 2000-2008	No.of strains
Total of O157 strains (People)	211 (210)
Causative strains (Cases)	146 (145)
(Sources)	
Group outbreaks	39 (8 cases)
Family outbreaks	**60 (26 cases)**
Sporadic cases	53 (52 cases)
Healthy carriers	59 (59 cases)

Cattle origin strains in 2008-2009	No.of strains
Total of O157 strains	8
(Sources)	
Fecal samples in slaughterhouse, 2007-2009	8

Table 2. Primers and probes for PCRs and real time PCR

A. PCR and RFLP

Primer pairs for indicated *stx* gene		Target gene	Size (bp) of PCR product	Reference
a. PCR for detection of all *stx* genes				
MK1	TTT ACG ATA GAC TTC TCG AC	*stx1* *stx2* *stx2c*	228	(75)
MK2	CAC ATA TAA ATT ATT TCG CTC	*stx2d* *stx2e* *stx2g*		
b. PCR to classify *stx* genes into *stx1*, *stx2*				
Stx1-F	GCA GTT CGT GGC AAG AGC G	*stx1*	505	(181)
Stx1-R	GCG TCG CCA GCG CAC TTG			
Stx2-F	AGA TAT CGA CCC CTC TTG AA	*stx2*	969	(181)
Stx2-R	GTC AAC CTT CAC TGT AAA TG			
G1-F	TAC GAT GAC GCC GGG AGA C	*stx2c* group	470	(112)
G1-R	GGC CAC TTT TAC TGT GAA TGT A			
G1'-F	ATT GTC ATC ATC AGG GGG CT	*stx2vhc* group	370	(112)
G1'-R	TAC TGG ACT TGA TTG TGA CTG			
G2-F	TAT ACG ATG ACA CCG GAA GAA G	*stx2d* group	302	(112)
G2-F	CCT GCG ATT CAG AAA AGC AGC			
G3-F	TTT ACT GTG GAT TTC TCT TTC GC	*stx2f* group	773	(112)
G3-R	TCA GTA AGA TCC TGA GGC TTG			
G4-F	CAG GAA GTT ATA TTT CCG TAG G	*stx2e* group	901	(112)
G4-R	GTA TTC TCT TCC TGA CAC CTT C			

B. PCR and subsequent RFLP analysis

Primer pairs for PCR		Predicted sizes of fragments produced				
a. PCR and RFLP for *stx2* variant		Target gene	Size (bp) of PCR product	by digestion with restriction enzyme: *Nci*I	*Rsa*I	*Hae*III	Reference
VT2-c	AAG AAG ATG TTT ATG GCG GT	*stx2*	285	285	216, 69	285	(168)
VT2-d	CAC GAA TCA GGT TAT GCC TC	*stx2c/stx2vha*	285	285	136, 80	161, 124	
		stx2vhb	285	159, 126	216, 69	161, 124	

b. PCR and RFLP for *stx2c*		Target gene	Size (bp) of PCR product	by digestion with restriction enzyme: *Eco*RV	Reference
stx2–F	GATGGCGGTCCATTATC	*stx2c*	529	329, 200	(40)
stx2-R	CGGTAGAAAGTATTTGTTG	*stx2vha*	529	529	

C. Real-time PCR

Primer pairs for indicated *stx* gene		Target gene	Size (bp) of PCR product	Reference
a. Real time PCR for *stx2*- related toxin				
vt2-F	GGG CAG TTA TTT TGC TGT GGA	*stx2* *stx2c*	107	(115)
vt2-R	GAA AGT ATT TGT TGC CGT ATT AAC GA			
vt2-Probe	ATG TCT ATC A GGC GCG TTT TGA CCA TCT T			
b. Real time PCR for tufA (internal standard)				
*mtufAq*F	CATGGTTGATGACGAAGAGCTG	*stx2* *stx2c*	200	(40)
*mtufAq*R	CACGCTCTGGTTCCGGAAT			(40)
tufA Probe	CGATCGTTCGTGGTTCTGCTCTGAA			(This study)

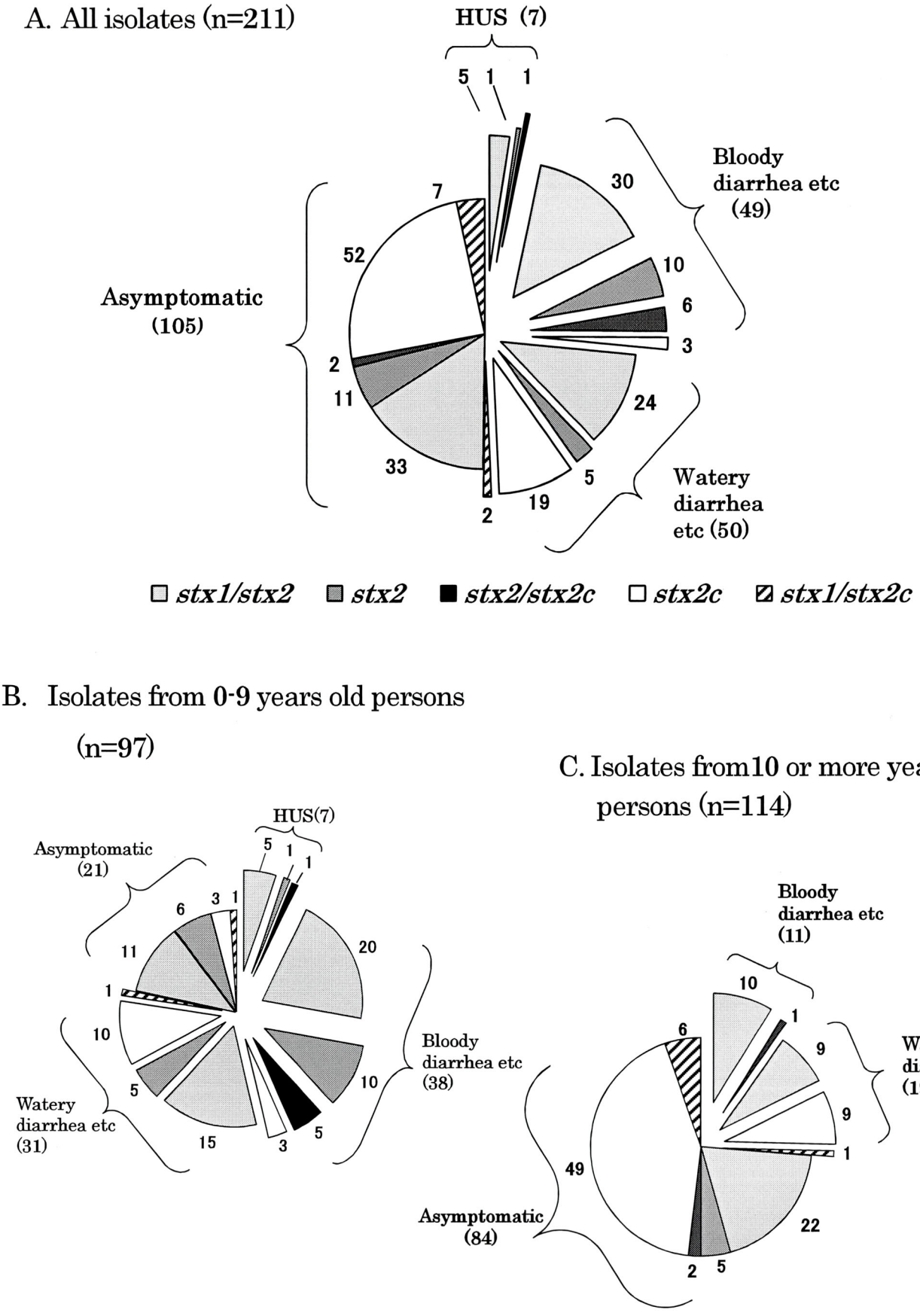


Fig.1 Relationship between *stx* genotype of STEC O157 strains and clinical manifestations in humans

Table 3. Relationship between *stx* genotype and clinical manifestations.

Age group	*stx* genotype	No. of strains	No. of people (%)				
			No.of people	HUS	Bloody diarrhea without HUS	Non-bloody diarrhea without HUS	Asymptomatic
0-9 years old	*stx2*	22	22* (100)	1 (4.5)	10 (45.5)	5 (22.7)	6 (27.3)
	stx2/stx2c	6	6* (100)	1 (16.7)	5 (83.3)	0 (0.0)	0 (0.0)
	stx1/stx2	51	51 (100)	5 (9.8)	20 (39.2)	15 (29.4)	11 (21.6)
	stx2c	16	16 (100)	0 (0.0)	3 (18.8)	10 (62.5)	3 (18.8)
	stx1/stx2c	2	2 (100)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)
	Total	97	97* (100)	7 (7.2)	38 (39.2)	31 (32.0)	21 (21.6)
10 or more years old	*stx2*	5	5 (100)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100)
	stx2/stx2c	3	3 (100)	0 (0.0)	1 (33.3)	0 (0.0)	2 (66.7)
	stx1/stx2	41	41 (100)	0 (0.0)	10 (24.4)	9 (22.0)	22 (53.7)
	stx2c	58	58 (100)	0 (0.0)	0 (0.0)	9 (15.5)	49 (84.5)
	stx1/stx2c	7	7 (100)	0 (0.0)	0 (0.0)	1 (14.3)	6 (85.7)
	Total	114	114 (100)	0 (0.0)	11 (9.6)	19 (16.7)	84 (73.7)
	Total	211	211 (100)	7 (3.3)	49 (23.2)	50 (23.7)	105 (49.8)

*: One strain with *stx2* genotype and one strain with *stx2/stx2c* genotype were isolated from the same individual.

Table 4 Relation between *stx* genotype and Stx activities by RPLA and Vero cell assay of extracts from STEC O157 isolates

stx genotype	No. of causative strains from 63 different outbreaks (%)	Stx activity titer (×) by					Stx activity titer (×) by		
		RPLA	RPLA for Stx1 (average)		RPLA for Stx2 (average)		Vero cell assay (average)		
		No. of strains tested	Polymyxin B treatment		Polymyxin B treatment		No. of strains tested	Polymyxin B treatment	
			Not done	Done	Not done	Done		Not done	Done
stx1/stx2	17	17	2^3-2^6 (2^5)	2^8-2^{10} (2^9)	2^6-2^9 (2^7)	2^6-2^{10} (2^8)	14	2^{15}-2^{19} (2^{17})	2^{16}-2^{19} (2^{18})
stx2	2*	2	$<2^0$	$<2^0$	2^5-2^{10} (2^8)	2^6-2^{10}≦ (2^8)	2	2^{14}-2^{17} (2^{16})	2^{14}-2^{17} (2^{16})
stx2/stx2c	2*	2	$<2^0$	$<2^0$	2^7-2^{10} (2^9)	2^{10}≦ (2^{10}≦)	2	2^{15}-2^{19} (2^{17})	2^{15}-2^{18} (2^{17})
stx2c	39	38	$<2^0$	$<2^0$	2^1-2^4 (2^3)	2^0-2^5 (2^3)	31	2^{10}-2^{15} (2^{13})	2^{10}-2^{15} (2^{13})
		1	$<2^0$	$<2^0$	≧2^{10}	≧2^{10}	1	2^{21}	2^{21}
stx1/stx2c	4	4	2^5 (2^5)	2^8-2^{10} (2^9)	2^1- 2^2 (2^2)	2^1-2^4 (2^2)	4	2^{14}- 2^{16} (2^{15})	2^{16}- 2^{18} (2^{17})
Total	64	64					54		

*: One strain with *stx2* genotype and one strain with *stx2/stx2c* genotype were isolated from the same individual.

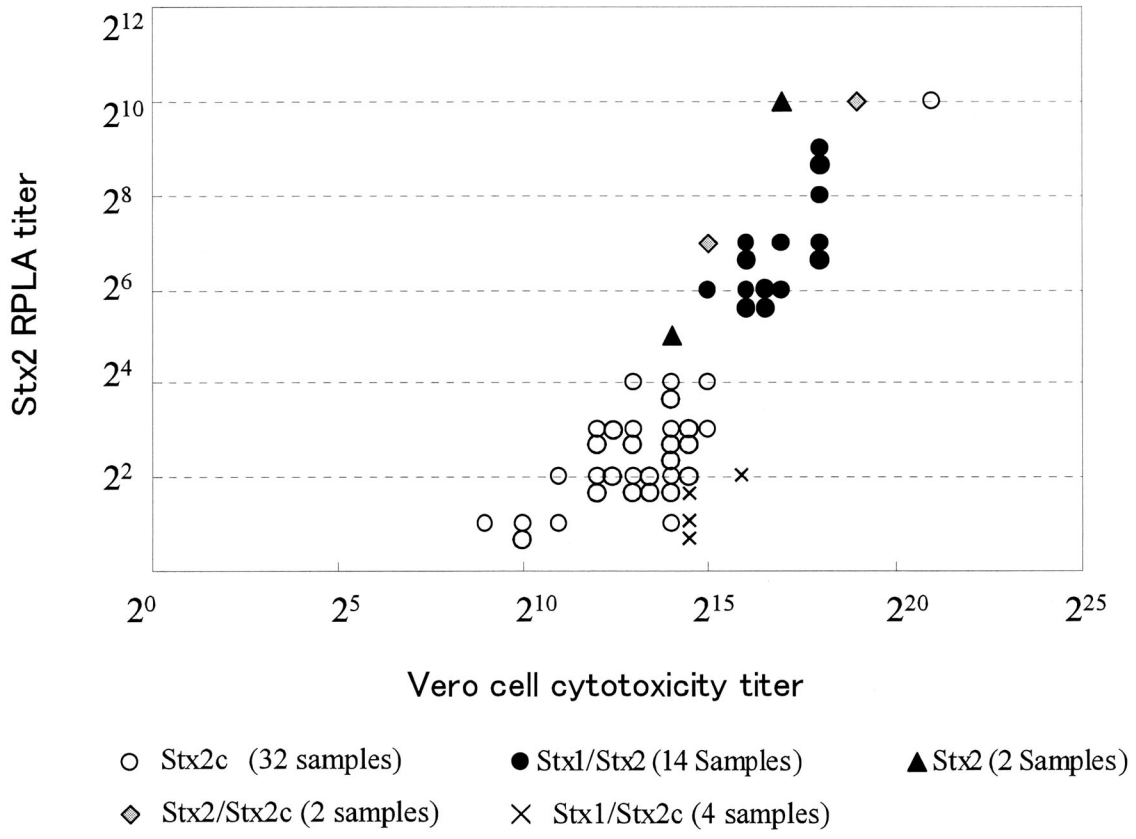


Fig. 2 Relation between Stx2 RPLA titer and Vero cell cytotoxicity of extracts from isolates not treated with polymyxin B.

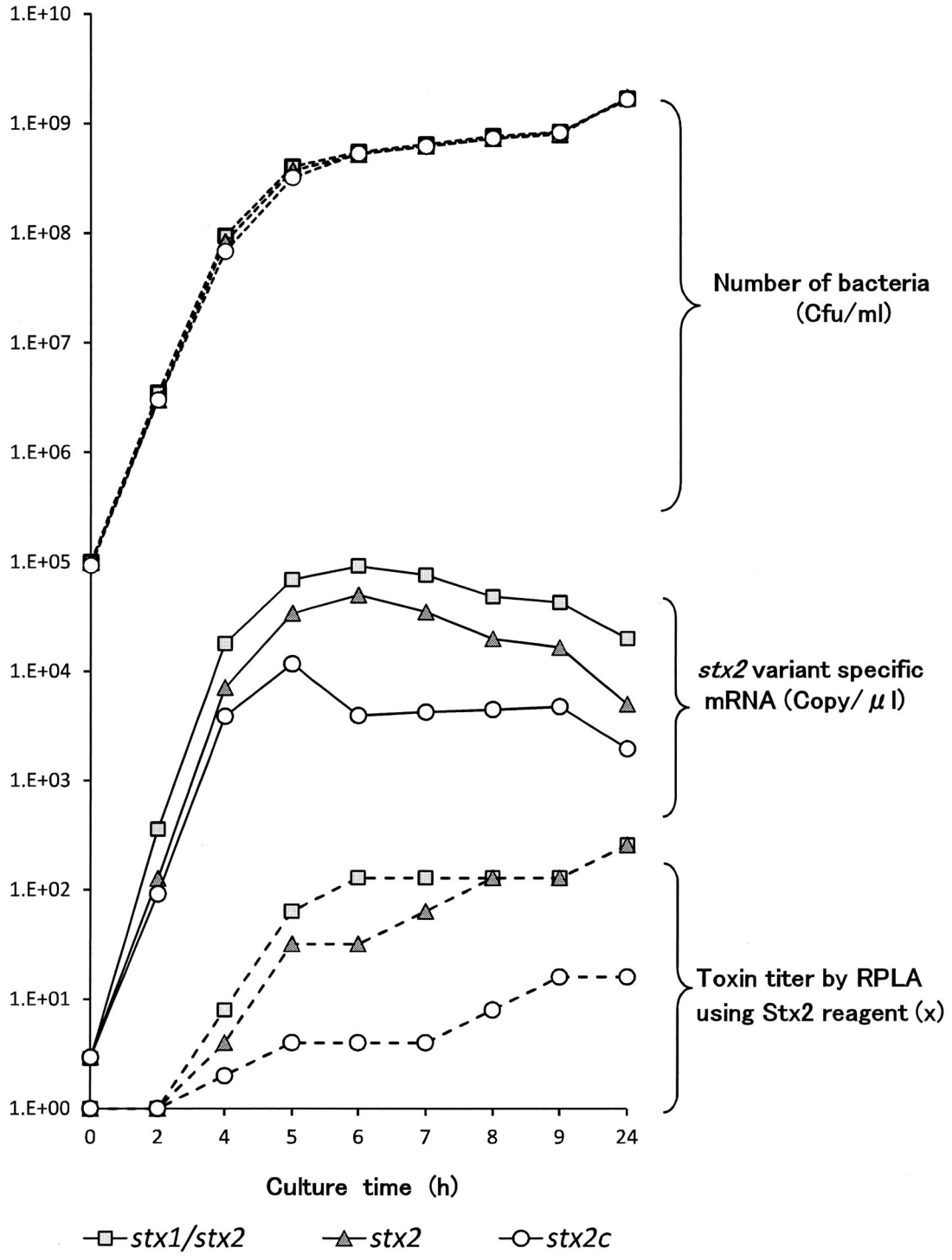


Fig.3. The kinetics of *stx2*-related mRNA expression and toxin production in STEC cultures. The small dotted line graphs show the number of bacteria. The line graphs show the amounts of *stx2* and *stx2c* mRNA measured by real-time PCR. The long dotted line graphs show the amounts of Stx2 and Stx2c measured by RPLA. Three bacterial strains are a *stx1/stx2*(□), a *stx2*(▲) and a *stx2c* genotype strain (○).

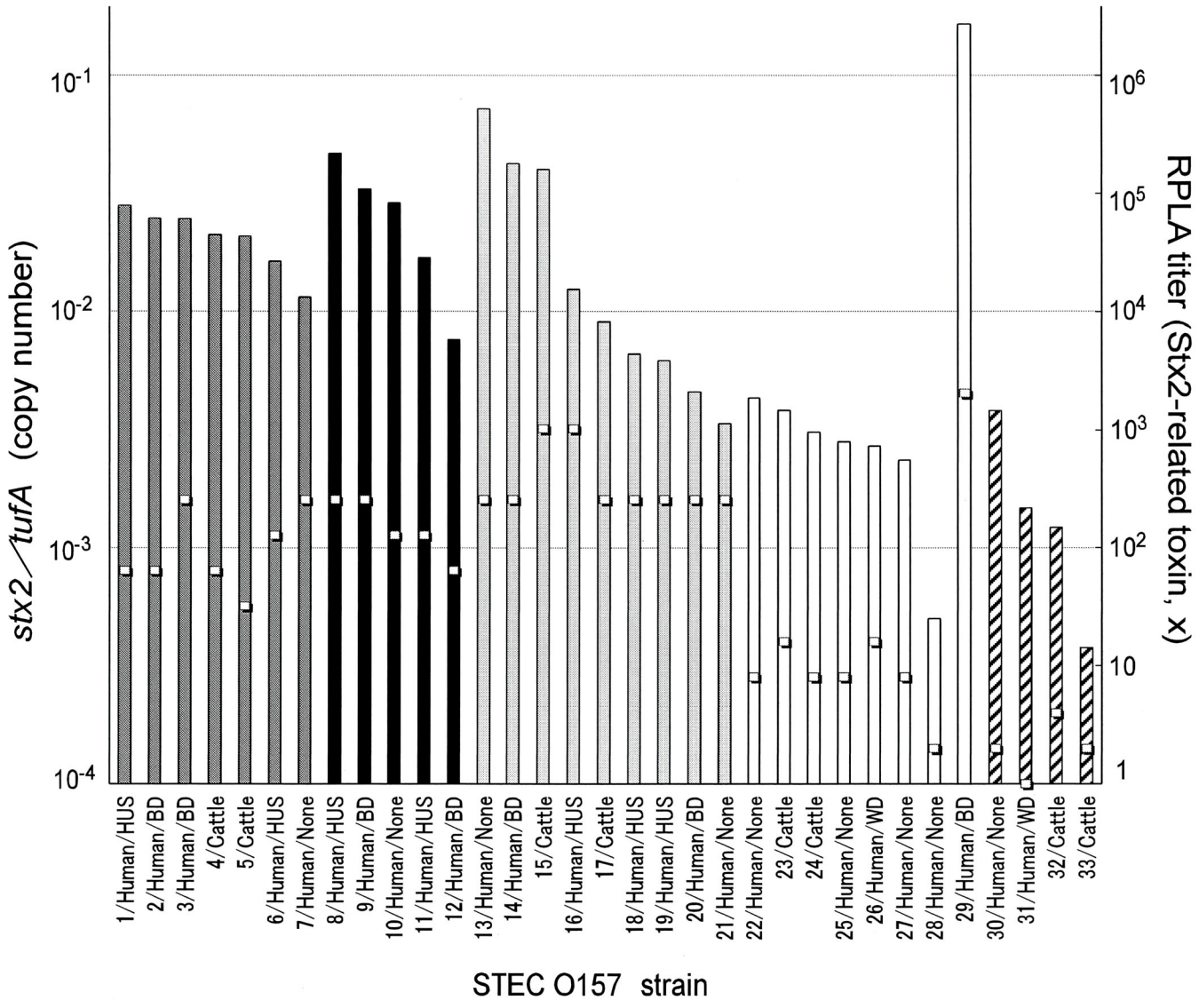


Fig. 4. Expressions of *stx2*-related mRNA and productions of Stx2 related toxins in 33 STEC isolates. Bar graphs show the relative amounts of *stx2* and *stx2c* mRNA to *tufA* mRNA measured by real-time PCR in bacterial cultures after 6 hour incubation of the *stx2* (▭), *stx2/stx2c* (▬), *stx1/stx2* (▭), *stx2c* (▭), and *stx1/stx2c* genotype strain (▨). The white square (□) indicates the Stx2 and Stx2 titers measured by RPLA in bacterial cultures after 24 hour incubation. HUS: Hemolytic uremic syndrome, BD: Bloody diarrhea, WD: Watery diarrhea, None: Asymptomatic

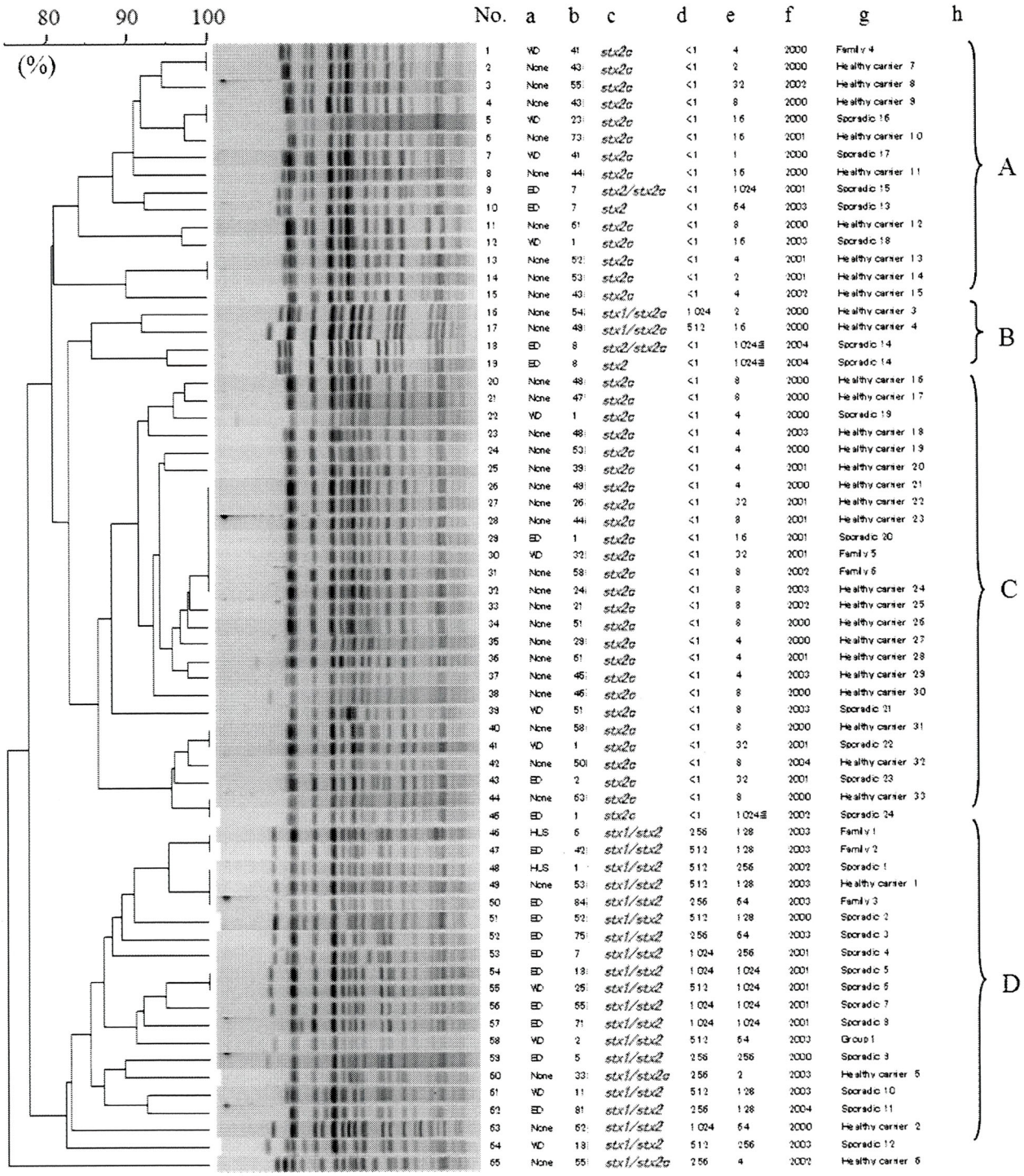


Fig. 5 Relation between clusters grouped on the basis of PFGE DNA fingerprints and the characteristics of 65 strains of STEC O157. a: Clinical manifestation (HUS, HUS; bloody diarrhea without HUS, BD; non-bloody watery diarrhea without HUS, WD; asymptomatic, None), b: Age, c: *stx* genotype, d: Stx1 RPLA titer (polymyxin B-treated), e: Stx2 RPLA titer (polymyxin B-treated), f: Year of isolation, g: Origin, h: PFGE cluster

Table 5 Comparison of *stx* genotypes of STEC O157 isolates derived from human in various area

stx genotype	No. of isolates (%)										
Country	Japan (Miyazaki)	Japan (Osaka)	Australia	Finland	Japan (Osaka)	Japan (Osaka)	Japan	Argentina	New Zealand	China	Belgium
Year	2000-2008	1996-1998	1993-1996	1990-2000	2000-2003	2000-2003	1995-2009	1993-1996	1993-1996	1999-2000	1987-1996
Subject	Patients, asymptomatic carrier	Patients, asymptomatic carrier	Patients, asymptomatic carrier	Patients, asymptomatic carrier	Asymptomatic carrier	Patients	patients, patient-linked persons	Patients	Patients	Patients	Unknown
stx1	0(0)	0(0)	2(10.0)	1(0.9)	0(0)	1(0.5)	2(2.6)	0(0)	0	ND ND	0(0)
stx2	27(12.8)	11(6.5)	3(15.0)	**21(18.9)**	3(4.2)	17(9.3)	8(10.3)	3(8.6)	**16(88.9)**	0(0)	16(16.8)
stx2c	**74(35.0)**	**25(14.9)**	1(5.0)	3(2.7)	**29(40.8)**	19(10.4)	6(7.7)	0(0)	2(11.1)	**22(73.3)**	**27(28.4)**
stx1/stx2	**92(43.6)**	**96(57.1)**	**6(30.0)**	1(0.9)	**27(38.0)**	**108(59.3)**	50(64.1)	0(0)	0(0)	**8(26.7)**	3(3.2)
stx1/stx2c	9(4.3)	11(6.5)	**4(20.0)**	5(4.5)	5(7.0)	12(6.6)	1(1.3)	0(0)	0(0)	0(0)	**20(21.1)**
stx2/stx2c	9(4.3)	23(13.7)	3(15.0)	**71(64.0)**	7(9.9)	**23(12.6)**	11(14.1)	**32(91.4)**	0(0)	0(0)	**28(29.5)**
stx1/stx2/stx2c	0(0)	2(1.2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(1.1)
stx2vhb	0(0)	0(0)	0(0)	3(2.7)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
stx2/stx2vhb	0(0)	0(0)	0(0)	1(0.9)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
stx1/stx2vhb	0(0)	0(0)	0(0)	2(1.8)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
stx1/stx2/stx2vhb	0(0)	0(0)	0(0)	1(0.9)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Others	0(0)	0(0)	1(5.0)	2(1.8)	0(0)	2(1.1)	0(0)	0(0)	0(0)	0(0)	0(0)
Total	211(100)	168(100)	20(100)	111(100)	71(100)	182(100)	78(100)	35(100)	18(100)	30(100)	95(100)
Referrence	This study	(117)	(92)	(45)	(111)	(111)	(89)	(92)	(92)	(187)	(128)

3. Epidemiological relationship between human and Cattle in STEC O157 infection

Molecular epidemiological analyses of STEC O157:H7/H- in human and cattle isolates

3.1 Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is an emerging pathogen of significant clinical and public health concern that causes various symptoms, such as diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS), in humans (143). Various factors influence the diversity of symptoms caused by STEC strains, and the Stx type (gene alleles are designated as *stx* genotypes) is the most important of these factors. The dominant serotype of STEC of human origin in Japan is O157, and many O157 strains have been reported to produce three distinct toxins, Stx1, Stx2, and Stx2c (40,168). In humans, Stx2-related toxins are more commonly associated with serious disease than are Stx1-related toxins (18, 80, 150, 151). Some reports indicate that the Stx type (*stx* genotype) is associated with the virulence of STEC (53), and further suggest that Stx2 is more virulent to humans than Stx2c (50, 80, 117).

Cattle are thought to be a major reservoir of STEC and often carry STEC in their intestinal flora (59). Humans are occasionally infected with STEC O157 through the ingestion of contaminated beef and related food products (120,138), or by direct contact with infected animals or with environmental materials, such as soil, water, vegetables, and farm equipment (109,173). Therefore, cattle infected with STEC might be one of a source of infection for humans; this also means that the *stx* genotypes present in cattle must affect the degree of seriousness of the human infectious disease (50, 80, 117).

In second chapter, we reported the *stx* genotype distribution of STEC O157 strains isolated from people in the Miyazaki area of Japan, where stockbreeding

is common; we showed that a strain carrying only *stx2c* was prevalent there, as well as a strain carrying *stx1/stx2* (80). Some studies have reported differences in the genotype and virulence of STEC O157 strains from human patients and cattle (4,89,116).

The purpose of this study was to survey the *stx* genotype distribution of STEC O157 strains isolated from cattle in the Miyazaki region, and to compare them with O157 strains from humans who are living in the same area. Furthermore, we evaluated molecular epidemiologically whether human O157 infections were actually caused by O157 strains originating in cattle. We performed two molecular epidemiological techniques, pulsed-field gel electrophoresis (PFGE) analysis (80) and insertion sequence (IS) printing analysis (124), to analyze O157 strains derived from humans and cattle.

3.2 Materials and methods

3.2.1 Bacterial Strains

STEC O157 strains (n=268) comprising 211 isolates from 210 human fecal samples collected in 2000-2008, and 57 isolates from 57 cattle samples collected in 1991-2009, were analyzed in the present study (Table1, 6). Human samples were derived from 145 occurrences from 2000 to 2008 in Miyazaki Prefecture, Japan; these occurrences comprised eight group outbreaks (n=39 isolates), 26 family outbreaks (n=60), 52 sporadic diarrhea cases (n=53), and 59 healthy carriers (n=59). Cattle samples were derived from 57 individual healthy animals; the

samples comprised 9 swab samples from carcass surfaces and 48 fecal samples collected in slaughterhouses and farms from 1991 to 2009 in Miyazaki (Table 6). All 211 strains were used for *stx* genotyping and quantitative analysis of Stxs using reversed passive latex agglutination (RPLA). The clinical manifestations in 210 STEC O157-infected people were also investigated.

Additionally, for genomic analysis using PFGE and O157 IS printing, a collection of 175 strains which comprised 118 isolates of human origin (the total of each representative isolate in 5 group cases, 23 family cases, 44 sporadic cases, 46 healthy carriers) and 57 isolates of cattle origin was used. .

3.2.2 Detection and typing of *stx* genes

PCR screening for detection of *stx* genes and typing of the *stx* genes were performed as described in the second chapter (40, 75, 81, 93, 112, 146).

3.2.3 PFGE

PFGE was performed according to the procedure described previously in the second chapter (81,102).

3.2.4 IS-printing analysis

IS-printing analysis was performed using a commercial multiple PCR-based typing method kit for STEC O157 strains based on the variable location of insertion sequence IS629 (IS-printing System, TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions (124).

3.2.5 Quantitative RPLA

STEC O157 bacteria were incubated at 37°C with shaking for 24 h in CAYE broth (Denka Seiken Co. Ltd., Tokyo, Japan), and treated with polymyxin B (final concentration of 5000 U/ml). Culture supernatants were used as the Stx extracts for RPLA for quantification of Stx1 and Stx2. RPLA was performed using a commercial RPLA kit (VTEC-RPLA, Denka Seiken) according to the manufacturer's instructions (76). The titers of RPLA for Stx1 and Stx2 were expressed as the reciprocal of the highest dilution (used "x" as a unit of dilution) that caused agglutination by the respective latex reagent.

3.3 Results

3.3.1 Frequency of *stx* genotypes of STEC O157 strains isolated from humans and cattle

The *stx* genotypes of 211 strains isolated from humans and those of 57 strains isolated from cattle are shown in Fig. 6. Both isolates were classified into five genotypes each, but the genotypes for each group were different. In human isolates, *stx1/stx2*, *stx2*, *stx2/stx2c*, *stx2c*, and *stx1/stx2c* were detected, and in cattle isolates, *stx1/stx2*, *stx2*, *stx2c*, *stx1/stx2c*, and *stx1* were detected.

In humans, *stx1/stx2* and *stx2c* genotypes were the most prevalent, accounting for 43.6% and 35.1% of the isolates, respectively, and *stx2*, *stx2/stx2c,* and *stx1/stx2c* genotypes accounted for 12.8, 4.3, and 4.3%, respectively, of all strains. In cattle, *stx2c* was the most prevalent genotype and accounted for 47.4% of

isolates. The other genotypes, *stx1/stx2*, *stx2*, *stx1/stx2c*, and *stx1*, accounted for 29.8, 8.8, 7.0, and 7.0%, respectively.

In addition, we confirmed that every O157 strain used produced Stx (i.e., a toxin titer by RPLA) which was corresponded to each *stx* genotype.

3.3.2 Molecular epidemiology

To investigate the potential epidemiological relationship between STEC O157 isolates derived from humans and those derived from cattle, a collection of 175 strains which comprised 118 isolates of human origin and 57 isolates of cattle origin was analyzed by PFGE. Furthermore, to investigate the epidemiological correlations with isolates from other areas in Japan, 16 publically available O157 PFGE patterns distributed widely throughout Japan during 2005-2008 were used. The publically available PFGE patterns are published on the internet at the National Institute of Infectious Diseases website, and the strains associated with these publically available PFGE patterns are likely to be *stx1/stx2* strains based on their PFGE patterns. However, the strain genotypes are not published on the website.

The isolates were divided into six groups based on genotype as follows and were analyzed: A, *stx1/stx2*; B, *stx2c*; C, *stx2*; D, *stx2/stx2c*; E, *stx1/stx2c*; and F, *stx1*. In several groups, there were some correlations among the patterns from human isolates, those from cattle isolates, and the publically available patterns. Strains that showed high homology (>95% identity by PFGE pattern) were analyzed further by IS printing to confirm the genetic similarity. The PFGE patterns could be organized into dendograms with clusters (strain groups which showed similar patterns with each other), sub-clusters (smaller clusters within a

strain cluster) marked by lower case letters, and sets (groupings within sub-clusters in which all strains share an identical PFGE pattern), as shown in Fig. 7-A-F.

A. *stx1/stx2* genotype strains

PFGE patterns of 64 *stx1/stx2* strains including 47 human isolates and 17 cattle isolates, and 16 publically available PFGE patterns, were compared. They were divided into 14 small clusters each of which consisted of 2 to 17 strains (Fig. 7A).

Six isolates with the same PFGE and IS printing patterns in the A-n sub-cluster were from cattle from the same farm. Among the six isolates, one strain was isolated first, and after about 80 days, five strains were isolated from five separate cattle on that same farm.

Notably, there was no human isolate with 100% identity to a cattle isolate by both PFGE and IS printing. In the A-f sub-cluster, two cattle isolates showed the 100% identity by both PFGE and IS printing, and they also had high homology (>95% identity by PFGE) with five human isolates.

In contrast, PFGE patterns from human isolates in four sets of each of the A-a, -b, -e, and -f sub-clusters, were very similar to publically available PFGE patterns from the strains collected throughout Japan. Specifically, the isolation year of the isolates in each set in the A-a and A-e sub-clusters was the same. However, we could not perform further comparisons between these strains by IS printing, because the strains associated with the publically available PFGE patterns were not available.

B. *stx2c* genotype strains

The PFGE patterns of 77 *stx2c* genotype strains, those of 50 human isolates and 27 cattle isolates, are shown in Fig. 7B. These patterns formed three large clusters and three smaller clusters. Each large cluster included isolates from cattle, patients, and healthy carriers.

Cattle strains in one set in the B-j sub-cluster were isolated at widely separated time points (1998 and 2008).

Comparing cattle isolates and human isolates, there were no strains with PFGE patterns and IS patterns with 100% identity, but 11 of 27 cattle isolates (41%) showed high homology (>95% identity by PFGE) with human isolates in each of the B-a, -c, -d, -f, -g, -h, -i, and -j sub-clusters as follows. The cattle isolates in 1998-2001 showed high homology with the human isolates in 2000-2008 in each of the B-a, -c, -d, -f and -j sub-clusters. The cattle isolate in 2007-2008 showed high homology with the human isolates in 2000-2003 and 2008 in each of the B-g, -h, -i and -j sub-clusters. In contrast, many strains isolated from cattle since 2007 seemed to show low homology to isolates from humans (B-n, -p, -q, -r, and -s sub-clusters).

C. *stx2* genotype strains

The PFGE patterns of 12 *stx2* strains (7 human isolates and 5 cattle isolates) are shown in Fig. 7C. There are no strains that both PFGE patterns and IS patterns accorded between human isolates and cattle isolates, but three strains in the C-a sub-cluster showed high homology (> 95% identity by both PFGE and IS printing) with each other.

D. *stx2/stx2c* genotype strains

A comparison of the PFGE patterns among six *stx2/stx2c* strains, all isolated from humans, is shown in Fig. 7D. Each overall PFGE pattern was unique.

E. *stx1/stx2c* genotype strains

A comparison of the PFGE patterns of 12 *stx1/stx2c* strains, including eight human isolates and four cattle isolates, is shown in Fig. 7E. No PFGE pattern from a human isolate had >95% identity to a pattern from a cattle isolate.

F. *stx1* genotype strains

No *stx1* strain was isolated from humans during this study. The PFGE patterns of four cattle isolates are shown in Fig. 7F. Each overall PFGE pattern was unique.

3.4 Discussion

Considering the relationship between *stx* genotypes and symptoms, our study in second chapter, suggested the following. STEC O157 strains carrying genotypes that include the *stx2c* gene without the *stx2* gene (i.e., *stx2c* or *stx1/stx2c* genotype) tend to show low pathogenicity; in contrast, strains with the *stx2* gene (i.e., *stx1/stx2*, *stx2*, or *stx2/stx2c* genotype) tend to show high pathogenicity in humans (80,50,117). Furthermore, most human infection cases involving *stx2c* genotype strains were asymptomatic (52 of 74 strains, 70%) or presented mild symptoms (19 of 74 strains, 26%), but 3 of 74 people infected by *stx2c* genotype strains (4%) developed serious symptoms such as bloody diarrhea,

and one strain derived from one of these three patients showed a very high toxin titer by RPLA, a high Vero cell cytotoxicity and a high expression of *stx2*-related mRNA (Fig.1, 2, 4, Table 4). Other researchers have reported that *stx2c* genotype strains can cause HUS (45,50). As described above, the *stx2c* genotype strain generally seems to be less virulent, but we must not ignore that it may cause severe symptoms. The report showed by Nakamura *et al.* are consistent with our data (111). They have shown that most isolates from patients with serious symptoms possess only the *stx2* gene, in contrast, most O157 strains isolated from bovines (possessing only the *stx2vha* (i.e., *stx2c*) gene) might not readily cause disease in humans. However, they noted that caution is necessary because *E. coli* strains carrying *stx2vha* (i.e., *stx2c*) were also isolated from patients with HUS.

Previously, we showed that the *stx2c* genotype was a common *stx* genotype of STEC O157 isolated from persons living in Miyazaki (80). Miyazaki is an area where the production of livestock for meat is very prolific and many cattle are bred. These cattle are a reservoir for STEC O157, and thus might be one of the important sources of infection to humans (2,58). In this study, among *stx* genotypes of STEC O157 isolates from humans, the *stx2c* genotype strain accounted for 35%. In cattle, the *stx2c* genotype was the most prevalent at 47% (27 of 57 strains). This prevalence in cattle isolates resembled findings from other areas in Japan, in China, and in Denmark (111,187,116). Taken together, the frequency of *stx2c* genotype strains isolated from both humans and cattle was significantly higher than other genotype strains in Miyazaki area.

Here, we investigated whether molecular epidemiologic correlations based on PFGE analysis and IS printing analysis between *stx2c* genotype strains from cattle and humans were evident (Fig. 7B). No strain isolated from humans showed

100% identity according to both PFGE patterns and IS printing patterns with any cattle isolates; therefore, we concluded that no cattle strains analyzed in this study were clear candidates for human infection.

However, 41% of the strains with *stx2c* genotypes isolated from 27 animals (11 of 27 strains) were highly homologous (>95% identity) as follows; the first, homology was recognized among some strains isolated from cattle (1998-2001) and from humans (2000-2008). The second, high homology was recognized in some strains isolated from cattle (2007-2008) and from humans (2000-2003 and 2008) significantly separated in time, as shown in Fig. 7B. These results suggested that strains showing those patterns had been preserved in cattle, people, or their surrounding environment continuously for more than several years, and there must have been some opportunities to transfer to humans directly or indirectly from them.

In addition, both PFGE and IS printing pattern of the strain isolated from cattle in 2008 accorded with those isolated from different cattle in 1998, although it is unknown whether the farms where the two cattle herds were bred is the same. This suggests that the strain represented by these patterns might have survived and was preserved in environments surrounding the cattle from 1998 to 2008.

As described above, we supposed that some STEC strains carrying *stx2c* could survive long-term in the gastrointestinal tracts of cattle or in the breeding environment, and might have an opportunity to infect people through various routes of infection. This is consistent with other reports (48, 91, 94), although none of them mention the *stx* genotypes of the bacteria. Ezawa *et al.* assumed that EHEC O157 did not remain in individual cattle for long periods, but contaminated farms long-term due to repeated infection (48). LeJeune *et al.* isolated EHEC

O157 exhibiting indistinguishable PFGE patterns from a feedlot over several years, and continued to isolate it from this and another feedlot in the same geographic area, suggesting that a certain type of strain might be adapted predominantly either to survival and persistence in the feedlot environment or to colonization of the bovine gastrointestinal tract (91). Other studies have shown that some clones can survive over a period of at least 17 months in the same farm or survive over a period of at least 7 months in the same animal, indicating that less prevalent clones may represent only transient episodes of contamination (94,29).

There are many reports that found direct and indirect transmission of STEC O157 strains from cattle to humans in infection cases by molecular epidemiological methods (58,98,120,138), but not so many reports that mentioned *stx* genotype of the causative O157 strain, even if a causative organism was a virulent genotype, such as *stx2*, *stx1/stx2*, and *stx2/stx2c*. Several cases of suspected infection from cattle by STEC (*stx2/stx2c* genotype) to humans were reported by Aspan *et al.* in Sweden (3). Whereas, there is no report demonstrating transmission of *stx2c* genotype O157 strains from cattle to humans by molecular epidemiological methods. In the present study, we compared *stx2c* genotype O157 strains derived from cattle with humans by PFGE and IS printing techniques, and found that there were some relations among them. Cattle seemed to be a possible source for the *stx2c* genotype strains as well as *stx2*-possessing genotype O157 strains.

The pathological effects of *stx2c* genotype strain seem to be mild or asymptomatic, and therefore, the detection of this genotype might be difficult in contrast to other genotype strains that cause severe symptoms in humans. In this

study, only food handlers and patients' families were used as healthy carriers; however, when a *stx2c* genotype O157 strain was detected randomly in such a healthy carrier, the same organism (clone) was occasionally detected in family members (data not shown). This suggests that *stx2c* genotype O157 strains may spread person-to-person without being noticed. Because surveys of STEC O157 in healthy persons are rarely performed in Japan, it is very hard to detect *stx2c* genotype bacterial infections from reservoirs, and might be difficult to find an expansion of cryptic infection in healthy carriers.

In contrast, among the strains isolated from cattle after 2007, some *stx2c* strains showed very low homology to isolates from humans and other cattle. This suggests that some STEC O157 strains might have been newly imported from other regions when, for example, calves were brought to local farms from other parts of the country, or occasionally from a foreign country, and raised over one or two years until they became adults, a cycle that is repeated every two or three years.

The genotypes that contain the *stx2* allele — *stx1/stx2, stx2*, and *stx2/stx2c* — might cause severe symptoms in humans, and these three genotype strains accounted for 61% of all isolates in total. In cattle, *stx1/stx2* and *stx2* isolates represented 39% of all isolates in total of both genotypes in the present study. The appearance of cattle that carry O157 strains with these genotypes at high frequency may affect the development of serious symptoms in human infections. The importation of calves suspected to be O157-contaminated from other areas every several years for breeding might be one cause for the increased frequency of these genotypes. Actually, six strains from cattle from the same farm sampled in 2008, including one strain isolated at first and five other strains isolated after

about 80 days, showed the same PFGE and IS printing patterns. These results suggested that the STEC O157 infection spread from the first animal to other animals and persisted on the farm. When we compared PFGE and IS printing patterns, the cattle and human origin isolates that showed high homology (>95% identity by PFGE and IS printing) with each other were recognized in one sub-cluster of *stx1/stx2* strains (Fig,7A-f) and the other one sub-cluster of *stx2* strains (Fig.7C-a). The finding suggests the possibility that these possibly high virulent genotype strains might transfer from cattle to human. However, on the whole, a close connection was not recognized so much between these genotype strains isolated from cattle and humans.

Comparing PFGE patterns of *stx1/stx2* genotypes of cattle isolates and human isolates, with publically available PFGE patterns from strains throughout Japan, showed that some patterns distributed throughout Japan correspond to patterns from isolates, from patients, and from healthy carriers in four sets (Fig. 7A; A-a, b, e, and f sub-clusters). These results suggested that some *stx1/stx2* strains might have spread throughout Japan via the circulation of meat and related food products.

In the present study, a survey of STEC O157 strains was carried out for cattle and humans, including healthy carriers who lived in the same geographic area, and *stx* genotypes of the isolated strains were determined by the molecular techniques. In both cattle and humans, *stx2c* genotype strains were isolated from healthy carriers frequently; however, this genotype was isolated from a few patients showing severe symptoms. This is the first report showing that *stx2c* genotype O157 is widely distributed in both cattle and humans in Miyazaki Prefecture, and that there is a correlation between these two as demonstrated by

molecular epidemiology.

In conclusion, STEC O157 strains are the most important pathogens in human STEC infectious diseases, both in terms of severity of clinical symptoms and frequency of incidence. Based on an analysis of the *stx* genotypes of 211 causative O157 strains and the symptoms of 210 infected people, the prevalent genotypes in isolates in this area were the *stx1/stx2* (44%) and *stx2c* genotypes (35%), and furthermore, strains with the *stx2, stx1/stx2,* or *stx2/stx2c* genotypes tended to be more virulent than strains with the *stx2c* or *stx1/stx2c* genotypes in humans. Cattle are clearly important as environmental reservoirs of STEC. The *stx2c* genotype was the most prevalent, accounting for 47% of all isolates derived from cattle in this area. PFGE and IS printing analysis showed that some genetic patterns of *stx2c* genotype strains are likely preserved in cattle or their surrounding environment for more than several years.

Generally, the virulence to humans of these *stx*2c strains is mild, and therefore most of the *stx*2c genotype strains would not readily contribute to serious disease or mass outbreaks. However, we should still be cautious with *stx2c* genotype strains because of the occasional occurrence of severe symptoms in human and the appearance of a high-toxin producing strain. Furthermore, it may be a public health concern that these strains have been transferred as infections from cattle to humans through various routes, and then spread person-to-person without being noticed. Accordingly, caution is warranted for infections by *stx2c* genotype O157 strains, in addition to *stx2*-possessing genotype O157 strains (81).

3.5 Tables and figures

Table6 STEC O157 strains used in this study

Human origin strains in 2000-2008	No.of strains (No. of people or cases)
Total of O157 strains (People)	211 (210)
Total of causative strains (Cases)	146 (145)
(Sources)	
Group outbreaks	39 (8 cases)
Family outbreaks	60 (26 cases)
Sporadic cases	53 (52 cases)
Healthy carriers	59 (59 cases)

Cattle origin strains in 1991-2009	No.of strains
Total of O157 strains	57
(Sources)	
Fecal samples in slaughterhouse, 1991	1
Fecal samples in slaughterhouse, 2007-2009	41
Fecal samples in farms,1998-2001	6
Swab samples in slaughterhouse, 1998-2000	9

Table 7 STEC O157 isolates from cattle used in this study

Year stx genotype	1991	1998-2001	2007	2008	2009	Total	(%)
stx1		1		2	1	4	(7.0%)
stx2				5		5	(8.8%)
stx1/stx2		4	4	9		17	(29.8%)
stx2/stx2c						0	(0.0%)
stx2c	1	8	2	12	4	27	(47.4%)
stx1/stx2c		2		1	1	4	(7.0%)
Total	1	15	6	29	6	57	(100%)

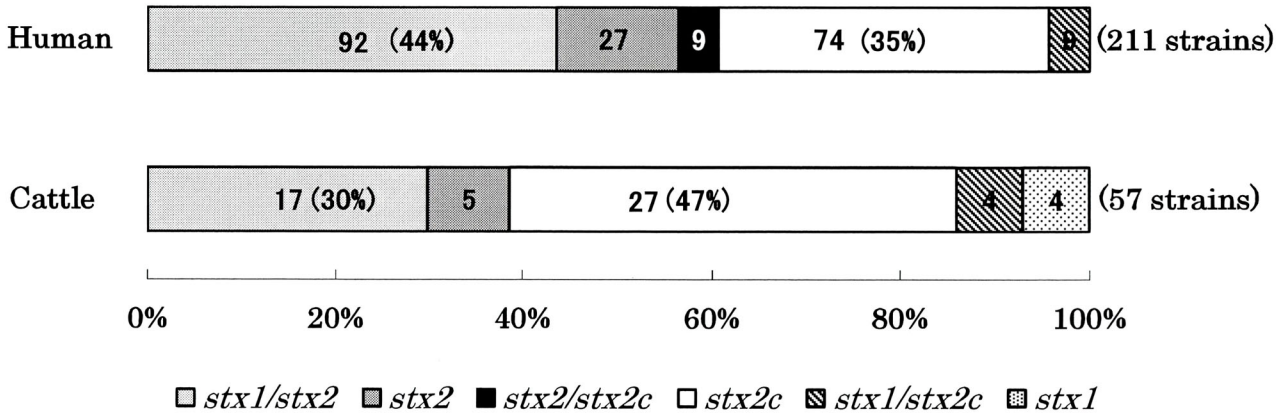


Fig.6 Frequency of *stx* genotypes in STEC O157 human and cattle islates

A. *stx1/stx2* genotype strains
n=64 strains (human:47,cattle:17)
and 16 patterns

B. *stx2c* genotype strains
n=77 (human:50,cattle:27)

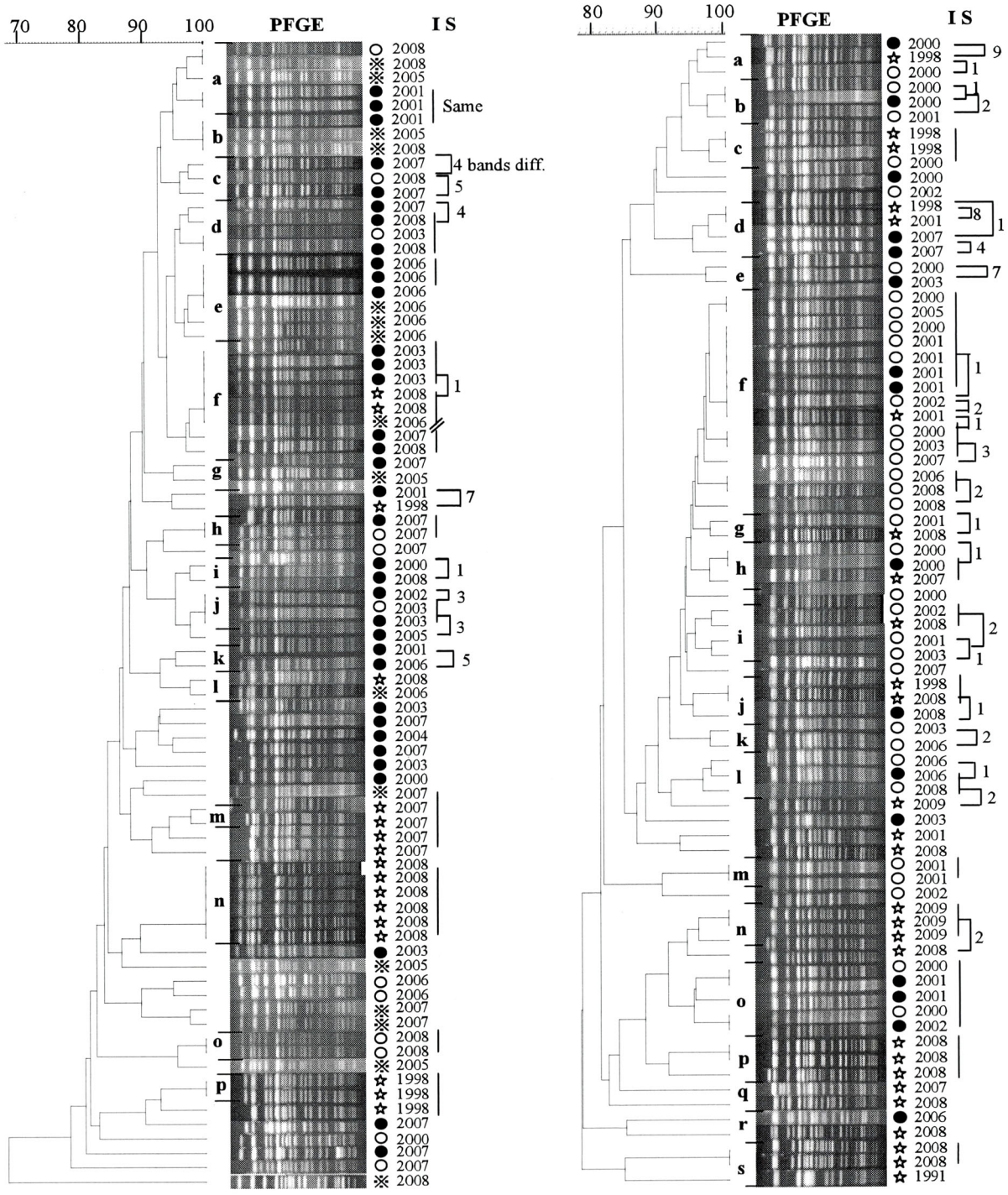
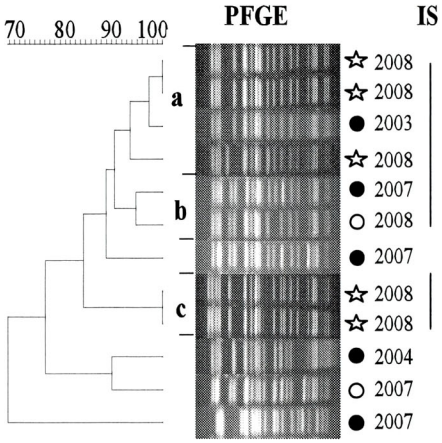


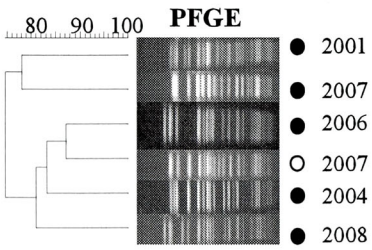
Fig.7—A,B Molecular epidemiologic correlation between STEC O157 isolates derived from human and cattle

○:A strain from a healthy carrier ●:A strain from a patient ★:A strain from cattle
※:A publically available PFGE pattern in Japan in 2005-2008

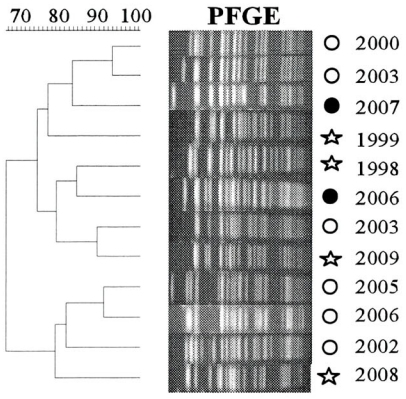
C. *stx2* genotype strains
n=12 (human:7,cattle:5)



D. *stx2/stx2c* genotype strains
n=6 (human:6)



E. *stx1/stx2c* genotype strains
n=12 (human:8,cattle:4)



F. *stx1* genotype strains
n=4 (cattle:4)

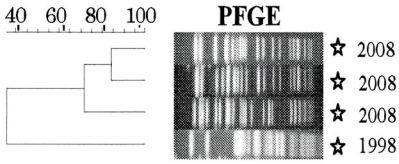


Fig.7—C,D,E,F Molecular epidemiologic correlation between STEC O157 isolates derived from human and cattle

○:A strain from a healthy carrier ●:A strain from a patient ★:A strain from cattle
※:A publically available PFGE pattern in Japan in 2005-2008

4. Conclusion

The aim of this study is to clarify the factors affecting pathogenicity of STEC O157 strains for a better understanding of diversity of symptoms including severe symptoms such as HUS and encephalitis (first research). In addition, we also aimed to clarify the epidemiological relationship between STEC O157 in the cattle population which had been prolific and STEC O157 in human population (second research).

In the first research, the 211 strains isolated from humans were found to carry the following *stx* genes (*stx* genotype): *stx1* and *stx2* (*stx1/stx2,* 92 strains, 43.6%), *stx2c* alone (*stx2c,* 74 strains, 35.1%), *stx2* alone (*stx2,* 27 strains, 12.8%), *stx1* and *stx2c* (*stx1/stx2c,* 9 strains, 4.3%), and *stx2* and *stx2c* (*stx2/stx2c,* 9 strains, 4.3%). No strain carried the *stx1* gene alone. The high frequent isolation of *stx1/stx2* and *stx2c* genotypes seemed to be the characteristic in some particular countries in the world. In Japan, a similar survey had been carried out in Osaka. *stx1/stx2* strains were detected at high frequency in both areas, but *stx2c* strains were detected more dominantly in Miyazaki than in Osaka. *stx2/stx2c* was prevalent in Finland and Argentina, unlike in Miyazaki (128,45,96).

Based on analysis of *stx* genotype and clinical manifestations, *stx2*-related toxins seemed to be more virulent than *stx1*-related toxins, as supported by previous reports (18,151). Generally, the virulence of the strains carrying *stx2c* but not *stx2* was mild, and therefore most of these strains would not readily contribute to serious disease. Since 2000

to 2008, there have been no mass outbreaks caused by *stx2c* strains in Miyazaki. Meanwhile, we isolated an unique *stx2c* strain from a patient who developed bloody diarrhea. The isolate was capable of developing cytotoxicity for cultured cells as a consequence of producing remarkably a large amount of toxin. These results indicate that a caution is warranted for infections caused by *stx2c* O157 strains, in addition to O157 strains with genotypes that included *stx2* gene.

By the dendrogram of genomic DNA by PFGE using restriction enzyme *Xba*I, we newly found that clusters identified by *Xba*I-PFGE, *stx* genotypes and clinical manifestations (pathogenesis of the bacteria) were correlated well each other.

RPLA and Vero cell assays might not measure the precise amount of each toxin variant, due to difference in either receptor affinity or specificity caused by difference in the composition of variants. In contrast, real-time PCR which used a common part of *stx2* and *stx2c* mRNAs as a target sequence can quantify both *stx2*- and *stx2c*-specific mRNA, and can conjecture toxin production levels from *stx*-related mRNA expressions. Based on measurements of *stx2*-related mRNA expressed in cultures of each of 33 O157 isolates, *stx2*-related mRNA expression in *stx2*-inclusive isolates (i.e., *stx2*, *stx1/stx2*, and *stx2/stx2c* strains) was significantly higher on average than that in isolates with genotypes that included *stx2c*, but not *stx2* (i.e., *stx2c* and *stx1/stx2c* strains) ($p<0.01$). This finding strongly indicated that O157 strains with a genotype that included *stx2* produced more toxin than those with a genotype that included *stx2c*, but not *stx2*.

From the above, we have found that severity of symptoms in human STEC O157

infections is closely related to *stx* genotypes, and that one reason to cause severe symptom is the amount of toxin produced by each *stx* genotype strain.

In the second research, the 57 cattle isolates carried *stx1/stx2* (17 strains, 29.8%), *stx2* (5 strains, 8.8%), *stx2c* (27 strains, 47.4%), *stx1/stx2c* (4 strains, 7.0%) and *stx1* genotypes (4 strains, 7.0%). No strain carried the *stx2/stx2c* genotype strain. *stx2c* was the most prevalent genotype in isolates derived from cattle.

PFGE and IS printing analysis of 57 cattle isolates and 118 representative isolates of human origin cases showed the possibility that some genetic patterns of *stx2c* genotype strains had been preserved in cattle or their surrounding environment for more than several years, and that the strains carrying those patterns were transmitted to humans, although the time and the route were unknown. Whereas, 4 PFGE patterns of *stx1/stx2* genotype strains isolated from human were very similar to each publically available PFGE patterns from the strains collected throughout Japan, 2005-2008, respectively. These findings indicate the possibility of spreading of *stx1/stx2* genotype throughout Japan via the distribution of meat and related food products.

Sum up my main points in conclusion, this study demonstrated that severity of symptoms is closely related to *stx* genotypes in human STEC O157 infections, and that the severity of the disease might be regulated by the amount of toxin produced by each *stx* genotype strain, although the amount of toxin might be one of several factors for developing severe symptoms. Accordingly, the determination of the *stx* genotype is helpful to assess the risk of developing severe symptoms in STEC O157 infection.

Cattle is thought to be a major reservoir of STEC. In Miyazaki area, stockbreeding is prolific and many cattle have been breeding, and the *stx2c* genotype was the most prevalent type in both cattle O157 isolates and human O157 isolates. Results of PFGE and IS printing analysis suggested that some strains carrying *stx2c* had been likely preserved in cattle or their surrounding environment for a long period, and during these periods, they might have opportunities for transmission to people through some kind of routes. Direct or indirect transmissions to human from cattle and/or its surrounding environment are suggested, as well as transmission to human from contaminated food (fresh meat of cattle). It may be a public health concern and caution is warranted.

5. Acknowledgments

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学 位 論 文 要 旨

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題　　目：志賀毒素産生性大腸菌 O157 の病原性およびヒト・牛分離株間の疫学的関連に関する研究

Studies on the virulence of Shiga toxin-producing *Escherichia coli* O157 and on the epidemiologic relationship between human and cattle isolates

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志賀毒素(Stx)産生性大腸菌（STEC）O157 が産生する志賀毒素（Stx）の型（Stx 型, 遺伝子は *stx* genotype）は、感染者の重症化に関連する要因の一つと考えられる。本研究では、まず Stx 型と病原性との関連を明らかにする目的で、2000 年~2008 年に宮崎県で発生した 145 事例の感染者から分離した STEC O157 211 株の *stx* genotype を決定し、これらの *stx* genotype と、症状、菌の遺伝子パターン（パルスフィールドゲル電気泳動解析:PFGE、IS-printing analysis:IS)、毒素産生量（逆受身ラテックス凝集試験:RPLA、Vero 細胞法)、および *stx* 特異的 mRNA 発現量との関連を調べた。次に、STEC O157 の牛分離株とヒト分離株の疫学的関連性についても調べた。

感染者から分離した 211 株の *stx* genotype は、*stx1/stx2* が 43.6%、*stx2c* が 35.8%、*stx2* が 12.8%、*stx2/stx2c* および *stx1/stx2c* が 4.3%の 5 type に分類された。*stx1/stx2* 株や *stx2c* 株が高頻度で分離されるのは一部の国の特徴と思われた。日本では、大阪で同様の調査が行われており、*stx1/stx2* 株は両地方で高頻度に分離されたが、*stx2c* 株は宮崎の方が高い頻度で分離された。

stx genotype と感染者の症状との比較結果から，*stx2* 関連の毒素が *stx1* 関連の毒素に比べヒトへの病原性が強いこと、さらに *stx2* 保有株(*stx2, stx1/stx2, stx2/stx2c*)は非保有株（*stx2c*，*stx1/stx2c*)より血便や溶血性尿毒症症候群(HUS)などの重篤な症状を呈する頻度が高いことが判明した。また *stx2c* 株に感染した 74 名のうち 3 名は血便を起こしたが、感染者のほとんどは、無症状又は軽い下痢症状の後、回復した。しかし、血便患者の一人から高い毒素活性を示す *stx2c* 株が分離され、*stx2c* 株であっても高い毒素産生性を有する場合があり、*stx2* 保有強毒株同様、注意が必要であると考えられた。

O157 分離株の PFGE による遺伝子パターンのデンドログラムにおいて、そのクラスターは *stx* genotype と関連が見られた。従って PFGE のクラスターは症状（病原性）とも関連すると考えられた。

RPLA およびベロ細胞法で各株の毒素産生量を調べたところ、*stx2* 保有株は非保有株より産生量が多いことが分かった。しかしこれらの方法による Stx2 と Stx2c の測定値は、毒素の構造の違いによるレセプターへの特異性又は親和性の違いが測定反応に影響している可能性も考えられ、正確な毒素量を反映していることが確認できない。そこで *stx2* と *stx2c* の共通配列部分を標的にしたリアルタイム PCR により *stx2* 関連毒素特異的 mRNA の発現量を測定し Stx2 関連毒素の産生量を推定した。mRNA の発現量は *stx2* 保有株の方が非保有株より有意に高く（$p<0.01$)、すなわち、前者は後者より毒素産生量が多いと推定された。

以上のことから、症状の重症化はSTECの保有する *stx* genotypeと密接に関連し,その一因は毒素産生量であることが示された。

宮崎県は畜産業が盛んで多くの牛が飼育されている。また牛がO157を保有していることはよく知られている。そこで本県在住の患者から分離された株と本県で飼育されている牛由来のO157株の疫学的関連性を調べた。まず、宮崎県の食肉検査所に出荷された牛のと体拭き取りおよび便から57株を得た。各株の *stx* genotypeを調べたところ、*stx2c*が47%(27株)、*stx1/stx2*が30%(17株)、残る13株は *stx2*(5株), *stx1/stx2c*(4株), および *stx1*(4株)で、*stx2c* genotypeが最も高率に検出された。

これらの牛分離株57株と、ヒト分離株211株のうち疫学的関連性を有しない118株をPFGEおよびISにより遺伝子解析した結果、牛分離 *stx2c*株の27株中11株が、ヒト分離 *stx2c*株と95%以上の相同性を示した。このことから、*stx2c*株は、牛またはその環境で長期間保持され、時期や経路は不明ながらヒトに感染したものと推定される。一方、*stx1/stx2*株のなかにもヒト株と牛株で95%以上の相同性が認められるものがあったがそれは1パターンのみであった。また興味あることに、2005年~2008年に我が国の広域で分離された株のPFGEパターンのうち4パターンが、宮崎在住のヒト分離株のそれと一致した。このことから、*stx1/stx2*保有株は、食品などの流通により日本の各地域に広がっている可能性が考えられた。

以上をまとめると、本研究により、ヒトのSTEC O157感染症において、症状の重症化はSTECの保有する *stx* genotypeと密接に関連し、その一因は毒素産生量であることが示された。従って、*stx* genotypeを同定することは，予後を判断するための有用な手段になると考えられる。

畜産の盛んな本地方において、牛由来株のなかで最も優勢な *stx2* genotype株が、ヒト由来株においても優勢であることが判明した。また、分子疫学的解析により、STECが、牛またはその環境で長期間保持され、時期や経路は不明ながらヒトに感染したと推測される例が、特に *stx2c* genotype株で認められた。従って、畜産が盛んな地方においては、食品からの感染やヒト-ヒト感染の他に、牛やその周囲の環境からの直接的、間接的感染も考えられ、公衆衛生上注意する必要がある。