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ABSTRACT Atypical enteropathogenic *Escherichia coli* (aEPEC) strains (36 Japanese and 50 Bangladeshi) obtained from 649 poultry fecal samples were analyzed by molecular epidemiological methods. Clermont's phylogenetic typing showed that group A was more prevalent (58%, 50/86) than B1 (31%, 27/86). Intimin type β1, which is prevalent among human diarrheal patients, was predominant in both phylogroups B1 (81%, 22/27) and A (70%, 35/50). However, about 95% of B1-β1 strains belonged to virulence group I, and 77% of them were Japanese strains, while 17% (6/35) of A-β1 strains did. Multi-locus variable-number tandem-repeat analysis (MLVA) distributed the strains into 52 distinct profiles, with Simpson's index of diversity (D) at 73%. When the data were combined with those of 142 previous strains from different sources, the minimum spanning tree formed five zones for porcine, poultry, healthy human, bovine and human patients, and the B1-β1 poultry strains. Antimicrobial resistance to nalidixic acid was most common (74%) among the isolates. Sixty-eight percent of them demonstrated resistance to ≥3 antimicrobial agents, and most of them (91%) were from Bangladesh. The strains were assigned into two groups by hierarchical clustering. Correlation matrix analysis revealed that the virulence genes were negatively associated with antimicrobial resistance. The present study suggested that poultry, particularly Japanese poultry, could be another reservoir of aEPEC (phylogroup B1, virulence group I, and intimin type β1); however, poultry strains seem to be apart from patient strains that were closer to bovine strains. Bangladeshi aEPEC may be less virulent for humans but more resistant to antibiotics.

IMPORTANCE Atypical enteropathogenic *Escherichia coli* is a diarrheagenic *E. coli* as it possesses the intimin gene (*eae*) for attachment and effacement on epithelium. Since aEPEC is ubiquitous even in developed countries, we previously used molecular epidemiological methods

to discriminate aEPEC as a human pathogen. The present study assessed poultry as another source of human diarrheagenic aEPEC. Poultry could be the source of aEPEC (phylogroup B1, virulence group I, and intimin type β1) found among patient strains in Japan. However, the MST suggested that the strains from Japanese poultry were far from Japanese patient strains compared to the distance between bovine and patient strains. Bangladeshi avian strains seemed to be less diarrheagenic but are hazardous as a source of drug resistance genes.

INTRODUCTION

Enteropathogenic *E. coli* (EPEC) is a leading cause of child diarrhea globally, especially in developing countries (1). EPEC is specific in having a locus of enterocyte effacement (LEE) pathogenicity island, which comprises a type III secretion system (T3SS) to inject effectors into intestinal epithelium (2), and in having the ability to produce distinctive attaching and effacing (A/E) lesions (3). This pathotype of *E. coli* is subdivided into typical EPEC (tEPEC), having the EPEC adherence factor (EAF) plasmid, and atypical EPEC (aEPEC), which does not have the EAF plasmid (4). This plasmid is generally not responsible for the pathogenicity of tEPEC, but the genes *bfp* (bundle-forming pili) and *perA* (plasmid-encoded regulatory) encoded by the plasmid enhance its virulence (5). Consequently, aEPEC is recognized as less virulent than tEPEC, since the contribution of the virulence plasmid has been proven in a volunteer study (6).

Atypical EPEC is more prevalent than tEPEC among the total EPEC cases in childhood diarrhea in both developed and developing countries (7). Since aEPEC is located in the intestinal epithelium (8), the bacteria seem to produce longer-lasting diarrhea than other organisms (9). Control of aEPEC could be critical in managing persistent diarrhea among children. Monitoring and identification of the actual source of aEPEC is crucial to expel this virulent pathogen from the human food chain. However, since aEPEC strains are prevalent not only among diarrheic patients but also healthy children (9), it is essential to further investigate the virulence genes responsible for its human enteropathogenicity (10, 11).

The genes pivotal for the diarrheagenicity of EPEC still remain to be elucidated in spite of vigorous studies using comparative genomics of strains isolated from humans (11, 12). In this study, we applied the scheme of Afset et al. to assign aEPEC strains (10). The genes encoded by the pathogenicity island OI-122 (*efa1*/*lifA*, *nleB*, *nleE*, and *set*/*ent*), and the three variants of the

71 long polar fimbriae (LPF) in aEPEC are reportedly associated with diarrhea. In the categorization
72 scheme based on the presence and absence of these virulence genes (10), aEPEC strains could be
73 distributed into group I, having an association with diarrhea, and group II, which does not.
74 The different virulence determinants in combination with the phylogeny of the bacteria could
75 contribute to the recognition of a new virulent subgroup of bacteria (13). Among the eight
76 phylogroups, A, B1, B2, C, D, E, F, and *E. coli* clade-1 (14), group A and B1 strains are
77 considered to be generalists, as they are prevalent in all vertebrates, while group B2 and D strains
78 are reported to be largely restricted to endothermic vertebrates (15). Along with virulence genes
79 and phylogeny, the type of intimin is another important virulence determinant in aEPEC strains,
80 as intimin is required for the colonization and pathogenesis of EPEC (16). Intimin is an
81 outer-membrane protein encoded by *eae* in EPEC and is assigned into 17 genetic variants, α1, α2,
82 β1, ξR/β2B, δ/κ/β2O, γ1, θ/γ2, ε1, νR/ε2, μR/ι2, ζ, η, μB, νB, ι1, λ, and ξB (17). Different
83 variants are likely responsible for the specific host and tissue tropisms (18). Multi-locus
84 variable-number of tandem repeat analysis (MLVA) has also been used as a suitable, rapid,
85 accurate, and cost-effective genotyping method (19).
86 The emergence of antimicrobial resistance among *E. coli* strains of animal and poultry origin
87 is a global public health issue that burdens successful antibiotic treatment. To limit the spread of
88 drug resistant bacteria efficiently and competently, investigating bacterial genotypes along with
89 drug resistance frequency is fundamental (20).
90 In our previous reports, we analyzed the molecular epidemiological markers of aEPEC from
91 different sources (foods, cattle, swine, healthy carriers, and diarrheic patients) and found that
92 bovines could be a reservoir of human diarrheagenic aEPEC (19, 21). However, poultry and its
93 products are being progressively documented as the main source of *E. coli* infection (22). EPEC

is highly prevalent (63%) in poultry fecal samples according to our previous report compared with other domestic animals (23). A group of researchers from South Korea (24) and Argentina (25) isolated aEPEC from poultry. The former group described the phylogenetic groups, intimin types, and serotypes, while the other group described the serotypes and intimin types of the isolated strains and claimed that the strains could be diarrheagenic to humans, though they did not screen the virulence genes among the isolated strains, which is helpful to determine whether the strains are diarrheagenic (10). Another research group (26) attempted to screen some virulence genes in aEPEC isolated from poultry in Canada, though the intimin types, phylogenetic groups, and O antigens of the isolated strains were unknown, and these seem to be associated with the virulence of aEPEC. These three studies also did not determine the status of aEPEC in diarrheal patients within their study areas, which is also an important indicator in epidemiological investigations, because the molecular markers within the pathotypes might differ based on geographical location.

The present study was designed to explore the role of an avian host as the source of virulent aEPEC in the human food chain. Analysis of virulence gene profiles (10), determination of phylogroups (14), EPEC-specific intimin typing (17), O antigen genotyping (27), MLVA (28), and the drug resistance pattern of the bacteria were all used for predicting potential health risks associated with aEPEC strains of poultry origin. A total of 86 aEPEC strains obtained from 649 poultry fecal specimens by our colony hybridization method (29) in combination with multiplex real-time PCR (30) were analyzed. The acquired data were compared to our previous data on humans, bovine, and swine (19) to assess the role of avian aEPEC in diarrheal diseases and to narrow down the target strains for sequence-based studies in the future.

RESULTS

Detection of *bfpA* and *perA* by PCR. None of the 86 strains isolated in this study had *bfpA* and *perA* genes amplified by PCR. Hence, all strains must be negative for the pEAF plasmid and were categorized as aEPEC for further analysis as suggested by previous research (31).

Phylogenetic distribution. Quadruplex PCR distributed 86 aEPEC strains into six groups among the eight groups of Clermont's new phylogenetic scheme (Table 1). Phylogroup A (58%) was predominant, followed by B1 (31%). The prevalence of phylogroups varied according to geographic location. About 50% of Japanese strains were in group B1, while group A strains were more abundant (68%) in Bangladesh. The prevalence of phylogroups C, D, E, and clade-1 was 2, 1, 6, and 1% respectively. Phylogroup D and clade-1 were found only in Japan, while groups C and E were recognized only in Bangladesh. We did not find any B2 or F strains in this study.

O-genotyping. Fifty-five (64%) of the 86 aEPEC strains belonged to 11 O genotypes, and the remaining 31 (36%) strains for which genotypes could not be determined were designated UT (untypeable). Most of the strains (48/50) isolated in Bangladesh were successfully assigned to O genogroups with the described method (27), whereas 29 of the 36 strains isolated in Japan were assigned to the UT group (Tables 1 and 2). Significant numbers of the O-typeable strains were of five genotypes: O177 (24 strains), O26 (eight strains), O49 (five strains), O80 (five strains), and O8 (four strains). Other O genotypes detected in the study were O55 (two strains) and O25, O87, and O116 (one strain in each genotype). This O-genotyping method was based on the detection of O-AGC; however, O123 and O186 share identical or very similar O-AGC, and hence these two genotypes could not be differentiated by this method. Three strains reacted to O123/O186 O-AGC. These three strains could be either O123 or O186.

The strains of phylogroups A and B1 were assigned to diverse O genotypes, while the C and E strains were restricted to O55 and O26, respectively (Table 1).

Typing of *eae*. By subtyping of *eae*, strains isolated from poultry were assigned into three groups (Table 2). Intimin type β1 was predominant (67%, 58/86) in poultry EPEC followed by ε1 (6%, 5/86). Intimin type β1 was highly prevalent in both geographic locations studied, but ε1 was only detected among Bangladeshi strains. Three strains (3.5%) showed positive reactions with two sets of typing primers (β1 and μB). The EPEC strains that did not produce amplicons with the typing primers used in this study were designated UT.

Strains having intimin type β1 belonged to seven different O serogroups, including O177 (23) O49 (5), O80 (4), O23/O186 (2), O55, and O87. Twenty-two β1 strains, of which 21 were Japanese strains, did not react to any serotype-specific primer. EPEC strains with intimin type ε1 were affiliated with serotype O26, and strains that reacted with PCR primers for both intimin β1 and μB did not respond to any serotype-specific primers (Table 2).

Simultaneous analysis of intimin types and phylogroups revealed that intimin type β1 was predominant (81%, 22/ 27) in phylogroup B1 compared to A (70%, 35/50). Ninety-four percent of B1 strains in Japan and 82% of group A strains in Bangladesh had intimin β1 (Fig. 1-a). Two other strains having intimin β1 belonged to phylogroup C and clade-1. However, intimin type ε1 fell into phylogroups E (3/5) and A (2/5). Double intimin-positive strains (β1/μB) belonged to phylogroups A (2/3) and B1 (1/3).

Virulence profile. According to the previously described scheme (10), 86 strains of aEPEC were assigned into three virulence groups. About 44% (38 of 86; Ia = 2; Ib = 36) of strains belonged to group I, among which 68% (26/38) were in phylogroup B1, and 81% (21/26) of them contained intimin type β1. Over half of the Japanese strains (61%) were in virulence group

Ib, and among them, 77% possessed intimin type β1. In contrast, the untypeable virulence group was predominant in Bangladesh, with 85% of them having intimin type β1 (Fig. 1-b). Simultaneous analysis of phylogroups, virulence groups, and intimin types revealed that subgroup B1-I-β1 (47%; 17/36) was predominant in Japan, while A-UT-β1 (48%; 24/50) was superior in Bangladesh (Fig. 2).

Multi-locus variable-number tandem-repeat analysis. The genotyping of aEPEC by MLVA delineated the 86 strains into 52 distinct MLVA patterns, with Simpson's index of diversity (D) at 73.1% (Table 3). The data were combined to make the minimum spanning tree (MST) with the previous data of aEPEC isolated from bovine, swine, food, healthy carriers, and patients (19, 21). The MLVA assigned most of the 16 Bangladeshi and Japanese strains (yellow and gray circles in Fig. 3a) to phylogroup A (yellow circles) of zone A shown in Fig. 3b. Numbers of A-Ib-β1 and A-UT-β1 poultry strains collected in Japan and Bangladesh were assigned to two sets of branches in zone A, while the other A-II and A-UT poultry strains possessing intimin θ/γ2 or α1/α2/μB belonged to another set of branches in the same zone. In contrast, 11 strains of the other 20 Japanese strains (Fig. 3a) were in zone D, shown in Fig. 3b as B1-Ib-β1. Zone B was mainly composed of phylogroup B2 strains (Fig. 3b) isolated from healthy carriers (white circles in Fig. 3a), and the strains belonged to virulence group II (black circles in Fig. 3c). Zone C included many patient strains (red circles) and bovine strains (black circles) of the phylogroup B1 (red circles in Fig. 3b); among the 19 strains of virulence group Ia (red squares in Fig. 3c), seven strains each of bovine and patients were in the zone. Although 14 swine strains were also in zone C, most of the intimin was θ/γ2 (green circles in Fig. 3d). Zone E included many swine strains of phylogroup A and virulence group II (black circles in Fig. 3c).

Antibiotic resistance pattern. The 86 aEPEC were examined for their antimicrobial resistance status against 12 antibiotics. Eighty percent of the strains (69/86) were resistant to one or more antimicrobial agents, and the remaining 17 isolates were sensitive to all antibiotics tested in this study. However, the resistance frequency and resistance pattern of the aEPEC strains isolated in Bangladesh were significantly higher than in the Japanese strains. About 53% (19/36) of strains from Japan were resistant to an antimicrobial agent, although only four strains showed resistance to ≥ 3 antibiotics (Tables S1 and S2). All Japanese strains were susceptible to CZ, C, ATM, GM, FOX, and AMC. In contrast, all strains from Bangladesh exhibited resistance to at least two antimicrobial agents and were susceptible to ATM and AMC. Among the multidrug resistant strains (64%, 55/86), phylogroup A was more prevalent (67%, 37/55) followed by B1 (20%, 11/55). Most of these multidrug resistant isolates were of the untypeable virulence group (52%, 29/55) followed by groups Ib (32%, 18/55) and II (16%, 9/55). About 67% (37/55) of the multidrug resistant strains contained intimin type β1. However, synchronized analysis of the antibiotic resistance pattern with phylogroup, virulence group, and intimin types revealed that subgroup B1-I-β1, which constituted a major part of aEPEC in Japan (47%; 17/36), was resistant to ≤ 2 antibiotics, except for one strain that showed resistance to four antibiotics. In the case of Bangladesh, the most prevalent subgroup was A-UT-β1 (48%; 24/50), and all of the strains in this group were resistant to ≥ 4 of the antimicrobial agents examined in this study. The most common resistance pattern observed in Bangladesh was AM-C-SXT-CIP-NA-GM-TE, and 20 of 50 strains exhibited resistance to these seven antibiotics (Table S2).

To analyze the comparative predominance of resistant aEPEC isolated from two geographical locations, MAR (multiple antibiotic resistance) indices were calculated. The MAR

index range for Japanese strains was 0.0-0.3 (0.06) and that for Bangladeshi strains was 0.2-0.7 (0.5).

Correlation analysis between virulence genes and antibiotic resistance. Associations among virulence genes and antibiotic resistance were recognized within the hierarchical clustering of the heatmap (Fig. 4), principal component analysis (Fig. S1), and correlation matrix analysis (Fig. S2). The hierarchical clustering divided the isolates into two clusters based on their virulence genes and antibiotic resistance pattern. Most of the Japanese strains were allocated to cluster A and Bangladeshi strains were in cluster B (Fig. 4). The PCA and correlation matrix indicated a stronger positive association between antibiotic resistance with one another and positive or negative correlation among the virulence genes (Figs. S1 and S2). The PCA and correlation matrices indicated that the presence of three variant genes of *lpf* (*lpfA*, *lpfAR141*, *lpfA0113*) had a strong positive correlation. The gene *astA* had a negative correlation with *lpfA*, *lpfAR141*, *lpfA0113*, *ureD*, *nleE*, and *efa1*. The *efa1*, *nleE*, and *ureD* genes had a positive correlation with each other. The co-resistance phenomenon was observed among TE, AM, C, SXT, CIP, and GM, and among CZ, CRO, and FOX; these two sets of antibiotics were positively associated in each group, resulting in co-resistance. The gene *astA* showed a weak positive association with the co-resistance of the former group. In contrast, the presence of *lpfA*, *lpfAR141*, *lpfA0113*, *ureD*, *nleE*, and *efa1* was negatively related to resistance against these antibiotics (Figs. S1 and S2).

DISCUSSION

Our previous reports (19, 21) suggested that aEPEC organisms, particularly of phylogroups B1, virulence group I, and intimin type β1, cause diarrhea in humans. Cattle have been shown to

be a source of infection; neither swine nor healthy people seemed to be a source. In this study, we explored the possibility of an avian host as the source of aEPEC infection, concurrently performing phylogenetic grouping, intimin typing, serotyping, virulence profiling, antibiotic resistance patterning, and MLVA of avian aEPEC strains.

The predominance (50%) of phylogroup B1 in Japanese poultry strains suggests poultry as another source of B1 strains in Japanese patients, because phylogroup B1 was prevalent among diarrheal patients and cattle while phylogroups A and B2 were more prevalent among pigs and healthy humans, respectively, in Japan than among patients in our previous studies (19, 21). According to the virulence scheme (10), most of the isolates from Japanese poultry were in virulence group Ib. The aEPEC of this virulence group are reportedly infective and cause diarrhea in humans (32). The combined use of phylogenetic grouping and virulence profiles confirmed that group B1-Ib was predominant in the avian hosts of Japan. This finding also indicates that avian aEPEC could play an etiologically important role in Japan since the B1-Ia and B1-Ib strains were specific among patients, while groups B2-II and A-II are prevalent among healthy individuals and swine, respectively (19, 21). Further, the analysis of the intimin types along with phylogenetic and virulence groups revealed that most of the β1 strains belonged to phylogroup B1 and virulence group I in Japan (Fig. 2). This subgroup (B1-I-β1) of aEPEC is highly prevalent among bovines and diarrheal patients in Japan (19, 21). Besides Japan, a large number of B1-β1 strains are also prevalent among diarrheal patients in Brazil (33).

In contrast, 68% of Bangladeshi aEPEC strains belonged to phylogroup A, and the untypeable virulence group (UT) was predominant. Most of the β1-intimin strains isolated from Bangladesh belonged to phylogroup A and the untypeable virulence group. This finding is consistent with previous reports in which aEPEC of phylogroup A was recovered from 36% of

poultry in Korea (24), and most of the β1 strains belonged to phylogroups A and B1 (34). Although aEPEC organisms of phylogroup A have also been detected from diarrheal patients in Brazil (35), it remains to be elucidated whether the subgroup (A-UT-β1) of aEPEC from poultry can be a causal agent for human diarrheal diseases in Bangladesh.

Most of the serotypes isolated in this study (O26, O55, O177) are included in the list of frequently reported clinical EPEC serotypes (36–38). O serotyping itself could not provide useful information about whether these strains are pathogenic to humans, because the atypical EPEC strains that are significantly associated with diarrhea belonged to many different serogroups or were untypeable (32). It might be generally assumed that a group of strains possessing the same O antigen could be assigned to the same phylogroup; however, each of the O177, O26 and O123 strains belonged to two phylogroups. Other researchers reported similar (39, 40).

As genotyping is a useful tool for epidemiological studies, we combined MLVA patterning of the isolates with other molecular typing methods, which successfully discriminated the isolates among different branches in the MST. Most of the B1-I-β1 strains fitted to the same zone C, since most of the bovine and diarrheal B1-I-β1 strains were located in cluster-2 of the previous report (19). The avian B1-I-β1 strains were also expected to be in zone C based on the type; however, 11 strains were in zone D, which is apart from zone C, and seven strains were in the periphery of zone C. Furthermore, the majority of poultry strains collected in Japan and Bangladesh were assigned to zone A. Since most poultry strains occupied the zones A and C with only a few strains having orignated in other animal species, these strains might exclusively circulate among chickens, unlike swine strains which were not only in zone E but also in B and C. Although the MST suggested that poultry aEPEC is less likely to be a causative agent for human diarrhea, the virulence of Bangladeshi poultry strains should be clarified by combining

them with another investigation of aEPEC among diarrheal patients, healthy carriers, and other sources of aEPEC in Bangladesh in the future.

Each phylogroup was scattered onto different branches on the MST in this study, as detailed above in our explanation of the relationship between O antigens and phylogroups. This suggests that the recombination occurred multiple times horizontally in their phylogenetic history (41); the complicated evolutionary background of aEPEC should be taken into consideration when studying its host adaptation and virulence, transmission networks, and zoonotic potential. We recognize that it is insufficient to analyze them only by genotyping methods, and must improve the analysis with genomic comparison using high throughput sequencers. Recently, the enteropathogenicity of aEPEC and tEPEC organisms isolated from humans was analyzed using advanced phylogenomic methods by Ingle et al. and Hazen et al., respectively (11, 12). Both groups reported that polyphylogenomic lineages were present even among strains isolated only from humans. We must therefore reevaluate the zones shown for swine, poultry, healthy carriers, and bovine and patient strains in this study to show the polyphyletic nature using genomic sequence-based analysis.

EPEC seem to persist in the intestine for extensive periods compared to other DEC pathotypes (42). This persistence can be associated with various factors, including multidrug resistance patterns of the pathogen. *E. coli* of poultry origin are potentially dangerous to humans from the perspective of antimicrobial resistance (43). About 80% of isolates in this study were resistant to at least one antimicrobial agent. NA resistance was most common along with TE, AM, C, SXT, and CIP, while most of the strains were sensitive to CZ, AMC, CRO, and FOX. Similar findings of common resistance to NA, TE, AM, SXT, and CIP among *E. coli* isolates from avian origin and other food animals have been reported by many researchers from China

(44), Egypt (45), France (46), Bangladesh (47), and Japan (19). Although phylogroups D and B2 were related to higher drug resistance patterns in previous reports (48), we did not find any B2 strains in poultry. Phylogroup D strains isolated from retail foods showed the highest antimicrobial resistant rate in our previous report (49). Most of the multidrug resistant strains in this study were in group A-UT-β1. A similar result with group A-MDR in poultry *E. coli* was reported by another research group (50).

Most of the multidrug resistant strains of aEPEC originated from Bangladesh in this study, and all of those were resistant to at least two antimicrobial agents, including quinolone and the third-generation cephalosporin. The high prevalence of resistance to quinolone and the third-generation cephalosporin is correlated with usage in the South Asia region including Bangladesh (51). It was previously reported that multidrug resistant *E. coli* were isolated from food animals and patients in Bangladesh (47, 52). Widespread use of broad-spectrum antibiotics in food animals could be an issue in the development of drug resistant bacteria (53). Frequent use of new antibiotics in the management of diarrhea in South Asian countries including Bangladesh has led to the emergence of multidrug resistant aEPEC, because using new antibiotics for the treatment of drug resistant aEPEC leads to the buildup of resistance determinants rather than their replacement (51). Conversely, the majority of aEPEC isolated from Japan were sensitive to all antimicrobial agents used in this study, and only four strains exhibited resistance to ≥ 3 antibiotics. The comparatively high MAR indices in Bangladeshi strains indicate a high-risk level of antibiotic resistant aEPEC in Bangladeshi poultry compared to Japanese poultry. New antibiotics are frequently used in Bangladesh, which has led to the development of multidrug resistant aEPEC in that region (51).

Overuse or improper use of antibiotics selects for antibiotic resistant mutants or bacterial populations that previously received plasmids encoding antibiotic resistance genes. Those resistant bacteria can provide resistance genes to other bacteria (54, 55). We analyzed the correlation between virulence genes and antibiotic resistance by PCA and correlation matrix analysis, because most Bangladeshi strains belonged to lower virulence groups II and UT. Indeed, the genes of virulence group I (*lpfA*, *lpfAR141*, *lpfA0113*, *ureD*, *nleE*, and *efa1*), which have a significant association with diarrhea (10), were negatively correlated with or unrelated to antibiotic resistance. There was no negative correlation among the antibiotics in aEPEC in this study, although we performed the analysis according to the method of Osman et al. (45), who found a negative correlation between gentamycin and amoxicillin in *Bacillus* spp. in Egypt (45). The selection of alternative antibiotics to treat infections with multidrug resistant aEPEC may be difficult.

This study suggested that not only bovines but also poultry may serve as the source of aEPEC B1-Ib-β1, which is potentially pathogenic to humans, in Japan (19, 21), but those strains are not multidrug resistant and were somewhat far from patient strains on the MST. In Bangladesh, poultry is a reservoir of multidrug resistant aEPEC; however, additional investigations are vital to discover whether the multidrug resistant aEPEC of A-UT-β1 are hazardous to humans in Bangladesh.

MATERIALS AND METHODS

Sample collection. A total of 600 poultry fecal samples were collected from 20 poultry farms (30 samples from each farm) in seven districts of Bangladesh, and 49 poultry cecal feces samples were from the Hyogo meat inspection center. Although a total of 358 poultry samples were

collected in Japan, and PCR screening suggested that 224 of them were positive for EPEC (23), 49 samples were chosen to represent each farm. Samples were collected using a convenient method without repetition from any bird. Bacteriological sample collecting media (pro-media FC-20, ELMEX, Tokyo, Japan) was used for the sample collection.

Isolation of EPEC from fecal specimens. Fecal samples were cultured in trypticase soy broth for 20 h at 37°C for bacterial enrichment. Extraction of the bacterial genomic DNA was carried out using a genomic DNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. We used our multiplex real-time PCR method (30) to screen the samples targeting *eae, stx1*, and *stx2* genes, and the EPEC strains were isolated from the *eae*-positive broths using the HGMF-CH method (29). Fifty EPEC strains were isolated successfully from poultry fecal samples in Bangladesh and 36 from the samples collected in Japan. A total of 86 EPEC strains were used in the molecular study by O antigen genotyping, phylogenetic grouping, virulence profiling, subtyping of *eae*, multiple locus variable number tandem repeat analysis, and antibiotic resistance status of the strains. DH5α was used as a non-diarrheagenic negative control throughout the experiment.

Phylogenetic grouping. The distributions of phylogroups amongst EPEC isolates were analyzed by quadruplex PCR assay based on Clermont's new method of phylogenetic grouping (14). This new phylogenetic grouping method enables an *E. coli* to be assigned into one of the eight phylogroups, A, B1, B2, C, D, E, F and clade-1 (14).

O antigen genotyping. The O antigens of EPEC strains were determined by the multiplex PCR method targeting the O-AGCs using 162 pairs of primers to detect 182 serogroups of *E. coli*, excluding O14 and O57 (which contain no O-AGCs at the typical locus): 145 serogroups had unique O-AGCs, and the other 37 shared identical or very similar O-AGCs, which were placed

into 16 groups. Finally 20 multiplex PCR was used to identify 182 O serogroups as described previously (27).

Intimin typing. Subtyping of the intimin gene (*eae*) was performed using 17 pairs of intimin type-specific PCR primers to detect 17 subtypes of intimin (α1, α2, β1, ξR/β2B, δ/κ/β2O, γ1, θ/γ2, ε1, νR/ε2, μR/ι2, ζ, η, μB, νB, ι1, λ, ξB) according to the published protocol (17).

Virulence profiling. Virulence profiles of EPEC were performed based on 12 virulence genes or markers, including OI-122 genes (*efa1 [lifA], set [ent], nleB,* and *nleE*) and genes in other locations (*lpfA, ehxA, ureD, paa, yjaA, ibeA, b1121,* and *astA*), which have been reported to be significantly associated with diarrhea (10). The scheme classified aEPEC strains into two main virulence groups: group I strains were distinguished by the presence of OI-122 genes and/or *lpfA* genes as well as the absence of the *yjaA* gene, while group II strains were categorized by the presence of the *yjaA* gene and the absence of OI-122 and *lpfA* genes. Group I strains were further divided into subgroups Ia and Ib depending on whether they contained the gene *efa1* (*lifA*), which has the strongest association with diarrhea.

Antimicrobial susceptibility test. The isolated EPEC strains were subjected to antibiotic susceptibility testing for 12 antibiotics (Becton, Dickinson and Company, Piscataway, New Jersey, USA) following the disc-diffusion method on Mueller-Hinton agar plates according to M100-S28 of the Clinical and Laboratory Standards Institute (56). The concentrations of the tested antibiotic discs were as follows: ampicillin (AM) 10 μg, amoxicillin-clavulanic acid (AMC) 30 μg, cefazolin (CZ) 30 μg, ceftriaxone (CRO) 30 μg, cefoxitin (FOX) 30 μg, aztreonam (ATM) 30 μg, gentamicin (GM) 10 μg, tetracycline (Te) 30 μg, ciprofloxacin (CIP) 5 μg, nalidixic acid (NA) 30 μg, chloramphenicol (C) 30 μg, and sulfamethoxazole-trimethoprim (SXT) 25 μg. The isolates were classified as susceptible (S), intermediate (I), or resistant (R)

according to the zone of diameter described in CLSI-M100-S28. Detection of ESBL-producing strains was carried out by a combination of disc diffusion test with clavulanic acid (56).

MAR $_{index}$. The MAR (multiple antibiotic resistance) index was calculated using the formula a/(b×c), where 'a' is the aggregate antibiotic resistance score of all isolates from the sample, 'b' is the number of antibiotics to which the isolates were exposed, and 'c' is the number of isolates from the sample (57).

Multiple locus variable number tandem repeat analysis. The generic *E. coli* MLVA (GECM10) was performed to clarify the genetic relationship between the isolated EPEC strains by ten tandem repeats (CVN001, CVN002, CVN003, CVN004, CVN007, CVN014, CVN015, CCR001, CVN016, and CVN017) using PCR with multiple dye colored primers (28). PCR products were exposed to capillary electrophoresis on an ABI-3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Each peak was recognized and rendered to color and size, and the allele number was allocated based on fragment sizes. The minimum spanning tree (MST) was constructed using BioNumerics ver. 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) according to a protocol described previously (28). The obtained result was linked to other molecular markers to explain the genetic relationship of the isolated EPEC.

Statistical analysis. The open statistical program R was used for statistical analysis (58). Numerical coding was implemented for correlation matrix analysis. The presence or absence of a target gene was indicated as 1 and 0, respectively. For antibiotic resistance, antibiotic sensitivity was designated as 0 and resistance as 1. The R packages 'FactoMineR (59)' and 'factoextra'(60) were used to perform and visualize principal component analysis (PCA). The 'cor' function was used to analyze correlations, and the 'cor.test' function was used to determine significance between variables. Significant correlations were visualized using the 'corrplot' function from the

413 'corrplot' package. The heatmap representations were performed by the function 'heatmap.2' in
414 the 'gplot' package. Significant differences between the prevalence of virulence markers or
415 antibiotic resistance were determined by χ^2 tests.
416
417
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423

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612

613

614 **FIGURE LEGENDS**

615 FIG 1. Distribution of intimin types among phylogroups and virulence groups in Japan and
616 Bangladesh. (a) Distribution of intimin types among different phylogroups. (b) Distribution of
617 intimin types among different virulence groups. JP indicates the strains isolated from Japan, and
618 BD indicates the strains isolated from Bangladesh.
619 * indicates significant at $P \leq 0.05$, and ** indicates highly significant at $P \leq 0.01$.

620

621 FIG 2. Distribution of phylogroups, virulence groups, and intimin types among the aEPEC
622 strains isolated from poultry fecal specimens in Japan and Bangladesh.

623

624 FIG 3. Population modelling using the minimum spanning tree (MST) method of 228 aEPEC
625 strains isolated from cattle, pig, poultry, foods, healthy carriers, and patients. The MST was
626 constructed using the highest number of single-locus variants as the priority rule with no creation
627 of hypothetical (or missing) types. The pale brown, green, pink, blue, and ivory clouds indicate
628 the zones A, B, C, D, and E, respectively. (a) Strains isolated from different hosts are shown in
629 different colors. White, red, green, blue, black, gray, and yellow indicate strains of healthy
630 carriers, patients, foods, pig, cattle, Japanese poultry, and Bangladeshi poultry, respectively. (b)
631 Associations of phylogenetic group and MLVA are shown in different colors. Yellow circles, red
632 circles, green circles, blue circles, purple squares, black circles, white circles, gray circles, and

633 light green circles indicate strains of phylogenetic groups A, B1, B2, C, D, E, F, clade-1, and
634 unknown phylogenetic group, respectively. (c) Associations of virulence group and MLVA are
635 shown in the figure. Red closed squares, blue closed squares, black circles, and white circles
636 indicate strains of virulence groups Ia, Ib, II, and unknown virulence group, respectively. (d)
637 Association of intimin types and MLVA are shown in the figure. Red circles, green circles, gray
638 circles, yellow circles, closed blue circles, closed white circles, purple squares, light blue squares,
639 pink circles, aqua circles, white squares, and black circles indicate the intimin type β1, θ/γ2, ζ,
640 δ/κ/β2O, ι1, ξR/β2B, νR/ε2, ε1, γ1, α1/α2/μB, η, and untypeable, respectively.
641
642 FIG 4. Heatmap and hierarchical clustering of aEPEC isolates based on virulent genes and
643 antibiotic resistance. Green indicates the presence and red indicates the absence of genes or
644 antibiotic resistance. The upper row of the heatmap is a color indication of the geographical
645 location of the strains. Letters A and B denote the two clusters formed by genotyping and
646 antibiotic resistance patterns of the isolates. The hierarchical clustering was implemented using
647 Wald's method and a binary distance matrix.
648
649 FIG S1. Principal component analysis of drug resistance and gene contribution, a) relationships
650 with genes and antibiotic resistance, b) geographic source of the isolates; ellipses represent 95%
651 confidence intervals. Two lines pointing in the same direction indicate a high correlation,
652 orthogonal lines indicate no relationship, and lines pointing in opposite directions indicate a
653 negative correlation.
654
655 FIG S2. Spearman correlation matrix of antibiotic resistance and virulent genes. The figure

shows only significant correlations ($p < 0.05$). Blue circles indicate significant positive correlations and red circles indicate negative correlations. The size and strength of the color are indications of the numerical value of the phi correlation coefficient.

(b)

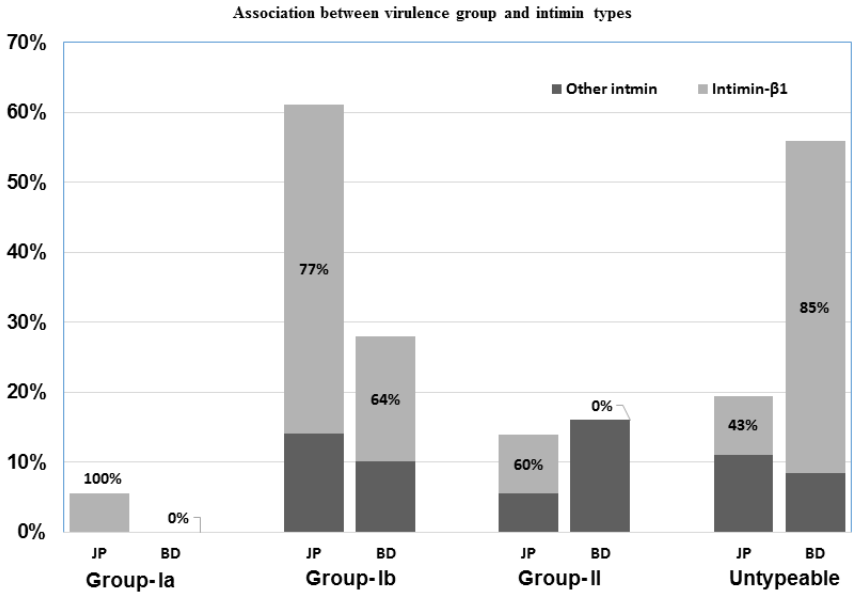


Fig. 1

(a)

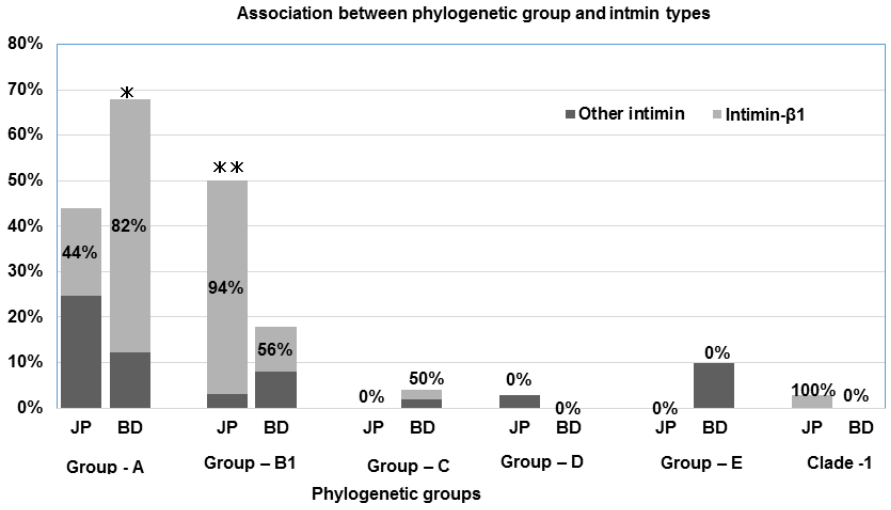


Fig. 1

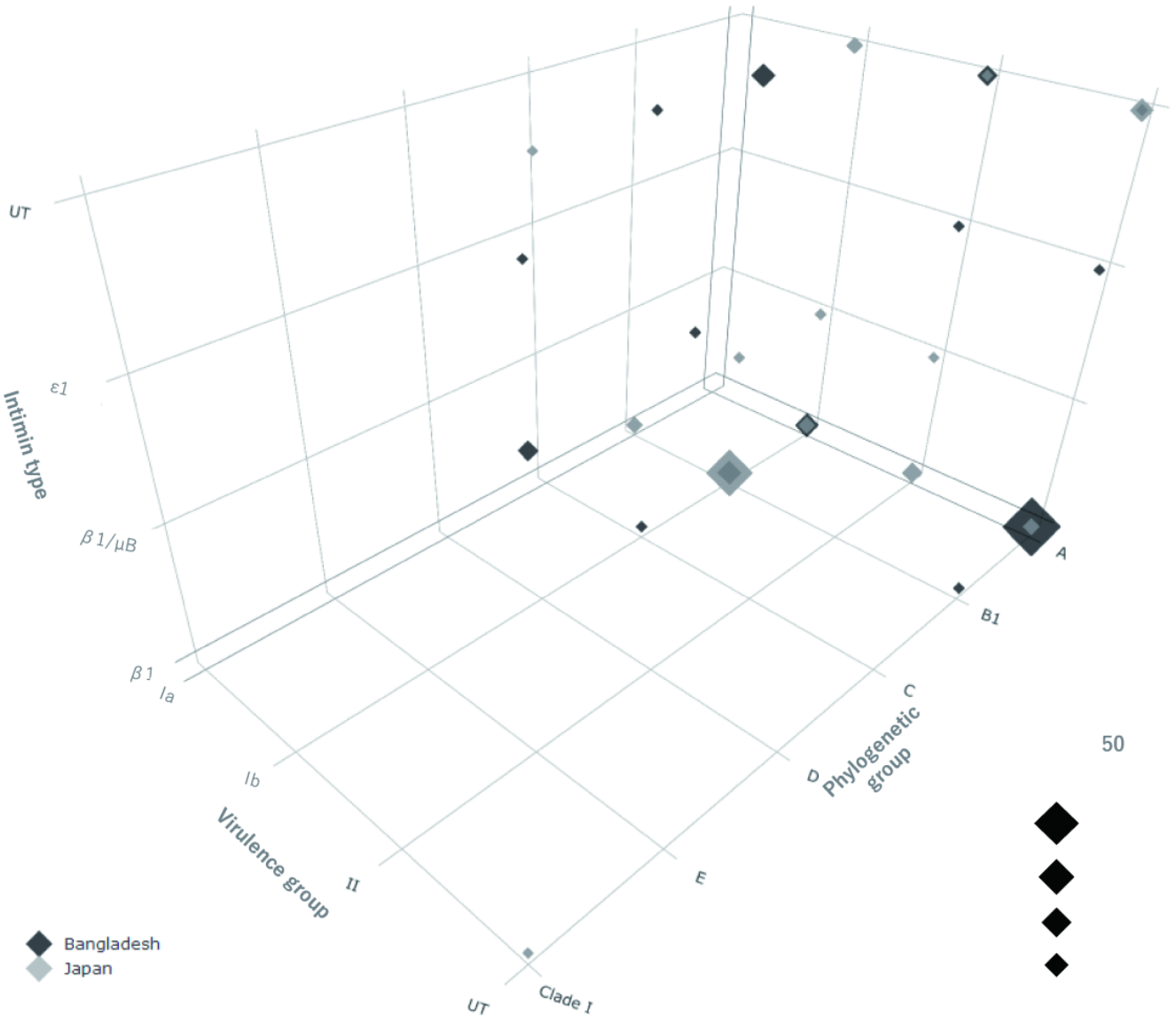


Figure 2

Figure 3 (a)

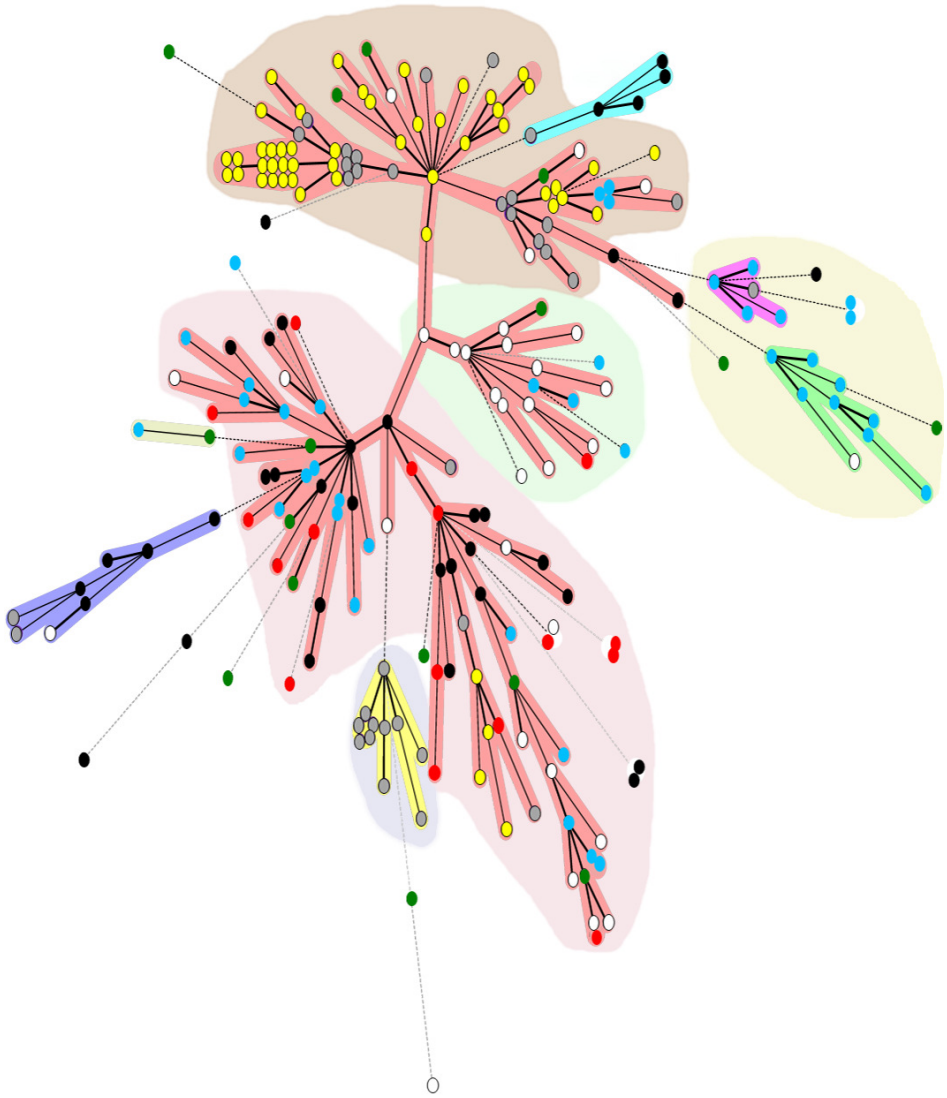
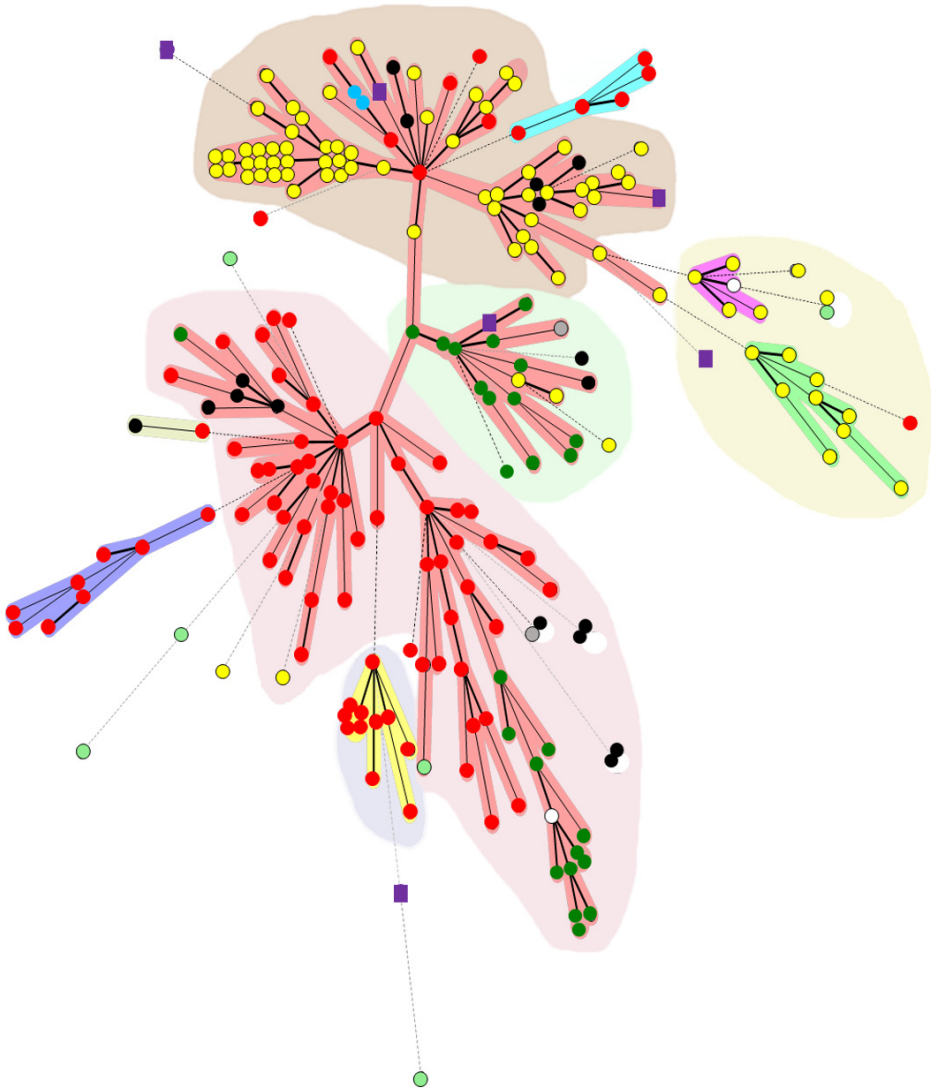


Figure 3 (b)



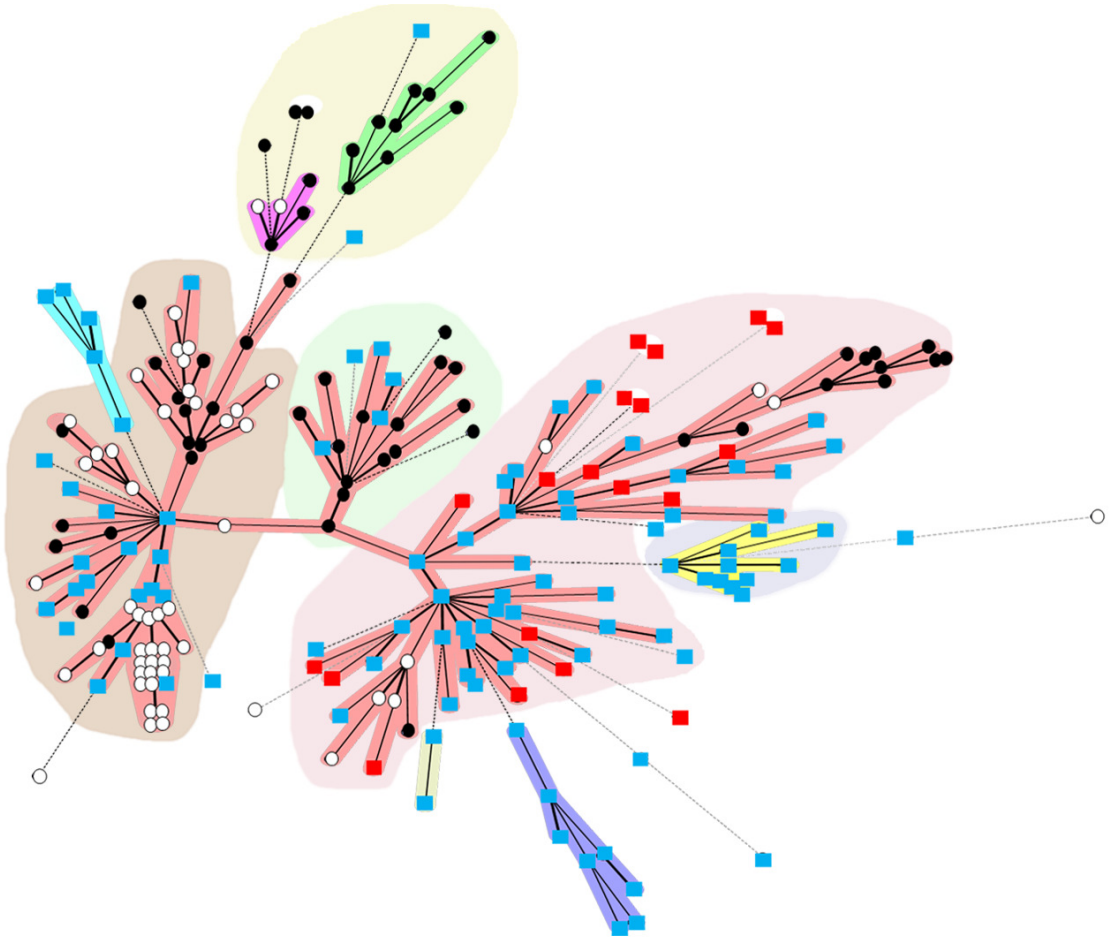
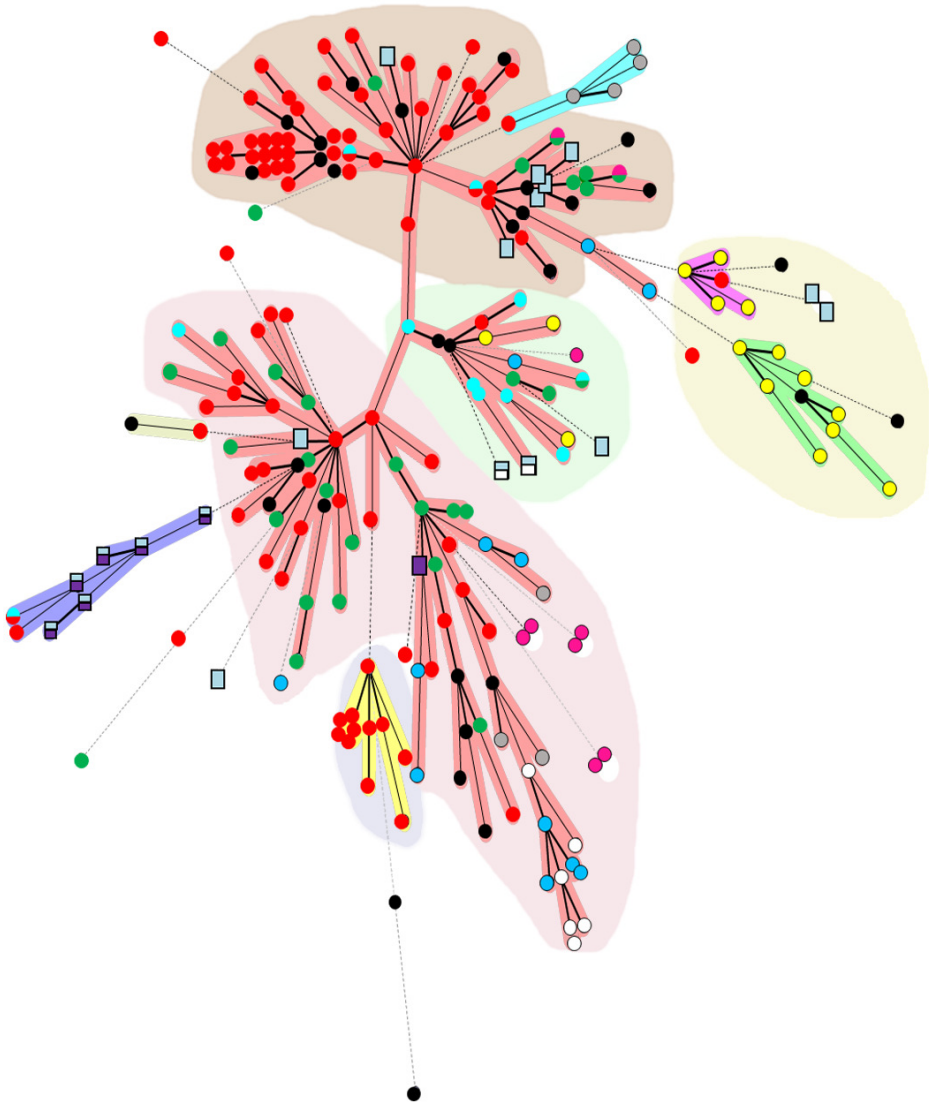


Figure 3 (c)

Figure 3 (d)



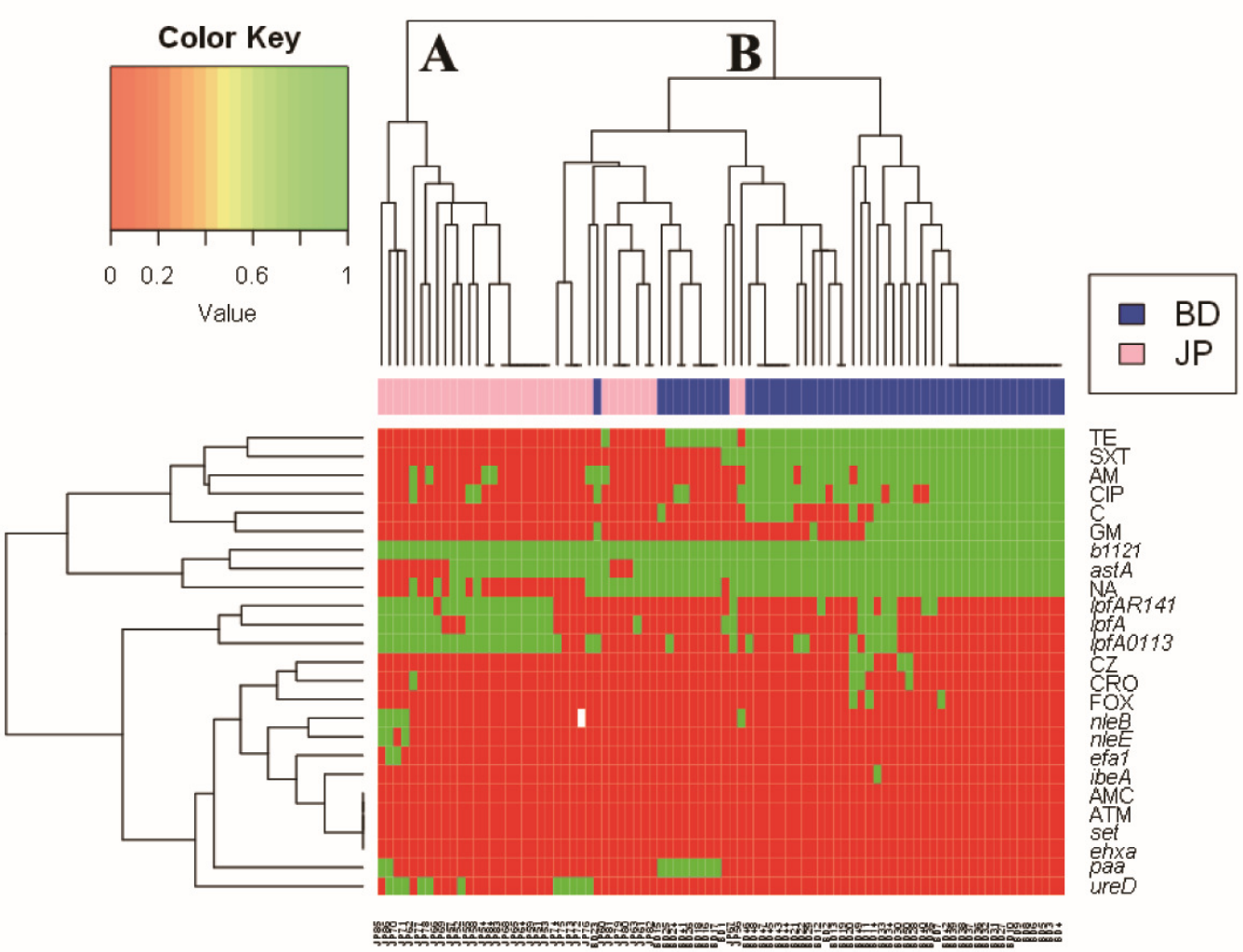


Figure 4

.

TABLE 1: Distribution of O serogroups among different phylogenetic groups

Phylogenetic groups (%)	O serotypes		
	Japanese poultry	Bangladeshi poultry	Subtotal (%)
A	16 (44.4) †; O123/O186 (2)‡, O25, O87, O116, UT (11)	34 (68.00); O26 (3), O49 (5), O80 (5), O177 (19), UT (2).	50 (58.1); O123/O186 (2), O25, O26 (3), O49 (5), O80 (5), O87, O116, O177 (19), (UT (13).
B1	18(50.0); O123/O186, UT (17)	09 (18.00); O8 (4), O177 (5).	27 (31.3); O8 (4), O123/O186, O177 (5), UT (17).
B2	ND	ND	ND
C	ND	02 (4.0); O55 (2).	2 (2.3); O55 (2)
D	1 (2.7); UT	ND	1 (1.1); UT
E	ND	05 (10.0); O26 (5).	5 (5.8); O26 (5)
F	ND	ND	ND
Clade - 1	1 (2.7); UT	ND	1 (1.1); UT
Total	36	50	86

ND: not detected

†: Subtotal number of strains and percentage among Japanese or Bangladeshi strains.

‡: Serogroup and number of strains

TABLE 2: Distribution of O serogroups among different intimin types

Intimin types (%)	O serotypes		
	Japanese poultry	Bangladeshi poultry	Subtotal (%)
β1	O123/O186(2)‡, O87, UT (21)	O177(23),O49(5), O80(4), O55, UT	O177(23), O49(5), O80 (4), O123/O186(2), O55, O87, UT (22)
ε1	00	O26 (5)	O26 (5)
β1/μB	UT (4)	ND	UT (4)
UT	O25,O123/O186,O56,O116, UT (4)	O8 (4), O26(3), O80, O177, O55, UT	O8(4), O26(3), O25, O123/O186, O56, O116, O80, O177, O55, UT (5)
Total	36	50	86

ND: not detected

‡: Serogroup and number of strains

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TABLE 3: Simpson's index of diversity among phylogenetic group, O antigen, intimin types, virulence group and MLVA type

Phylogenetic group		O antigen		Intimin types		Virulence group		MLVA	
Type	No.	Type	No.	Type	No.	Type	No.	Type	No.
A	50	O8	4	β1	58	Ia	2	Type-1	42
B1	27	O123/O186	3	ε1	5	Ib	36	Type-2	12
C	2	O25	1	β1/μB	4	II	13	Type -3	8
D	1	O26	8	UT	19	UT	35	Type -4	5
E	5	O49	5					Type -5	4
Clade-1	1	O55	2					Type -6	4
		O80	5					Type -7	3
		O87	1					Type -8	2
		O116	1					Type -9	2
		O177	24					Type -10	2
		UT	32					Type -11	2
D =	56.58%		77.3%		49.7%		64.3%		73.1%

UT, untypeable; D, Simpson's index of diversity.

*42 strains formed unique MLVA profiles; Other 44 strains formed 10 different MLVA patterns.

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