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Running head: Discrimination of diarrheagenic *Escherichia coli*

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ABSTRACT

Diarrheagenic *E. coli* (DEC) isolates were recovered from local retail markets and the Osaka Municipal Central Wholesale Market in Japan. Retail food samples were collected for analysis in Osaka Japan from 2005 to 2008 and consisted of 32 beef, 28 pork, 20 poultry, 136 fish, 66 fruits and vegetables and 51 ready–to-eat (RTE) food samples. A total of 82 DEC strains were recovered from 64 (19%) food samples with the highest prevalence in poultry (100%, 20/20), followed by pork (54%, 15/28), beef (28%, 9/32), fruits and vegetables (12%, 8/66), fish (6.6%, 9/136) and RTE foods (5.9%, 3/51). Most of the strains belonged to *E. coli* possessing the enteroaggregative *E. coli* (EAEC) heat-stable enterotoxin 1 (EAST1) gene (EAST1EC; n=62, $P<0.0001$) and enteropathogenic *E. coli* (EPEC; n=16, $P<0.01$), whereas only 1 strain belonged to Shiga toxin-producing *E. coli* (STEC), 1 to EAEC and 2 to enterotoxigenic *E. coli* (ETEC) strains. Of the 82 DEC isolates, 22 O and 13 H serogroups were detected, including some specific serogroups (O91, O103, O115, O119, O126, and O157) which have been associated with human diarrheal infections. Phylogenetic group A and B1 were predominant among the DEC isolates. Antimicrobial resistance to tetracycline was most common (49%), followed by nalidixic acid (28%), ampicillin (24%), sulfamethoxazole/trimethoprim (20%), and cephalothin (18%). All isolates were susceptible to aztreonam. Of the resistant strains, 44% (22/50) demonstrated resistance to more than 3 antimicrobial agents. Isolates resistant to more than 5 antimicrobials were only found in the meat samples, while isolates from the fruits and vegetables as well as RTE foods showed resistance to only 1 or 2 antimicrobial agents. Sixty one percent of EAST1EC, 56% of EPEC and all of the EAEC and ETEC were resistant to at least 1 antimicrobial agent. Multiple-locus variable-number tandem repeat analysis (MLVA) was used in this study for genotyping of DEC. The 82 isolates collected for this study showed 77 distinct MLVA profiles

45 located among 3 branches. The Simpson's Index of Diversity (*D*) was 99.9% at its highest. The
46 high diversity of these food strains would suggest their originating from a variety of sources and
47 environments. In conclusion, retail food samples in Japan were contaminated with DEC;
48 EAST1EC, a putative DEC, were detected at high rates in poultry, pork and beef. Isolates
49 resistant to more than 3 antimicrobials were found only in raw meat and fish. Food animals may
50 act as the reservoir for multi-resistant bacteria. Due to the finding that nearly 1/3 of EAST1EC
51 strains were resistant to more than 3 antimicrobials, additional surveillance for EAST1EC should
52 be initiated.
53
54 Keywords: diarrheagenic *Escherichia coli*, EAST1EC, MLVA, food, antimicrobial resistance

1. Introduction

Escherichia coli are widespread commensal bacteria found in humans and animals and some of them cause both animal and human infections (Kalita et al., 2014). *E. coli* causing intestinal diseases have been categorized as diarrheagenic *E. coli* (DEC) and classified into six well-described categories including EPEC, STEC or enterohemorrhagic *E. coli* (EHEC), ETEC, EAEC, enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004).

The *astA* gene encoding EAEC heat-stable enterotoxin 1 (EAST1) was initially detected in EAEC (Nataro and Kaper, 1998) and subsequently has been detected quite frequently in other DEC pathotypes including EPEC, ETEC, and EHEC (Kameyama et al., 2015; Sirikaew, 2014; Wang et al., 2013). Although the role of EAST1 in human disease still remains to be clarified, EAST1EC, defined as an *E. coli* possessing only the *astA* as a possible virulence gene, has been implicated in several outbreaks (Nishikawa et al., 1999; Zhou et al., 2002).

Food borne diseases are an important public health problem not only in developing countries but also in developed countries. Numerous outbreaks have been reported to the national surveillance and reporting systems of many countries (Crowe et al., 2015; Inatsu et al., 2015; Moon et al., 2014). Animal meat and dairy products can be easily contaminated by *E. coli* during slaughter and subsequent handling. Numerous studies have revealed that animal meat and dairy products are important transmission routes of DEC to cause human infections. DEC strains were present on 2.84 and 0.75% of dairy products and meat products including beef, pork goat, lamb meat and poultry, respectively (Canizalez-Roman et al., 2013). Additionally, raw fish such as Sushi and ready–to-eat (RTE) foods are consumed without further cooking which is considered to be a potential risk because of contamination by microorganisms during processing and storage (Jain et al., 2008; Terajima et al., 1999; Yu et al., 2016). Furthermore, food borne disease

78 outbreaks associated with fresh fruits and vegetables, especially leafy green vegetables, have
79 been increasing in occurrence worldwide (Herman et al., 2015; Hyde R. 2011; Kozak et al.,
80 2013).
81 According to our previous studies, EPEC and EAST1EC are frequently isolated from
82 diarrheal patients (Nishikawa et al., 2002) and are most prevalent among healthy individuals
83 (Fujihara et al., 2009) and domestic animals (unpublished data). These bacteria seem to play
84 important roles in the cause of sporadic diarrheal illnesses. However, the transmission routes of
85 foodborne DEC have not yet been clarified. The aim of this study was to reveal the current
86 condition of DEC contamination in Japanese retail food products and the possible role of food
87 acting as a vehicle for the pathogens, particularly EPEC and EAST1EC, based on the prevalence
88 of antimicrobial resistance and their phylogenetic relationship. To the knowledge, this is the first
89 survey concerned with the extensive isolation of DEC strains from various Japanese food
90 products, including poultry, pork, beef, fruits and vegetables, fish and RTE foods.
91
92 **2. Materials and methods**
93 *2.1. Sampling and DEC isolation*
94 A total of 333 food samples (136 fish, 66 fruit and vegetables, 51 RTE foods, 32 beef, 28 pork
95 and 20 poultry samples) were obtained from local retail markets and the Osaka Municipal Central
96 Wholesale Market in Osaka, Japan from 2005 to 2008. The samples were transported in a cooling
97 bag and examined immediately after arrival at the laboratory. Food samples (10 g) were
98 homogenized in 90 ml of Brain Heart Infusion Broth (BHI, Nissui Pharmaceutical Company,
99 Tokyo, Japan) using a Masticator (IUL Instrument, Barcelona, Spain). The BHI was then
100 decanted into a 200 ml Erlenmeyer Flask through a paper strainer attached to a Stomacher bag,

and then incubated for 3 h at 37°C to resuscitate damaged cells. The cultured BHI was transferred to a 500 ml flask and mixed with an equal amount of double-strength Tryptone Phosphate Broth (TP, prepared according to the FDA manual), and the mixture was then incubated for 20 h at 44°C in a water bath. This enrichment broth was streaked onto Tricolor Agar Plates (Elmex, Tokyo, Japan) and/or Eosin Methylene Blue Plates (EMB, Nissui) to assess the presence of coliform and fecal *E. coli*. Enrichment broth cultures were screened for the 7 pathogenic *E. coli* (EPEC (*eae*), STEC (*stx1, stx2*), ETEC (*elt*, *est* for STh and *est* for STp), EIEC (*virB*), EAEC (*aggR*), EAST1EC (*astA*), and DAEC (*afaB*)) by the Multiplex Real-Time PCR method (Hidaka et al., 2009), and DEC strains were isolated by the previously developed HGMF-Colony Hybridization method (Wang et al., 2011).

2.2. Serotyping

Eighty-two DEC isolates (62 EAST1EC, 16 EPEC, 2 ETEC, 1 STEC and 1 EAEC) were serotyped with 50 specific O antisera and 22 specific H antisera designed for pathogenic *E. coli* (Denka Seiken Company, Tokyo, Japan), according to the manufacturer's protocol, and the bacterial motility was confirmed using the method described by Arikawa et al. (2010). Isolates that did not react with any of the O and H antisera examined were classified as OUT (O antisera untypeable) and HUT (H antisera untypeable), and non-motile strains were denoted as HNM (non-motile).

2.3. Phylogenetic group determination

Eighty-two DEC strains were classified into the four major phylogenetic groups (A, B1, B2, or D) as proposed by Clermont et al. (2000) based on the presence or absence of the genes *chuA and yjaA* and the DNA fragment tspE4C2 determined using a two-step triplex PCR protocol.

124

125 *2.4. Antimicrobial susceptibility testing*

126 Antimicrobial susceptibility testing to 12 antimicrobials was carried out in the 82 DEC
127 isolates by the Disk Diffusion Method on Mueller Hinton II Agar (Becton Dickinson, Franklin
128 Lakes, NJ). The standard procedure of the Clinical and Laboratory Standards Institute (CLSI)
129 M100-S25 (CLSI, 2015) was followed throughout the testing procedure.

130 The concentration of the disks (Becton Dickinson, Franklin Lakes, NJ) and the abbreviations
131 of the antimicrobial agents which were used throughout this study are amoxicillin/clavulanic acid
132 (AMC: 20/10 μg), ampicillin (AMP: 10 μg), aztreonam (ATM: 30 μg), cefoxitin (FOX: 30 μg),
133 ceftriaxion (CRO: 30 μg), cephalothin (CEP: 30 μg), chloramphenicol (CHL: 30 μg),
134 ciprofloxacin (CIP: 5 μg), gentamicin (GEN: 10 μg), nalidixic acid (NAL: 30 μg),
135 sulfamethoxazole/trimetroprim (SXT: 23.75/1.25 μg) and tetracycline (TET: 30 μg). The isolates
136 were classified as susceptible, intermediate, or resistant according to the zone diameter
137 interpretative standard recommendations by CLSI-M100-S25. Confirmation of ESBL production
138 was carried out by the Combination Disc Diffusion Test with clavulanic acid (CLSI-M100-S25,
139 2015) while AmpC DEC production was confirmed according to the description in a previous
140 study (Yagi et al., 2005).

141

142 *2.5. Multiple-locus variable-number tandem-repeat analysis (MLVA) typing*

143 The relationship among the DEC strains was determined by exploring polymorphisms in 10
144 variable-number tandem repeat (VNTR) loci, and primers were constructed in order to amplify
145 the targets in all species where the loci were present as described by Lindstedt et al. (2007) and
146 Løbersli et al. (2012). The PCR reactions were performed with GoTaq Flexi DNA Polymerase

147 and dNTPs set (Promega, Madison, WI) and the KAPA2G Fast Multiplex PCR Kit (Kapa
148 Biosystems, Cape Town, South Africa) according to the manufacturer's recommendations and the
149 previous description (Lindstedt et al., 2007; Løbersli et al., 2012). After the PCR amplifications,
150 samples were prepared for capillary electrophoresis on an ABI-3130 Genetic Analyzer (Applied
151 Biosystems, Foster City, CA) as described by Lindstedt et al. (2007) and Løbersli et al. (2012).
152
153 *2.6. Proposed allele designations*
154 For each locus, the following formulae were varied in order that the strains gave the best
155 conversion to actual repeat numbers: CVN001: ([OP (observed PCR product size)+3]−250)/39,
156 CVN002: (OP−272)/18, CVN003: (OP−404)/15, CVN004: (OP−231)/15, CVN007:
157 (OP−314)/18, CVN014: ([OP+2]−111)/6, CVN015:(OP−189)/6, CCR001: (OP−131)/59,
158 CVN016: ([OP+2]−478)/6 and CVN017: ([OP+3]−202)/6. To best fit the data, all VNTR repeat
159 numbers were rounded to the nearest whole repeat, while the CRISPR repeat numbers were
160 rounded down to the nearest full repeat as described by Lindstedt et al. (2007) and Løbersli et al.
161 (2012). Absence of PCR product is designated with a negative number (−2), and zero (0) is used
162 to describe a positive PCR product containing no repeats. The results are always reported in the
163 following order: CVN001, CVN002, CVN003, CVN004, CVN007, CVN014, CVN015,
164 CCR001, CVN016 and CVN017. All Dendrograms and Minimum Spanning Trees (MST) were
165 constructed using BioNumerics Version 5.10 (Applied Maths, Saint-Martens-Latem, Belgium).
166
167 *2.7 Statistics*
168 The differences between the DEC strains isolated from the different food sources were tested
169 for significance by performing a Chi-squared Test with Fisher’s Exact Probability Test. Simpson's

Index of Diversity (*D*) was calculated according to the formulas described by Hunter and Gaston (1988).

3. Results

3.1. DEC detection and isolation from food samples

A total of 82 DEC strains were isolated from 333 food samples. Overall, 19% (n=64) of the 333 samples were positive for DEC (Table 1). The isolation rate from poultry was significantly higher than those from other types of food samples ($P<0.0002$). Similarly, DEC isolates were more prevalent among pork than fish, fruits and vegetables and RTE foods samples ($P<0.0001$). Beef samples showed a higher DEC isolation rate than fish and RTE food samples ($P<0.01$). Most of the strains isolated from the 333 food samples belonged to EAST1EC (n=62, $P<0.0001$) and EPEC (n=16, $P<0.01$), whereas only 1 belonged to STEC, 1 to EAEC and 2 to ETEC strains.

3.2. Prevalence of serotypes

Of the 82 DEC isolates, 24 (29%) strains didn't respond to any of the O and H antisera, and the remaining isolates belonged to 22 O and 13 H serogroups (Table 2 and Fig. 1). In the 31 O-identifiable strains, O8 (four strains) and O18 (four strains) were the most frequent serogroups, followed by O91, O103 and O126 with 2 strains each while 1 strain each was detected in the other 17 O serogroups. In contrast, H16, H12, H6, H9, H34 and H40 were identified in 12, 9, 3, 3, 3, and 3 DEC isolates of 44 H-discriminable strains, respectively. Interestingly, 1 EAST1EC isolate from pork responded to both O20 and O157 antisera.

3.3. Phylogenetic grouping

193 Phylogenetic grouping of the 82 DEC isolates showed that 37 (45%), 27 (33%), 5 (6.1%), and
194 13 (16%) belonged to the phylogenetic groups A, B1, B2 and D, respectively, as shown in Table
195 3. Phylogenetic group A was predominant among EAST1EC (50%), while group B1 was
196 prevalent in EPEC (38%), and all of the pathogenic strains of STEC, EAEC and ETEC belonged
197 to the phylogroups A and B1. Phylogenetic group A was predominant among almost 50% of the
198 strains obtained from beef (45%), pork (50%), poultry (54%) and RTE foods (50%), while group
199 B1 was most prevalent in fish (38%) and fruit and vegetable (50%) samples (date not shown). No
200 B2 strains were isolated from beef, poultry or RTE samples. Statistically significant differences
201 were not obtained in the distribution of phylogenetic groups among the isolates from each food
202 source.
203
204 *3.4. Antimicrobial susceptibility testing*
205 A total of 50 (61%) isolates from the food samples were resistant to at least 1 antimicrobial
206 agent, while 32 (39%) isolates were sensitive to all the 12 antimicrobials tested in this study
207 (Table 4). Poultry isolates showed a higher resistant rate than pork ($P<0.05$) and fish ($P<0.01$).
208 Antimicrobial resistant strains were significantly more prevalent in poultry ($P<0.001$) and pork
209 ($P<0.05$) samples than fruits and vegetables.
210 Resistance was observed to TET (49%), NAL (28%), AMP (24%), SXT (20%), and CEP
211 (18%) as shown in Table 5. A low prevalence of resistance (from 1.2 to 7.3% of strains) was
212 detected for the remaining agents and no ATM resistant strains were detected in the DEC isolates
213 in this study. The poultry isolates had the greatest resistance to TET (77%, $P<0.001$), followed by
214 beef (55%, $P<0.05$), pork (50%, $P<0.05$), RTE foods (50%) and fish (15.4%) while no TET
215 resistant strains were isolated from fruits and vegetables. A significant difference in TET resistant

properties was also observed between strains isolated from poultry and fish (P<0.001). Similarly, 6 (55%) isolates from beef and 14 (54%) isolates from poultry were resistant to NAL and both of these were significantly higher than the isolates obtained from fish (7.7%), pork (5%) and fruits and vegetables (0%) with P values of P<0.05. Additionally, significant differences in SXT resistant properties were observed between strains isolated from pork and fish (P<0.05).

Only 30% of the drug resistance strains (15/50) were resistant to only 1 antimicrobial agent (Table 4), while 35 (70%) showed multi-resistance (resistance to 2 or more antimicrobials). The highest degree of resistance was exhibited by an EAST1EC strain in a beef sample, which was resistant to 9 antimicrobial agents. Most of the drug resistant strains from beef (5/7, 71%) and fish (3/5, 60%) were resistant to more than 3 antimicrobial agents. Isolates resistant to more than 5 antimicrobials were only found in meat samples, while isolates from fruits and vegetables and RTE foods showed resistance to only 1 or 2 antimicrobial agents.

ESBL and AmpC Producing Conformation Tests were performed for 3 DEC strains (1 EAST1EC from poultry and beef, and the EAEC from fish), which are marked with underline in Fig 1. Two EAST1EC strains showed the results for ESBL minus but AmpC plus, including the one which displayed the highest degree of resistance to 9 antimicrobial agents.

From the point of view of DEC pathotypes, 61% of EAST1EC, 56% of EPEC and all of the EAEC and ETEC were resistant to at least 1 antimicrobial agent (Table 6). However, the only 1 STEC, which was positive for *stx2*, wasn't resistant to any of the antimicrobials tested. About 31% of EAST1ECs were resistant to more than 3 antimicrobials. In contrast, ETEC strains and a high rate of 44% EPEC strains showed resistance to 1 or 2 antimicrobials. The only 1 EAEC strain was resistant to 4 antimicrobials.

Combined with the results of phylogenetic grouping (Table 7), antimicrobial resistant strains

were prevalent in the phylogenetic groups D (69%), A (68%) and B1 (56%). However, only 1 resistant strain was recognized in group B2. All group A and B1 strains from poultry were resistant to 1 or more antimicrobials; except for 1 EAST1EC strain in group A (Table 4 and 7).

3.5. Multiple-locus variable-number tandem-repeat analysis (MLVA) typing

MLVA typing divided the 82 DEC strains into 78 distinct profiles with 74 of unique and four pairs of identical MLVA loci pattern marked in black squares, indicating high polymorphism in the samples tested (Fig. 1). The resolution for DEC isolates was determined by GECM10 Assay with a Simpson's Index of Diversity (*D*) value of 99.9%. The isolates could be principally discriminated by alleles of locus CVN014 (*D* = 90.3%), followed by those of loci CVN016 (*D* = 69.9%) and CVN001 (*D* = 68.2%).

The MLVA typing was independent from the serotyping since 12 strains of serogroup H16, 9 of H12, 4 each of O8 and O18, and 2 O126 strains were widely scattered on the dendrogram (Table 2 and Fig. 1). Ten of 16 EPEC strains centered around 2 adjacent regions. Four pairs of isolates were assigned to the same MLVA types. Two pairs consisted of isolates of the same O serogroup or the virogroup ETEC. However, 1 pair was composed of an EAST1EC strain obtained from fruits and vegetables and 1 STEC strain obtained from beef.

A Minimum Spanning Tree (MST) was constructed to investigate the phylogenetic relationship of the 82 food-borne DEC isolates in Fig. 2. The central stem strain marked in the red circle belonged to EAST1EC, which was isolated from a beef sample and resistant to 9 antimicrobial agents. Two fish, 1 pork and 1 fruit and vegetable isolates were branched in the distinctly green group clearly separated from the other isolates. The strains which demonstrated resistance to 1 or 2 antimicrobials were prevalent in the periphery of the MST, while multi-drug

resistant strains were more predominant near the central branch with the core (red circle) of a 9-antimicrobial resistant strain from beef, although some of the strains were located outwards from the core.

4. Discussion

The epidemiological properties of 82 DEC strains from 333 food samples were examined to determine the risk of foods being used as a vehicle for transmission by DEC. The prevalence of DEC was 6.0% (20/333) in this study (Table 1), which did not include EAST1EC because the enterovirulence was not confirmed yet. This rate was higher than that reported from Mexico (1.1%) (Canizalez-Roman et al., 2013), Iran (4.0%) (Mazaheri et al., 2014) and Colombia (2.1%) (Amézquita-Montes et al., 2015), although higher prevalence (45%) than this study (30%, 6/20) in poultry has been reported from Finland (Kagambèga et al., 2012). These results suggested that the higher DEC isolation rate observed in this study may not necessarily imply serious DEC contamination in food in Japan, but rather, the efficient isolation procedure (colony hybridization) used (Wang et al., 2011) might have contributed to the relatively higher recovery of the DEC strains.

All the 82 DEC strains were analyzed for O and H antigens. Although the isolates of EAST1EC and EPEC belonged to various O and H groups, most of them did not belong to the epidemiologically important O serogroups. However, some EPEC strains were identified as O103 and O115, which have been reported to be clinically important serogroups and are associated with outbreaks of gastroenteritis (MacDonald et al., 2012; Saito et al., 2005), and some EAST1EC strains were recognized as O119, O126, and O157, which are considered to be specific serotypes of EPEC or STEC (Kobayashi et al., 2013; Tozzoli et al., 2014). The only

285 STEC isolated from beef in the present investigation was serotype O91:H21. The only EAEC
286 strain isolated from fish was serotype O126: HUT, which contains the heat stable enterotoxin
287 gene *astA* and is usually found among EAEC that cause diarrhea in humans (Silva et al., 2014).
288 The serotyping results of the 2 ETEC isolates in this study revealed that a strain from poultry was
289 untypeable, while the ETEC isolated from a RTE food sample was serotype O127a: HUT that
290 was reportedly associated with a food poisoning outbreak in China (Hao et al., 2012). Therefore,
291 a few DEC stains isolated from this study with specific O serogroups appeared to be potentially
292 harmful to consumers. Additionally, phylogenetic grouping could be effective for assessing the
293 pathogenicity of these strains further. All hemolytic uremic syndrome (HUS)-associated STEC
294 strains (Haugum et al., 2014) and more than half of the EPEC strains (Staples et al., 2013; Wang
295 et al., 2013) isolated from diarrhea patients are from the phylogroup B1. Phylogenetic grouping
296 examined in this study and other surveys indicated that group A and B1 were predominant among
297 the DEC isolates from foods, consistent with the findings reported by previous studies (Koo et
298 al., 2012; Siriwan-Sirikaew et al., 2015; Ombarak et al., 2016). The same results have also been
299 found in the studies of domestic animals (Ishii et al., 2007; Coura et al., 2015). It is suggested that
300 DEC, especially the group B1 of EPEC, contaminating food products might have a potential of
301 intestinal infection.
302 Antimicrobial susceptibility testing of isolates by the Disc Diffusion Method indicated that
303 DEC isolated from foods in this study showed a high resistant rate of 61% (50/82), while 43%
304 (35/82) of the DEC strains were resistant to multiple antimicrobial agents. Similar results were
305 reported from Mexico (Canizalez-Roman et al., 2013; Gómez-Aldapa et al., 2016) Greece
306 (Solomakos et al., 2009) and Thailand (Siriwan-Sirikaew et al., 2015) with the high resistant rate
307 of 66-100%. Resistances to TET, AMP, and SXT among DEC strains isolated from food samples

308 are also common in other reports (Canizalez-Roman et al., 2013; Teophilo et al., 2002). Despite
309 the high isolation rates in chicken (Abdallah et al., 2015; Pacholewicz et al., 2015), no ESBL
310 producing *E. coli* was found in this study. However, EAST1EC showed a high multi-drug
311 resistant rate of 31% (more than 3 antimicrobials) including 2 strains of AmpC-producing
312 EAST1EC. Since EAST1EC has been associated with outbreaks in Japan (Nishikawa et al., 1999;
313 Zhou et al., 2002; Ishiguro et al., 2005), foodborne EAST1EC could be important not only as an
314 enteric pathogen, but also as a source of the resistance genes.
315 The phylogenetic group D strains showed the highest antimicrobial resistant rate of 69%
316 (9/13), although the isolation rate (16%) of the DEC of group D was lower than groups A (45%)
317 and B1 (33%). The level of resistance to different antimicrobials varied according to the source
318 of the isolates and phylogenetic grouping. DEC isolates from meat and poultry showed a higher
319 resistance rate than those from other sources, which might be due to the common use of
320 antimicrobials for the prevention and treatment of diseases in the animals in which the food have
321 come from (Canizalez-Roman et al., 2013).
322 The phylogenetic relationship and the distance of the 82 DEC isolates in this study were
323 further discriminated by the GECM10 assay of Lindstedt et al. (2007) and Løbersli et al. (2012)
324 and the *D* value reached 99.9%. Similarly, diversity index values as high as 97% and 96% for the
325 GECM10 assay was observed in Argentina in a set of 72 non-O157:H7 VTEC isolates analyzed
326 (González et al., 2014) and 32 shigatoxin-producing *E. coli* O26:H11 strains isolated from
327 animals, food and clinical samples (Krüger et al., 2015), respectively. The high *D* values
328 observed in this study and previous reports suggest the sufficient discriminating power for the
329 GECM10 assay in DEC epidemiological surveys. The isolates could be principally discriminated
330 by the alleles of the locus CVN014 (*D* = 90.3%), CVN016 (*D* = 69.9%) and CVN001 (*D* =

68.2%), which also reconfirmed the primary discrimination power in previous reports (González et al., 2014; Krüger et al., 2015). The high diversity of DEC strains found in the current study suggested that these contaminating bacteria may have come from a variety of sources and environments. Multiple DEC strains could be transferred through food manufacturing, transportation and handling processes. Four pairs of isolates presented identical MLVA loci pattern (Fig. 1), suggesting that these are sets of homologous strains isolated from different sources. However, 1 pair was composed of different pathotypes of DEC (EAST1EC and STEC). Similar results were also reported in a raw milk and raw milk cheese analyzed by PFGE in Egypt (Ombarak et al., 2016). The distribution of strains in MST was independent of the food sample (Fig. 2).

In conclusion, retail food samples in Japan could be contaminated with DEC, particularly EAST1EC and EPEC, and high contamination rates were observed in poultry, pork and beef. It is suggested that fur and gastrointestinal tracts of pre-slaughter animals, especially chicken might carry a great number of bacteria. In addition, multiple antimicrobial resistances were among DEC isolated from retail foods, especially meat samples; food animals should act as the reservoir for multi-resistant bacteria. The prevalence of EAST1EC and EPEC among human including healthy carriers must be a reflection of their prevalence in foods. The finding that nearly 1/3 of EAST1EC being resistant to more than 3 antimicrobials suggests that EAST1EC is presumably worth being monitored nevertheless the diarrheagenicity has not been recognized yet.

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Table 1. Isolation of DEC from food samples[*]

DEC	Number (%) of DEC strains						
	Total (n=333)	Beef (n=32)	Pork (n=28)	Poultry (n=20)	Fish (n=136)	Fruits and vegetables (n=66)	RTE foods (n=51)
EAST1EC	62 (19)[§]	9 (28)	15 (54)	20 (100)	7 (5.1)	8 (12)	3 (5.9)
EPEC[#]	16 (4.8)	1 (3.1)	5 (18)	5 (25)	5 (3.7)		
STEC	1 (0.3)	1 (3.1)					
EAEC	1 (0.3)				1 (0.7)		
ETEC	2 (0.6)			1 (5)			1 (2.0)
Total samples[‡]	64 (19)	9 (28)[ac‡]	15 (54)[a]	20 (100)[b]	9 (6.6)[d]	8 (12)[cd]	3 (5.9)[d]
Total[†]	82	11	20	26	13	8	4

[*] DEC: diarrheogenic *E. coli*; EAST1EC: EAEC heat-stable enterotoxin 1 possessing *E. coli*; EPEC: enteropathogenic *E. coli*; STEC: Shiga toxin-producing *E. coli*; EAEC: enteroaggregative *E. coli*; ETEC: enterotoxigenic *E. coli*; RTE foods: ready to eat foods

[§] Numbers in brackets mean the percentage of DEC from food samples

[#] Phylogroups of the strains are as follows: 1 B1 strain from beef; 3 B1, 1 B2, and 1 D strains from pork; 3 A, 1 B1, and 1 D strains from poultry; 1 A, 2 B1, 1 B2, and 1 D strains from fish

[‡] Number of samples that DEC strains were isolated from

[†] Number of DEC strains

[‡] Treatments with different lower case letters (a-d) in the same line are significantly different (P <0.05)

Table 2. Serotypes of DEC isolated from different sources of food[*]

Serotype	Number of isolates	Serotype	Number of isolates	Serotype	Number of isolates
OUT:HUT	20 (1 ETEC, 3 EPEC)[#]	O8:HUT	1	O91:H21	1 (STEC)
OUT:HNM	4 (1 EPEC)	O8:H16	1	O103:HUT	1 (EPEC)
OUT:H2	1	O8:H40	2 (1 EPEC)	O103:H2	1 (EPEC)
OUT:H6	2	O15:H6	1	O115:HUT	1 (EPEC)
OUT:H7	1	O18:HUT	1	O119:H45	1
OUT:H10	2 (1 EPEC)	O18:H9	1	O124:HUT	1
OUT:H12	4 (2 EPEC)	O18:H12	2	O126:HUT	2 (1 EAEC)
OUT:H16	10 (3 EPEC)	O20:HUT	1 (EPEC)	O127a:HUT	1 (ETEC)
OUT:H34	3 (1 EPEC)	O20/157:H12	1	O136:H12	1
OUT:H40	1	O27:HNM	1	O148:HUT	1
OUT:H41	1	O28ac:H16	1	O152:H12	1
OUT:H42	1	O63:H42	1	O153:H9	1
OUT:H45	1	O74:HUT	1	O158:H9	1
O1:HUT	1	O91:HUT	1		

[*] DEC: diarrheogenic *E. coli*; OUT: O antisera untypeable; HUT: H antisera untypeable; HNM: non-motile strains

[#] Numbers and the pathogenic types of DEC were shown in brackets, and remains are EAST1EC.

Table 3. Distribution of phylogenetic groups among 82 DEC isolates obtained from different food sources[*]

Pathotypes	Number (%) of DEC strains			
	A	B1	B2	D
EAST1EC (n=62)	31 (50)[#]	18 (29)	3 (5)	10 (16)
EPEC (n=16)	4 (25)	7 (44)	2 (13)	3 (19)
STEC (n=1)		1 (100)		
EAEC (n=1)		1 (100)		
ETEC (n=2)	2 (100)			
Total (n=82)	37 (45)	27 (33)	5 (6)	13 (16)

[*] DEC: diarrheogenic *E. coli*; RTE foods: ready to eat foods
[#] Numbers in brackets mean the percentage of DEC from food samples

Table 4. Antimicrobial resistance patterns of DEC isolates obtained from different food sources [*]

Resistance pattern (29 profiles)	Number (%) and phylogenetic groups of DEC strains						
	Total (n=82)	Beef (n=11)	Pork (n=20)	Poultry (n=26)	Fish (n=13)	Fruits and vegetables (n=8)	RTE foods (n=4)
AMP	2	1, B1		1, D			
CEP	3				2, A,B1	1, B1	
TET	6		2, AB1	3, AAB1			1, A
NAL	2			2, AA			
CHL	1		1, A				
SXT	1		1, B1				
Subtotal	15 (18) [†]						
AMP-TET	1			1, A			
GEN-TET	1			1, D			
TET-NAL	6	1, D		4, AAAD			1, D
TET-SXT	5		4, AAAB2	1, A			
Subtotal	13 (16)						
AMP-CEP-TET	1		1, D				
AMP-TET-NAL	2	1, A			1, D		
CEP-TET-NAL	2	1, A		1, A			
Subtotal	5 (6.1)						
AMP-AMC-CEP-TET	1				1, A		
AMP-AMC-CEP-FOX	1				1, B1		
AMP-CEP-TET-NAL	1			1, B1			
AMP-CEP-TET-SXT	1		1, D				
AMP-GEN-TET-SXT	1		1, A				
AMP-TET-NAL-SXT	1			1, A			
AMP-TET-CHL-SXT	1			1, B1			

CEP-TET-NAL-SXT	1			1, B1			
GEN-TET-CIP-NAL	1			1, B1			
TET-NAL-CHL-SXT	1	1, B1					
Subtotal	10 (12)						
AMP-AMC-CEP-TET-SXT	1			1, A			
AMP-CEP-TET-CIP-NAL	2		1, B1	1, B1			
AMP-TET-CIP-NAL-CHL	1			1, B1			
AMP-TET-NAL-CHL-SXT	1	1, D					
Subtotal	5 (6.1)						
AMP-GEN-TET-CIP-NAL-SXT	1			1, A			
Subtotal	1 (1.2)						
AMP-AMC-CEP-CRO-FOX-TET-NAL-CHL-SXT	1	1, A					
Subtotal	1 (1.2)						
Total	50 (61)	7 (64)[abc‡]	12 (60)[a]	23 (88)[b]	5 (38)[ac]	1 (13)[c]	2 (50)[abc]

[*] DEC: diarrheogenic *E. coli*; RTE foods: ready to eat foods; AMP: Ampicillin, AMC: Amoxicillin - Clavulanic acid, CEP: Cephalothin, CRO: Ceftriaxion, FOX: Cefoxitin, ATM: Aztreonam, GEN: Gentamicin, TET: Tetracycline, CIP: Ciprofloxacin, NAL: Nalidixic acid, CHL: Chloramphenicol, SXT: Sulfamethoxazole - Trimethoprim

[†] Numbers in brackets mean the percentage of DEC from food samples

[‡] Treatments with different lower case letters (a-c) in the same line are significantly different (P <0.05)

Table 5. Antimicrobial resistance rate of DEC isolates obtained from different food sources[*]

Antimicrobial resistance	Number (%) of DEC strains						
	Total (n=82)	Beef (n=11)	Pork (n=20)	Poultry (n=26)	Fish (n=13)	Fruits and vegetables (n=8)	RTE foods (n=4)
AM	20 (24) [†]	4 (36)	4 (20)	9 (35)	3 (23)		
AMC	4 (4.9)	1 (9.1)		1 (3.8)	2 (15)		
CF	15 (18)	2 (18)	3 (15)	5 (19)	4 (31)	1 (13)	
CRO	1 (1.2)	1 (9.1)					
FOX	2 (2.4)	1 (9.1)			1 (7.7)		
ATM							
GM	4 (4.9)		1 (5.0)	3 (12)			
Te	40 (49)	6 (55)^{ac‡}	10 (50)^{ac}	20 (77)^{a}	2 (15)^{bc}	0(0)^{b}	2 (50)^{abc}
CIP	5 (6.1)		1 (5.0)	4 (15)			
NA	23 (28)	6 (55)^{a}	1 (5.0)^{b}	14 (54)^{a}	1 (7.7)^{b}	0(0)^{b}	1 (25)^{ab}
C	6 (7.3)	3 (27)	1 (5.0)	2 (7.7)			
SXT	16 (20)	3 (27)^{ab}	7 (35)^{a}	6 (23)^{ab}	0(0)^{b}		

[*] DEC: diarrheogenic *E. coli*; RTE foods: ready to eat foods; AMP: Ampicillin, AMC: Amoxicillin - Clavulanic acid, CEP: Cephalothin, CRO: Ceftriaxion, FOX: Cefoxitin, ATM: Aztreonam, GEN: Gentamicin, TET: Tetracycline, CIP: Ciprofloxacin, NAL: Nalidixic acid, CHL: Chloramphenicol, SXT: Sulfamethoxazole - Trimethoprim

[†] Numbers in brackets mean the percentage of DEC from food samples

[‡] Treatments with different lower case letters (a-c) in the same line are significantly different (P <0.05)

Table 6. Antimicrobial resistance rate of DEC isolates[*]

Antimicrobial resistance	Number (%) of DEC strains					
	Total (n=82)	EAST1EC (n=62)	EPEC (n=16)	STEC (n=1)	EAEC (n=1)	ETEC (n=2)
Resistant to 1 antimicrobial	15 (18)[#]	10 (16)	4 (25)			1 (50)
Resistant to 2 antimicrobials	13 (16)	9 (15)	3 (19)			1 (50)
Resistant to 2 or less antimicrobials	28 (34)	19 (31)	7 (44)	0	0	2 (100)
Resistant to 3 antimicrobials	5 (6.1)	4 (6.5)	1 (6.3)			
Resistant to 4 antimicrobials	10 (12)	8 (13)	1 (6.3)		1 (100)	
Resistant to 5 antimicrobials	5 (6.1)	5 (8.1)				
Resistant to 6 antimicrobials	1 (1.2)	1 (1.6)				
Resistant to 9 antimicrobials	1 (1.2)	1 (1.6)				
Resistant to 3 or more antimicrobials	22 (27)	19 (31)	2 (13)	0	1 (100)	0
Resistant to antimicrobials	50 (61)	38 (61)	9 (56)	0	1 (100)	2 (100)

[*] DEC: diarrheogenic *E. coli*; EAST1EC: EAEC heat-stable enterotoxin 1 possessing *E. coli*; EPEC: enteropathogenic *E. coli*; STEC: Shiga toxin-producing *E. coli*; EAEC: enteroaggregative *E. coli*; ETEC: enterotoxigenic *E. coli*

[#] Numbers in brackets mean the percentage of DEC from food samples

Table 7. Antimicrobial resistance rate of DEC isolates in each phylogenetic group obtained from different food sources[*]

Phylogenetic group	Number (%) of DEC strains						
	Total (n=82)	Beef (n=11)	Pork (n=20)	Poultry (n=26)	Fish (n=13)	Fruits and vegetables (n=8)	RTE foods (n=4)
A (n=37)	25 (68)[#]	3 (60)[ab†]	6 (60)[ab]	13 (93)[a]	2 (50)[ab]	0 (0)[b]	1 (50)[ab]
B1 (n=27)	15 (56)	2 (50)[abc]	3 (50)[abc]	7 (100)[a]	2 (40)[b]	1 (25)[bc]	0 (0)[abc]
B2 (n=5)	1 (20)		1 (50)				
D (n=13)	9 (69)	2 (100)	2 (100)	3 (60)	1 (50)		1 (100)
Total	50 (61)	7 (64)	12 (60)	23 (88)	5 (38)	1 (13)	2 (50)

[*] DEC: diarrheogenic *E. coli*; RTE foods: ready to eat foods
[#] Numbers in brackets mean the percentage of DEC from food samples
[†] Treatments with different lower case letters (a-c) in the same line are significantly different (P <0.05)

Figure legends

Fig. 1. Dendrogram of DEC was investigated in this study by GECM10. Four pairs of isolates that showed the identical MLVA loci pattern are marked in black squares. Three strains marked with underline were performed with ESBL and AmpC Producing Conformation Test. EAST1EC was represented by EASTEC in this figure.

Fig. 2. Population modelling using the Minimum Spanning Tree (MST) Method on a set of 82 DEC isolates. Each circle is noted with source and phylogenetic group while the different color of the circles indicates the antimicrobial resistant property of each strain. B: beef, P: pork, C: chicken?, F: fish, V: Fruits and vegetables, R: RTE foods. Black circle: resistant to 1 antimicrobial agent, blue circle: 2 agents, green circle: 3 agents, yellow circle: 4 agents, yellow triangle: 5 agents, yellow star: 6 agents, red circle: 9 agents, original purple circle with black edging: susceptible strains.

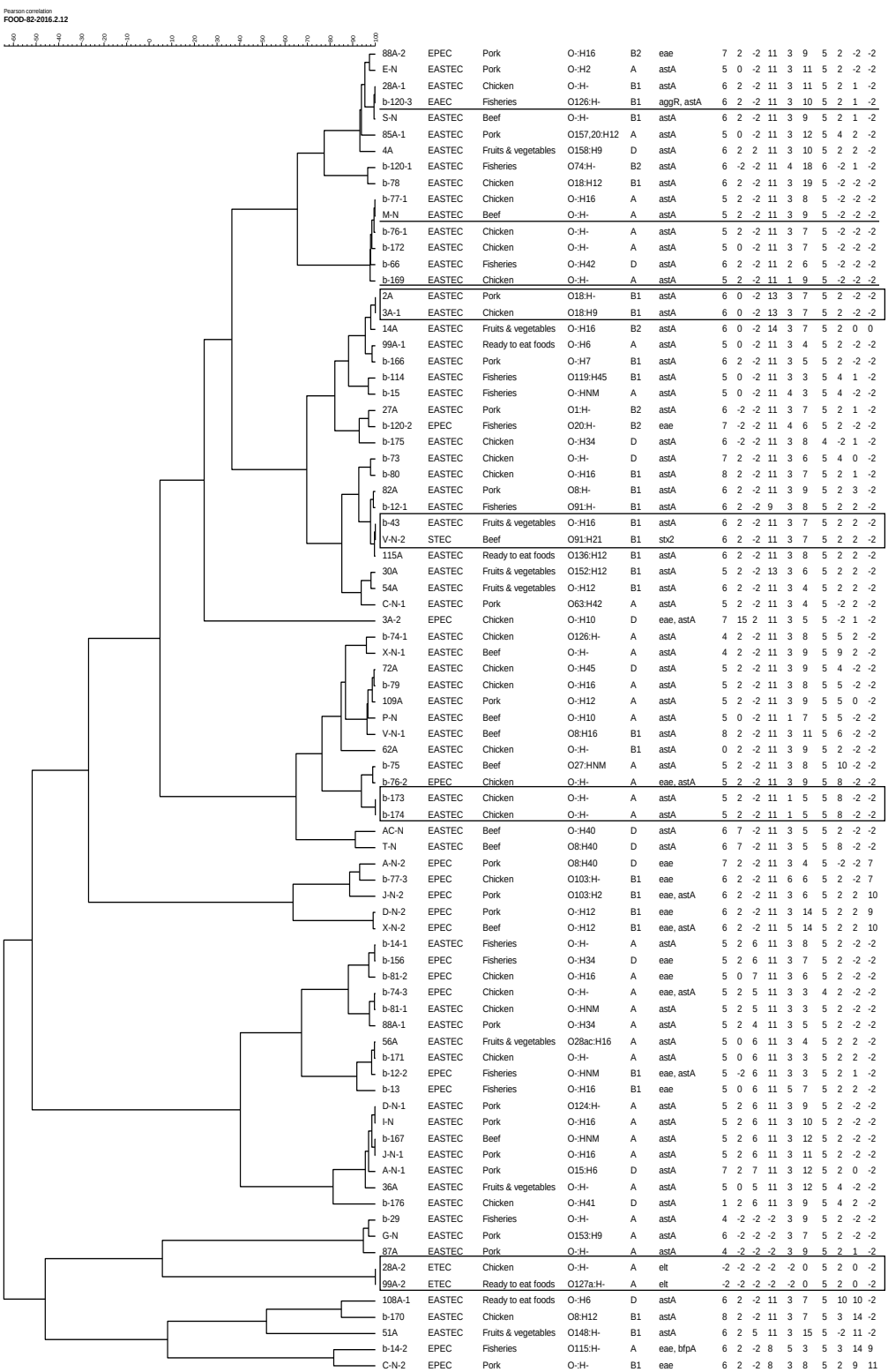
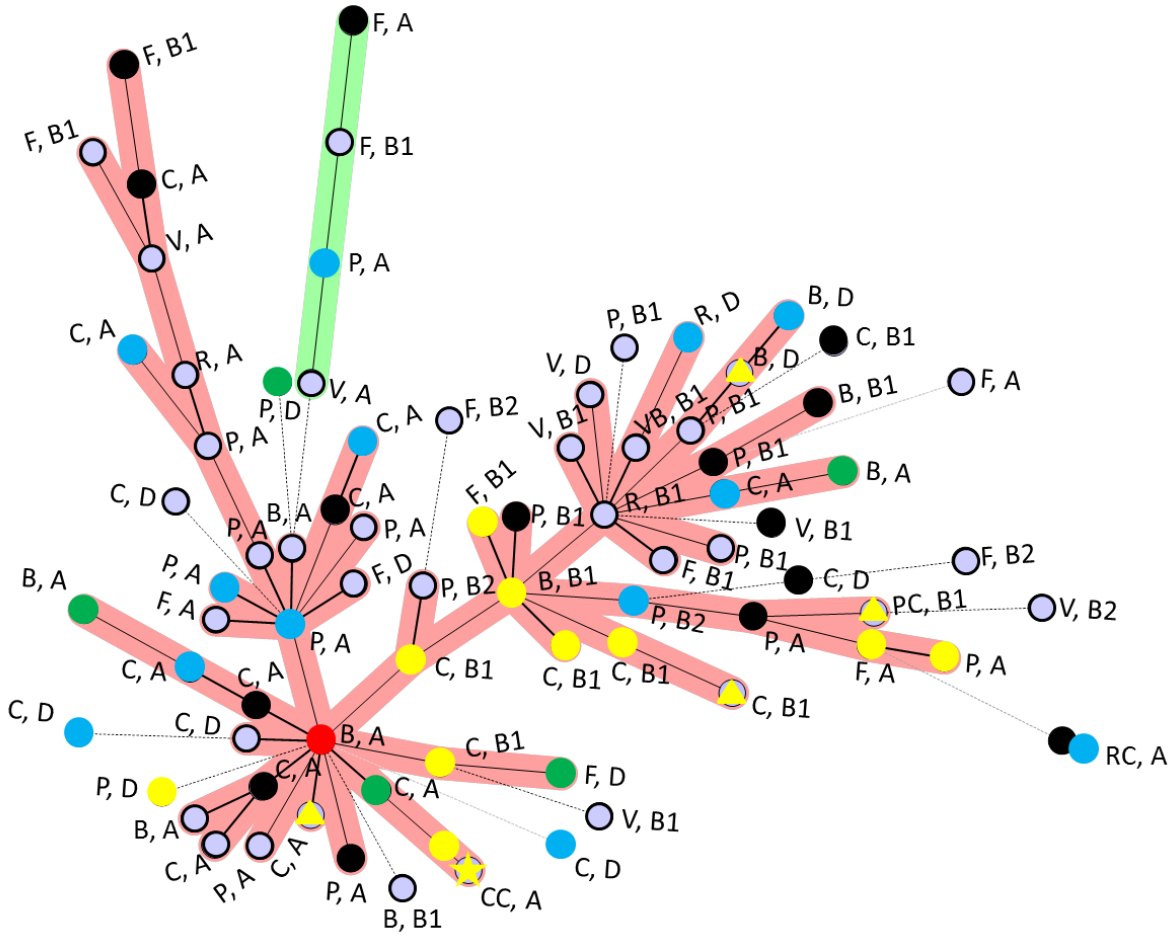

Pearson correlation
FOOD-82-2016.2.12
100 90 80 70 60 50 40 30 20 10 0
88A-2 EPEC Pork O-:H16 B2 eae 7 2 -2 11 3 9 5 2 -2 -2
E-N EASTEC Pork O-:H2 A astA 5 0 -2 11 3 11 5 2 -2 -2
28A-1 EASTEC Chicken O-:H- B1 astA 6 2 -2 11 3 11 5 2 1 -2
b-120-3 EAEC Fisheries O126:H- B1 aggR, astA 6 2 -2 11 3 10 5 2 1 -2
S-N EASTEC Beef O-:H- B1 astA 6 2 -2 11 3 9 5 2 1 -2
85A-1 EASTEC Pork O157,20:H12 A astA 5 0 -2 11 3 12 5 4 2 -2
4A EASTEC Fruits & vegetables O158:H9 D astA 6 2 2 11 3 10 5 2 2 -2
b-120-1 EASTEC Fisheries O74:H- B2 astA 6 -2 -2 11 4 18 6 -2 1 -2
b-78 EASTEC Chicken O18:H12 B1 astA 6 2 -2 11 3 19 5 -2 -2 -2
b-77-1 EASTEC Chicken O-:H16 A astA 5 2 -2 11 3 8 5 -2 -2 -2
M-N EASTEC Beef O-:H- A astA 5 2 -2 11 3 9 5 -2 -2 -2
b-76-1 EASTEC Chicken O-:H- A astA 5 2 -2 11 3 7 5 -2 -2 -2
b-172 EASTEC Chicken O-:H- A astA 5 0 -2 11 3 7 5 -2 -2 -2
b-66 EASTEC Fisheries O-:H42 D astA 6 2 -2 11 2 6 5 -2 -2 -2
b-169 EASTEC Chicken O-:H- A astA 5 2 -2 11 1 9 5 -2 -2 -2
2A EASTEC Pork O18:H- B1 astA 6 0 -2 13 3 7 5 2 -2 -2
3A-1 EASTEC Chicken O18:H9 B1 astA 6 0 -2 13 3 7 5 2 -2 -2
14A EASTEC Fruits & vegetables O-:H16 B2 astA 6 0 -2 14 3 7 5 2 0 0
99A-1 EASTEC Ready to eat foods O-:H6 A astA 5 0 -2 11 3 4 5 2 -2 -2
b-166 EASTEC Pork O-:H7 B1 astA 6 2 -2 11 3 5 5 2 -2 -2
b-114 EASTEC Fisheries O119:H45 B1 astA 5 0 -2 11 3 3 5 4 1 -2
b-15 EASTEC Fisheries O-:HNM A astA 5 0 -2 11 4 3 5 4 -2 -2
27A EASTEC Pork O1:H- B2 astA 6 -2 -2 11 3 7 5 2 1 -2
b-120-2 EPEC Fisheries O20:H- B2 eae 7 -2 -2 11 4 6 5 2 -2 -2
b-175 EASTEC Chicken O-:H34 D astA 6 -2 -2 11 3 8 4 -2 1 -2
b-73 EASTEC Chicken O-:H- D astA 7 2 -2 11 3 6 5 4 0 -2
b-80 EASTEC Chicken O-:H16 B1 astA 8 2 -2 11 3 7 5 2 1 -2
82A EASTEC Pork O8:H- B1 astA 6 2 -2 11 3 9 5 2 3 -2
b-12-1 EASTEC Fisheries O91:H- B1 astA 6 2 -2 9 3 8 5 2 2 -2
b-43 EASTEC Fruits & vegetables O-:H16 B1 astA 6 2 -2 11 3 7 5 2 2 -2
V-N-2 STEC Beef O91:H21 B1 stx2 6 2 -2 11 3 7 5 2 2 -2
115A EASTEC Ready to eat foods O136:H12 B1 astA 6 2 -2 11 3 8 5 2 2 -2
30A EASTEC Fruits & vegetables O152:H12 B1 astA 5 2 -2 13 3 6 5 2 2 -2
54A EASTEC Fruits & vegetables O-:H12 B1 astA 6 2 -2 11 3 4 5 2 2 -2
C-N-1 EASTEC Pork O63:H42 A astA 5 2 -2 11 3 4 5 -2 2 -2
3A-2 EPEC Chicken O-:H10 D eae, astA 7 15 2 11 3 5 5 -2 1 -2
b-74-1 EASTEC Chicken O126:H- A astA 4 2 -2 11 3 8 5 5 2 -2
X-N-1 EASTEC Beef O-:H- A astA 4 2 -2 11 3 9 5 9 2 -2
72A EASTEC Chicken O-:H45 D astA 5 2 -2 11 3 9 5 4 -2 -2
b-79 EASTEC Chicken O-:H16 A astA 5 2 -2 11 3 8 5 5 -2 -2
109A EASTEC Pork O-:H12 A astA 5 2 -2 11 3 9 5 5 0 -2
P-N EASTEC Beef O-:H10 A astA 5 0 -2 11 1 7 5 5 -2 -2
V-N-1 EASTEC Beef O8:H16 B1 astA 8 2 -2 11 3 11 5 6 -2 -2
62A EASTEC Chicken O-:H- B1 astA 0 2 -2 11 3 9 5 2 -2 -2
b-75 EASTEC Beef O27:HNM A astA 5 2 -2 11 3 8 5 10 -2 -2
b-76-2 EPEC Chicken O-:H- A eae, astA 5 2 -2 11 3 9 5 8 -2 -2
b-173 EASTEC Chicken O-:H- A astA 5 2 -2 11 1 5 5 8 -2 -2
b-174 EASTEC Chicken O-:H- A astA 5 2 -2 11 1 5 5 8 -2 -2
AC-N EASTEC Beef O-:H40 D astA 6 7 -2 11 3 5 5 2 -2 -2
T-N EASTEC Beef O8:H40 D astA 6 7 -2 11 3 5 5 8 -2 -2
A-N-2 EPEC Pork O8:H40 D eae 7 2 -2 11 3 4 5 -2 -2 7
b-77-3 EPEC Chicken O103:H- B1 eae 6 2 -2 11 6 6 5 2 -2 7
J-N-2 EPEC Pork O103:H2 B1 eae, astA 6 2 -2 11 3 6 5 2 2 10
D-N-2 EPEC Pork O-:H12 B1 eae 6 2 -2 11 3 14 5 2 2 9
X-N-2 EPEC Beef O-:H12 B1 eae, astA 6 2 -2 11 5 14 5 2 2 10
b-14-1 EASTEC Fisheries O-:H- A astA 5 2 6 11 3 8 5 2 -2 -2
b-156 EPEC Fisheries O-:H34 D eae 5 2 6 11 3 7 5 2 -2 -2
b-81-2 EPEC Chicken O-:H16 A eae 5 0 7 11 3 6 5 2 -2 -2
b-74-3 EPEC Chicken O-:H- A eae, astA 5 2 5 11 3 3 4 2 -2 -2
b-81-1 EASTEC Chicken O-:HNM A astA 5 2 5 11 3 3 5 2 -2 -2
88A-1 EASTEC Pork O-:H34 A astA 5 2 4 11 3 5 5 2 -2 -2
56A EASTEC Fruits & vegetables O28ac:H16 A astA 5 0 6 11 3 4 5 2 2 -2
b-171 EASTEC Chicken O-:H- A astA 5 0 6 11 3 3 5 2 2 -2
b-12-2 EPEC Fisheries O-:HNM B1 eae, astA 5 -2 6 11 3 3 5 2 1 -2
b-13 EPEC Fisheries O-:H16 B1 eae 5 0 6 11 5 7 5 2 2 -2
D-N-1 EASTEC Pork O124:H- A astA 5 2 6 11 3 9 5 2 -2 -2
I-N EASTEC Pork O-:H16 A astA 5 2 6 11 3 10 5 2 -2 -2
b-167 EASTEC Beef O-:HNM A astA 5 2 6 11 3 12 5 2 -2 -2
J-N-1 EASTEC Pork O-:H16 A astA 5 2 6 11 3 11 5 2 -2 -2
A-N-1 EASTEC Pork O15:H6 D astA 7 2 7 11 3 12 5 2 0 -2
36A EASTEC Fruits & vegetables O-:H- A astA 5 0 5 11 3 12 5 4 -2 -2
b-176 EASTEC Chicken O-:H41 D astA 1 2 6 11 3 9 5 4 2 -2
b-29 EASTEC Fisheries O-:H- A astA 4 -2 -2 -2 3 9 5 2 -2 -2
G-N EASTEC Pork O153:H9 A astA 6 -2 -2 -2 3 7 5 2 -2 -2
87A EASTEC Pork O-:H- A astA 4 -2 -2 -2 3 9 5 2 1 -2
28A-2 ETEC Chicken O-:H- A elt -2 -2 -2 -2 -2 0 5 2 0 -2
99A-2 ETEC Ready to eat foods O127a:H- A elt -2 -2 -2 -2 -2 0 5 2 0 -2
108A-1 EASTEC Ready to eat foods O-:H6 D astA 6 2 -2 11 3 7 5 10 10 -2
b-170 EASTEC Chicken O8:H12 B1 astA 8 2 -2 11 3 7 5 3 14 -2
51A EASTEC Fruits & vegetables O148:H- B1 astA 6 2 5 11 3 15 5 -2 11 -2
b-14-2 EPEC Fisheries O115:H- A eae, bfpA 6 2 -2 8 5 3 5 3 14 9
C-N-2 EPEC Pork O-:H- B1 eae 6 2 -2 8 3 8 5 2 9 11

Fig. 1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C, B1
RC, A

Fig. 2