

Nationwide investigation of Shiga toxin-producing *Escherichia coli* among cattle in Japan revealed the risk factors and potentially virulent subgroups

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Received 9 September 2016; Final revision 10 January 2017; Accepted 9 February 2017;
first published online 6 March 2017

SUMMARY

A nationwide study of Shiga toxin-producing *Escherichia coli* (STEC) was performed to determine the prevalence, characteristics and risk factors for fecal shedding of STEC among cattle in Japan. Information on rearing practices was also collected to identify risk factors for fecal shedding of STEC. STEC was isolated from 24·1% of samples (133/551) collected from 59·1% of farms (65/110). Bayesian clustering using the virulence marker profiles of the isolates subdivided the isolates into four genetically distinct groups, two of which corresponded to *eae*- or *saa*-positive STEC, which can cause severe disease in human. Both STEC groups exhibited characteristic phylogeny and virulence marker profiles. It is noteworthy that the tellurite resistance gene was not detected in all *saa*-positive STEC isolates, suggesting that the standard isolation method using tellurite might lead to an underestimation of the prevalence of *saa*-positive STEC. A multivariate logistic regression model using epidemiological information revealed a significantly ($P < 0\cdot01$) high odds ratio on STEC fecal shedding in tie-stall housing and a low odds ratio in flat feed box and mechanical ventilation. Information on isolate characteristics of the two major pathotypes and risk factors in rearing practices will facilitate the development of preventative measures for STEC fecal shedding from cattle.

Key words: Cattle, Japan, prevalence, Shiga toxin-producing *E. coli*, virulence factor.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is an important cause of foodborne disease and causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans [1]. Large outbreaks of

STEC are often linked to limited O serogroups, such as O157 and O26, and are caused by the consumption of contaminated food or water. By contrast, direct contact with cattle is important as a cause of sporadic cases [2].

Cattle are the principal reservoir of STEC. STEC prevalence in cattle ranges from 0·4 to 74·0% [3, 4]. This variation would be attributable to differences in farm environments and isolation methods. Temporal factors also contribute to fecal shedding of the bacterium [5]. Identifying risk factors that affect the prevalence of STEC can lead to developing intervention strategies for decreasing the fecal shedding. In other

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countries, various risk factors have been identified, including age of cattle, drinking water and feed ingredients [6–8]. However, in Japan, nationwide studies of the prevalence of STEC and associated rearing practices to identify risk factors for fecal shedding are lacking with only surveillance reports on the prevalence of a limited number of serogroups and regions being available [9, 10]. Therefore, the current study attempted to identify the risk factors for developing the intervention strategies.

As well as the prevalence, the strain characteristics are important. The principal virulence factor of STEC is Shiga toxin (Stx), which plays an important role in developing bloody diarrhea and HUS [11]. STEC secretes Stx1, Stx2 or both, and there are multiple Stx subtypes [11, 12]. Stx1a, Stx2a, Stx2c, and Stx2d are frequently detected from the human clinical isolates. However, the role in pathogenicity remains unclear. In addition to Stx, certain characteristics of STEC isolates are important for the development of vaccines, as well as elucidating its pathogenicity. Effector proteins secreted by a type III secretion system in STEC are essential for attachment to the intestinal epithelium in the initial stage of infection and thus are used in the vaccine against STEC O157 [13]. The type III secretion system is encoded by a genomic island known as the locus of enterocyte and effacement (LEE) region. This secretion system is responsible for the development of the characteristic attaching/effacing (AE) lesion, and STEC harboring this lesion is called AE-STECS [14]. In addition to AE-STECS, STEC can be divided into several other pathotypes according to their adhesins: Agg-STECS (which produces aggregative adherence fimbria, i.e., AAF/Hda adhesins) and Saa-STECS (which produces STEC auto-agglutinating adhesion, i.e., Saa) [14]. Most of severe cases in humans are attributable to these pathotypes. In addition, *E. coli* immunoglobulin-binding (Eib) protein was identified as an adhesin in LEE-negative STEC isolated from HUS patients [15]. Because STEC carrying more than two of these adhesins has not been observed, these pathotypes would be phylogenetically distinct. The potential relationships between phylogeny and various virulence factors are of great interest because adhesins themselves and other related markers can be potential targets for vaccine development. However, the relationships among the pathotypes, phylogeny and various virulence markers of STEC [16–18] in cattle isolates have not been fully elucidated.

In this study, we performed a nationwide investigation of the prevalence of STEC and rearing practices

in cattle farms in Japan for the following purposes: (i) to characterize virulence factors and phylogenies of STEC isolates among cattle in Japan, and (ii) to identify the risk factors for fecal shedding based on the rearing practices of farms. This information will facilitate the development of techniques to reduce fecal shedding of important STEC isolates from cattle.

METHODS

Sample collection

Japan consists of 47 prefectures located in eight districts. Cattle feces were collected from 110 farms in 15 prefectures, which were selected randomly, located in seven districts in Japan from May 2013 to February 2014 by veterinarians at local livestock hygiene service centers. One-third of domestic cattle population in Japan are kept in these 15 prefectures. Two to 15 farms from each prefecture were selected by convenience sampling and each farm was selected not to be close to other sampling farms. Because the reported STEC prevalence in cattle ranges from 0.4 to 74.0% [3, 4], we assumed that the prevalence of STEC in each farm was 50% in this study. Under this assumption, the sample size required to detect at least one case of STEC in each farm with a probability of 95% was four or five [19]. Therefore, five or six fecal samples were collected from each farm. In total, 551 fecal samples were collected. During sampling, a questionnaire (Table S1) was used to collect information about the farms and cattle for epidemiological analyses as described below.

STEC isolation

Because a previous study suggested that vancomycin and cefsulodin do not suppress the growth of STEC belonging to various serogroups [20], these antibiotics were added to the enrichment medium. Nine milliliters of mEC broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) containing 10 mg/L vancomycin (Wako Pure Chemical Industries, Osaka, Japan) and 3 mg/L cefsulodin (Sigma Aldrich, St. Louis, Missouri, USA) was added to one gram of a fecal sample and incubated for 20 h at 42 °C. One milliliter of the culture was subjected to DNA extraction by an alkaline boiling method as described by Beige *et al.* [21]. Using this template DNA, *stx*₁, *stx*₂, an O157-specific gene (*rfbE*_{O157}) and an O26-specific gene (*wzx*_{O26}) were amplified by PCR [12, 22].

In *stx*-positive samples, STEC isolation was attempted by the colony hybridization method. Briefly, the culture medium was spread onto a MacConkey agar plate (Becton, Dickinson and Company) and incubated at 37 °C overnight. The plate was then precooled to 4 °C, and a positively charged nylon membrane (Hybond N+, GE health care, Little Chalfont, UK) was placed on the plate. The membrane was then transferred to a fresh LB agar plate (Becton, Dickinson and Company) and incubated at 37 °C for an hour. The cultured membrane was placed on a filter paper soaked with lysis solution (0.5 M NaOH, 1.5 M NaCl), followed by soaking with neutralization solution (3 M NaCl, 0.5 M Tris-HCl). The membrane was then gently shaken in 3 × SSC solution with 0.1% SDS at 68 °C for an hour. The membrane was then baked at 80 °C for 2 h. The membrane was hybridized in PerfectHyb hybridization solution (Toyobo, Osaka, Japan) with PCR-generated digoxigenin (DIG)-labeled *stx* probes, *stx*₁, *stx*₂, and *stx*_{2f} [12] according to the manufacturer's instructions (PCR DIG Labeling Mix, Roche, Basel, Switzerland). The DIG signal was detected by a DIG Nucleic Acid Detection Kit (Roche), and the positive colonies were picked to obtain pure culture.

In *rfbE*_{O157}- or *wzx*_{O26}-positive samples, isolation of STEC O157 and O26 was attempted using Dynabeads anti-*E. coli* O157 and Dynabeads EPEC/VTEC O26 (Life Technologies, Carlsbad, CA, USA), respectively according to the manufacturer's instructions. Isolates were identified as *E. coli* by production of the β-galactosidase, β-glucuronidase and indole. Isolates that exhibited atypical characteristics were identified as *E. coli* by further biochemical tests using API ID 32E (bioMérieux, Marcy l'Etoile, France).

Serotyping, determination of the phylogenetic lineage and virulence marker profiling

Serogroup identification was performed by O-genotyping PCR [23] followed by slide agglutination using corresponding antisera obtained from the Statens Serum Institute (Copenhagen, Denmark) or from Denka Seiken Co., Ltd. (Tokyo, Japan). Major phylogenetic groups (A, B1, B2 and D) were determined by PCR as described by Doumith *et al.* [24]. Subtypes of *stx* were determined by the method of Scheutz *et al.* [12]. PCR was employed for the detection of 21 virulence markers (Table S2), including adhesins and its regulator (*eae*, *saa*, *bfpA*, *aggR*, *lpfA*_{O113}, *lpfA*_{O157/O1-141}, *f5*, *f17*, *f41*, *fedA*, *clpG*), enterohemolysin (*ehxA*), plasmid-borne

virulence markers (*stcE* and *katP*), and markers of pathogenicity islands (OI-43/48, OI-50, OI-57, OI-122, locus of proteolysis activity (LPA), pathogenicity island of CL3 (PAI I_{CL3}), HPI (high-pathogenicity island)). Eib was detected by Western blotting as described previously [15]. Subtypes of *eae* were determined by sequencing [25].

Antimicrobial susceptibility test

The Kirby-Bauer disc diffusion test was performed using Mueller-Hinton agar plates (Becton, Dickinson and Company) according to the manufacturer's instructions using the following antimicrobials: ampicillin (10 µg), cefoxitin (30 µg), cefotaxime (30 µg), kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), fosfomycin (50 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), nalidixic acid (30 µg), gentamicin (10 µg), and ciprofloxacin (5 µg) (Becton, Dickinson and Company).

Bayesian clustering

To group isolates according to their virulence marker profiles, a Bayesian clustering method implemented in the STRUCTURE program [26] was used. Markov Chain Monte Carlo searches consisting of 100 000 'burn-in' steps followed by 1 000 000 iterations were employed. The number of clusters (*K*) was evaluated from 1 to 20, with 20 replicate runs each under the admixture model with correlated allele frequencies. Inference for the best *K* was obtained by the Δ*K* method [27].

Epidemiological analysis

For epidemiological analyses, a questionnaire (Table S1) was designed to identify risk factors on farm management described in previous studies, including herd size [28], farm type [6], housing type [28, 29], ventilation [30], and other animals in the same farm [9, 30]. We assumed that inappropriate management of feces and feed could lead to continuous infection and transmission of STEC to other animals. Therefore, type of bedding, feedbox, and watering were also asked. As potential control measures, use of probiotics and several biosecurity measures [9] were investigated. In addition, because individual factors can influence the STEC prevalence [31], information on sex, age, and breed of individual cattle was collected. The collected information was subjected to logistic regression models. First, univariate logistic regression analysis was performed using the

glm function of *R* version 3.1.2 [32]. The presence of STEC in the farm or cattle was considered a binary outcome variable. Data on potential risk factors collected through the questionnaire were used as explanatory variables in the models. Continuous data were transformed to categorical data (i.e., farm size was categorized into <50, <100, <300, \geq 300 head per farm; cattle <8 months old was regarded as juvenile and the other was regarded as adult.). Second, all the categorical data were transformed to dichotomous data and subjected to logistic regression analyses using glm function of *R* and determination coefficient between all variables was calculated by *R*. Third, explanatory variables that generated *P* values of <0.2 in univariate logistic regression analyses were subjected to multivariate logistic regression with a generalized linear mixed model using the glmmML function of *R*. Explanatory variables were selected for the final model to generate minimum variance by analysis of variance. Absence of collinearity among variables to be included in the multivariable models was investigated and if two variables were collinear only the variable with the lowest *P* value in the univariable model was included in the analysis.

RESULTS

Prevalence of STEC

STEC was isolated from 133 samples (24.1%) collected from 65 farms (59.1%). In STEC-positive farms, the mean prevalence was 40.9%. At this prevalence, the calculated probability to detect at least one STEC in five samples was 98.9%, which indicated that the sample size for each farm was valid. Up to three and four isolates were detected in one sample and one farm, respectively. More than two isolates were isolated from 31 farms (27.9%). A total of 112 STEC isolates were used for further analyses, as isolates with the same O serogroup and *stx* subtype originating from the same farm were regarded as the same clone.

Serogroups and phylogenetic groups of STEC isolates

Among the 112 STEC isolates, 51 O serogroups were identified (Table 1). Among them, the common O serogroups observed in human infections were O157, O26, O103, O121, and O145. STEC O157 was isolated from four samples (0.7%) collected from two farms (1.8%). STEC O26 was isolated from nine samples (1.6%) collected from five farms (4.5%). The most

Table 1. Distribution of *O* serogroups in STEC isolates

O serogroup	No.
O113	10
O2	9
O109	7
O163	6
O26, O130	5
O168, O174	4
O6, O8, O116, O136, O178	3
O71, O74, O87, O103, O156, O157, O179	2
O3, O22, O28ac, O55, O76, O82, O88, O96, O100, O104, O108, O115, O121, O123, O132, O140, O145, O150, O153, O171, O185, O183, OSB9*	1
OUT	10

* OSB9: *Shigella boydii* O serogroup 9.

prevalent phylogenetic group was B1 (69 isolates, 61.6%), followed by A (34 isolates, 30.4%) (Fig. 1). The serogroup and phylogenetic group mostly correlated. However, in some serogroups, including O6, O74, O109, O113, and O116, the isolates belonged to two phylogenetic groups (Fig. 1). One and two *E. coli* isolates of O157 and O26, respectively, without *stx* were isolated by immunomagnetic separation. Among them, one *eae*-positive O157 and O26 isolate each belonged to phylogenetic groups D and B1, respectively, whereas the *eae*-negative O26 isolate belonged to phylogenetic group B2. These isolates were not studied further.

Prevalence and subtypes of *stx* and adhesins

Various *stx* subtypes, including *stx*_{1a}, *stx*_{1c}, *stx*_{2a}, *stx*_{2b}, *stx*_{2c}, *stx*_{2d}, *stx*_{2e}, and *stx*_{2g}, were detected among the isolates (Fig. 1). The most prevalent subtypes in *stx*₁ and *stx*₂ were *stx*_{1a} (86.4%) and *stx*_{2a} (64.7%), respectively. Several types of adhesins were detected in the isolates. Sixteen isolates (14.3%) were positive for *eae*, and 39 isolates (34.8%) were positive for *saa*. Isolates with both *eae* and *saa* positive were not found. Most of the *eae*-positive isolates belonged to serogroups commonly involved in human infections, including O157, O26, O103, O121, and O145. The subtypes of *eae* in these isolates were beta, epsilon, or gamma (Fig. 1), as reported in human isolates [33, 34], while isolates in uncommon serogroups, O108, O115, O150, and O156, possessed theta and zeta. Most the isolates (73.2%) possessed *lpfA*_{O113}, whereas five isolates that belonged to phylogenetic group D were positive for *lpfA*_{O157/O1-141}. Only one isolate each was positive for *f17* or *f41*. Other adhesins investigated

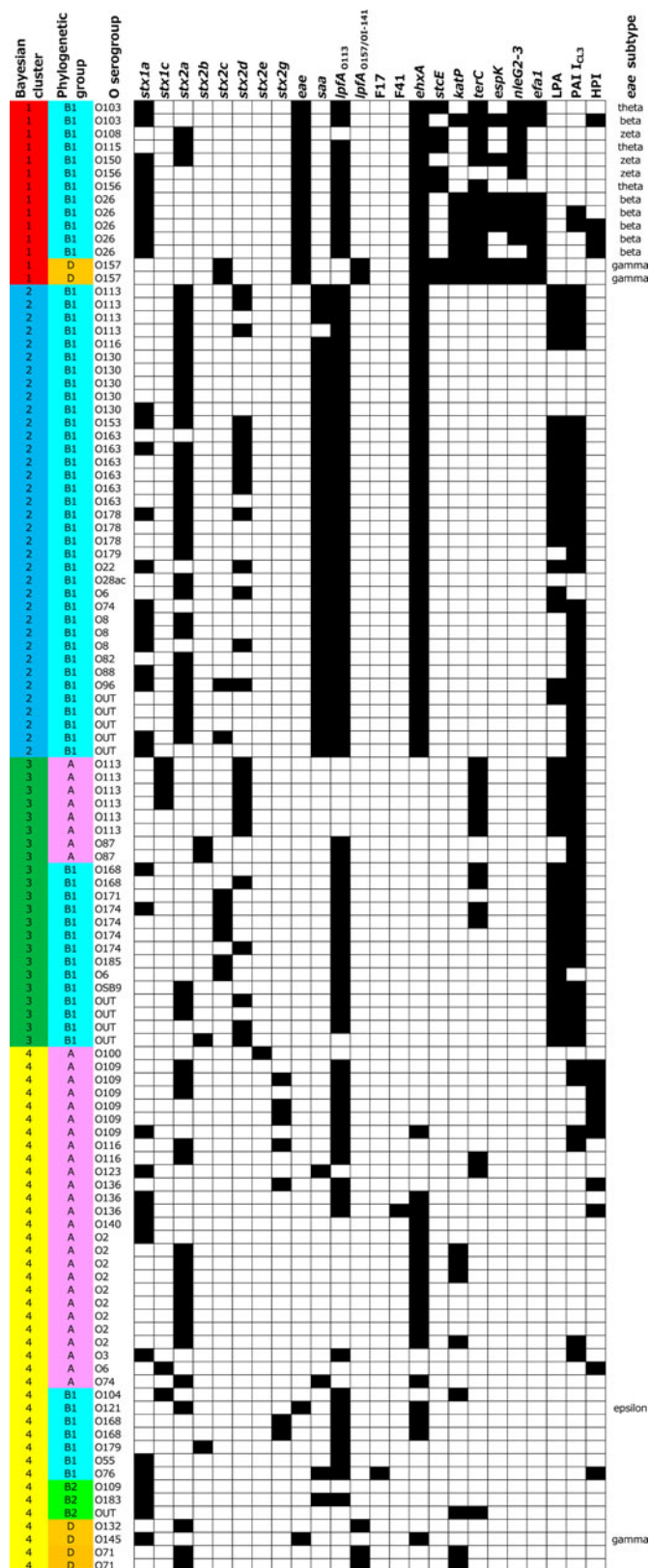


Fig. 1. Bayesian clustering results of STEC isolated from cattle in Japan. Each cluster or phylogenetic group is highlighted by a different color in the first and second columns. The distribution of virulence markers is shown in columns 4–27; black = presence; white = absence. The results for *stx1d*, *stx2f*, *bfpA*, *aggR*, *f5*, *fedA*, *clpG*, and Eib are omitted because no isolate was positive for these markers.

Table 2. Risk factors for STEC fecal shedding in multivariable logistic regression analyses with mixed models

Variable	No. of positive farm/sample	Total	Prevalence	Odds ratio (95% confidence interval)	P value
Farm level					
Housing: tie stall	26	36	0.72	3.5 (1.7–7.1)	0.001
Feed box: flat	17	35	0.49	0.3 (0.1–0.6)	0.001
Ventilation: mechanical	39	72	0.54	0.4 (0.2–0.8)	0.005
Housing: free barn	23	35	0.66	2.1 (1.1–4.1)	0.032
Bedding: manure	2	7	0.29	0.2 (0.0–1.0)	0.045
Presence of pigs on the same farm	2	3	0.67	4.5 (0.8–25.6)	0.093
Sample level					
Female	117	415	0.28	2.5 (1.1–5.5)	0.021
Adult	119	501	0.24	0.3 (0.1–0.9)	0.034

in this study, including *bfpA*, *aggR*, *f5*, *fedA*, *clpG*, and *Eib*, were not detected in any isolates (Fig. 1).

Virulence marker profiles and Bayesian clustering

To determine significant clusters based on the distribution of virulence markers, the virulence marker profiles were subjected to a Bayesian clustering approach. The ΔK method revealed that the most appropriate number of clusters was four (Fig. S1).

The results of clustering and the distribution patterns of the virulence markers are shown in Fig. 1. Bayesian cluster 1 consisted of *eae*-positive STECs, and their phylogenetic group was either B1 or D. In this group, *lpfA*_{O113}, *ehxA*, *terC*, *nleG2-3*, and *efal* were highly prevalent. The virulence marker profiles of isolates belonging to the O121 and O145 serotypes, which were also *eae* positive, differed from those in cluster 1, and thus these isolates fell into cluster 4. Bayesian cluster 2 consisted of phylogenetic group B1 and exhibited highly homogeneous virulence marker profiles. All but one isolate possessed *saa*. Most of the isolates possessed *stx*_{2a}, *lpfA*_{O113}, and *ehxA* and two pathogenicity islands, LPA and PAI I_{CL3}. Bayesian cluster 3 was similar to cluster 2 in that LPA and PAI I_{CL3} were prevalent. This cluster could be subdivided into two groups. One was a group belonging to phylogenetic group B1. This group exhibited a virulence marker profile similar to that of cluster 2 except for the absence of *saa* and *ehxA*. The other group consisted of mainly O113 isolates belonging to phylogenetic group A. This group exhibited a high prevalence of *stx*_{2d} and *terC*. Bayesian cluster 4 was the most heterogeneous group. This cluster consisted of the isolates with various *stx* subtypes and were from all four phylogenetic

groups. A few isolates carried *eae* or *saa* but had different virulence marker profiles than clusters 1 or 2, respectively.

Antimicrobial resistance

Antimicrobial resistance was observed for 7 of 12 antimicrobials used in this study. The resistance rate in the STEC isolates was highest against tetracycline (31.3%), followed by streptomycin (24.1%) (Fig. S2). When the results were stratified by the Bayesian clusters, differences were apparent. No antimicrobial-resistant isolates were present in cluster 2, whereas a relatively high rate of resistance was observed in cluster 3.

Risk factors associated with fecal shedding of STEC

Several risk factors for fecal STEC shedding were identified (Table 2). In the final model, high STEC prevalence was associated with tie-stall housing, and low prevalence was associated with flat feed boxes and mechanical ventilation ($P < 0.01$). Weak associations ($0.01 \leq P < 0.05$) were observed between high prevalence and free barn housing or female cattle and between low prevalence and manure bedding or adult cattle. Meanwhile, associations between STEC shedding and other known risk factors, including season, breed and water equipment, was not significant.

DISCUSSION

Our nationwide investigation revealed that more than half of the farms were contaminated by STEC in Japan. Kobayashi *et al.* [10] isolated STEC from 19% to 31% of samples collected from 69% of

sampling farms in a limited area of Japan, consistent with our results. In another nationwide study, the prevalence of O157 and O26 was much higher than that in the present study [9]. This discrepancy could be due to differences in isolation methods. The cited study used novobiocin, cefixime, and potassium tellurite in the enrichment broth and agar plates, whereas we used mEC broth supplemented with cefsulodin and vancomycin and MacConkey agar plates without antimicrobials. Although our method might underrepresent the prevalence of O157, it should have contributed to the isolation of a wide variety of STEC. In fact, 76.8% of the isolates did not possess *terC*, which is responsible for tellurite resistance. If potassium tellurite had been used in this study, many of these *ter*-negative isolates would not have been isolated.

Virulence marker profiling and Bayesian clustering revealed that STEC in the cattle were genetically heterogeneous, although AE- and Saa-STECC isolates showed similar virulence marker profiles. This result is important because the majority of severe infection in humans is responsible for these pathotypes. In AE-STECC, most of the isolates belonged to the common O serogroups isolated in human infection, including O157. The other O serogroups, O108, O115, O150, and O156, are not commonly isolated in human infection. The *eae* subtypes of these isolates, *eae*-theta and *eae*-zeta, are often detected in animal isolates [35]. Most of the Saa-STECC isolates fell into cluster 2 via Bayesian clustering in this study (Fig. 1). This cluster has a characteristic high prevalence of *stx*_{2a}, *lpfA*_{O113}, *ehxA*, LPA, and PAI I_{CL3} and a low prevalence of other virulence markers. LPA and PAI I_{CL3} encode homologs of adherence-mediated Iha [36] and *Yersinia pestis*-like adhesion [37], respectively. Because these pathogenicity islands are more likely to be detected in animal isolates, genes in the islands may have a role in the colonization of animal guts. No *saa*-positive isolates were resistant to any of the antimicrobials used in this study (Fig. S2). These results suggest that the Saa-STECC observed in this study might consist of phylogenetically related isolates. In Saa-STECC, O113:H21, O48:H21, and O91:H21 have been implicated as causative agents of HUS [38]. Some of our Saa-STECC isolates displayed virulence marker profiles similar to those of the above-mentioned serotypes, such as the presence of *ehxA*. These Saa-STECC isolates might have the virulence potential to cause severe disease in humans. These findings could be useful to select candidate markers for vaccine development

against AE- and Saa-STECC. Vaccines against type III secreted proteins [13] and siderophore receptor/porin proteins [39] have been reported for STECC O157, but no successful vaccine against Saa-STECC is available. Thus, immunological responses against Saa or other virulence markers should be further studied to improve vaccine development. Surprisingly, all Saa-STECC isolates but one did not possess *terC*. This result implies that a standard isolation method for STECC using cefixime and potassium tellurite would fail to isolate the Saa-STECC isolates as described above.

The virulence potential of the *eae*- and *saa*-negative STECC isolates in this study remains unclear. Isolates in cluster 3 were genetically homogeneous, with a high prevalence of LPA and PAI I_{CL3} (Fig. 1). Cluster 3 differed from cluster 2 by the absence of *saa* and *ehxA*, and thus acquisition of plasmids carrying those genes may lead to a gain in virulence. In contrast to the other clusters, the virulence marker profiles of the isolates from cluster 4 were highly heterogeneous. In this cluster, non-pathogenic *E. coli* might acquire *stx* occasionally and play a role as a reservoir of *stx*.

The prevalence of antimicrobial-resistant isolates was comparable to that observed in previous investigations of commensal *E. coli* and STECC (Fig. S2) [40, 41]. The relatively higher prevalence of isolates resistant against tetracycline and streptomycin can be attributed to frequent usage of those agents in cattle [42]. However, risk against public health of antimicrobial resistance STECC would be limited so far because no isolates showed resistance against fosfomicin, which is commonly used as first-line treatment for human STECC infection. Interestingly, the Bayesian clustering also elucidate biased distribution of antimicrobial resistance. In cluster 2, no isolates exhibited resistance, whereas isolates in cluster 3 exhibited a higher prevalence of resistance to tetracycline and streptomycin, regardless of the phylogenetic group. The isolates in these clusters may have different mechanisms in acquisition of resistance genes. These results support the hypothesis that the Bayesian clusters reflect not only the phylogeny of STECC but also their distinct phenotypes.

In epidemiological analyses, several risk factors associated with the fecal shedding were identified. The results were in accordance with previous studies indicating that the incidence rates of diseases are lower in free stall than in tie-stall [28, 29] housing and that the prevalence of STECC O157 is lower in farms with mechanical ventilation compared with

natural ventilation (Table 2) [30]. Flat feed boxes also contributed to a lower prevalence of STEC, possibly due to the ease of cleaning compared with other feeding mechanisms, including indent and box types. We expected the bedding substance to be important for the persistence of STEC in the environment, but only manure appeared to have a weak ($P=0.045$), negative association with STEC shedding. Because only seven farms used manure as bedding, this association requires further investigation. Interestingly, female cattle displayed a higher odds ratio for STEC shedding in this study. Female breeding cattle were previously identified as a risk factor for fecal shedding in Scottish farms [31]. Calves <8 months old had a weak association with fecal shedding. Because some serogroups can cause diarrhea in calves [43, 44], calves may be more likely to shed STEC. In contrast, statistical significances were not detected from some known risk factors, including the presence of other animals and farm type (cow or beef) [9]. The aim of our study was to investigate risk factors on different types of cattle farm. However, management practices would differ widely in farm type and region. To confirm risk factors revealed in our results, intervention studies are essential. Studies on intervention strategies such as a management of fecal wastes may be helpful to find out effective control measures, because feces and hides or environment contaminated by feces can promote continuous infection and transmission of STEC to other animals in cattle farms [45].

In conclusion, unbiased sampling and isolation method contributed to the effective isolation of a wide variety of STEC from more than half of sampled farms in Japan. Clustering analysis of the isolates revealed that AE- and Saa-STECS are widely distributed among cattle in Japan. These adhesins might play an important role in the colonization of cattle as well as severe disease in humans. The virulence characteristics of the AE- and Saa-STECS isolates presented may provide insight into controlling STEC on farms and for developing detection methods and vaccines.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at <https://doi.org/10.1017/S0950268817000474>.

ACKNOWLEDGEMENTS

This study was supported by grants-in-aid from the Japan Society for the Promotion of Science fellows

(25–5234), and the Japan Agency for Medical Research and Development, AMED, a Grant-in-Aid for the Research Program on Emerging and Re-emerging Infectious Diseases (15fk0108021h0002). The authors are grateful to the veterinarians at the local livestock hygiene service centers for collecting fecal samples and questionnaires. They are also grateful to Atsushi Iguchi, University of Miyazaki, for O-genotyping.

DECLARATION OF INTEREST

None.

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