NEW MICROBIOLOGICA, **36**, 385-394, 2013

Phylogenetic analysis of multidrug-resistant *Escherichia coli* clones isolated from humans and poultry

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SUMMARY

Chicken products represent a source for multidrug-resistant *Escherichia coli* causing extraintestinal infections (ExPEC) in humans. We applied phylogenetic analysis to a collection of *E. coli* strains from both hosts (poultry/humans) to improve our understanding of the origin and spread of ExPEC in humans. The dataset consisted of 58 sequences among 172 *E. coli* strains from human extraintestinal infections and avian species. Human phylogenetic tree analysis showed a major clade, within which ST clones belonging to groups A and B1 were largely intermixed, and two clusters, each exclusively including B2 or D clones. The avian tree exhibited greater heterogeneity between and within clades/clusters. In the Bayesian tree, consisting of sequences from both human and avian *E. coli*, the B2 and D human ST clones were clustered together separate from the avian strains, whereas B1 and A ST clones (frequently associated with multidrug resistance) were intermixed with avian strains. This study suggests that a subgroup of *E. coli* clones, A and B1, associated with multidrug resistance, is potentially exchangeable between poultry and humans. Such a subgroup may be of public health concern. On the contrary, *E. coli* clones included in B2 and D appeared clearly separate between human and avian sources, suggesting a minor zoonotic potential of these phylotypes.

KEY WORDS: Escherichia coli, Phylogeny, Zoonosis, Multidrug resistance.

Received May 31, 2013 *Accepted* August 11, 2013

INTRODUCTION

Escherichia coli extraintestinal infections (urinary tract infections and bloodstream infections) represent a significant public health burden worldwide (Johnson and Russo, 2002; Russo and Johnson, 2003). Since the 2000s, antimicrobial resistance among *E. coli* isolates has increased contributing to the complexity in management of such infections (Karlowsky *et al.,* 2006; Pallett and Hand, 2010). Prevention and control of the spread of *E. coli*-causing infections in humans requires an understanding of the population genetics of this pathogen. In the past, multilocus enzyme electrophoresis (MLEE) demonstrated that the *E. coli* population is largely clonal and strains fall into four major phylogenetic groups (A, B1, B2 and D) (Selander *et al.,* 1987; Goullet and Picard, 1989; Clermont *et al.,* 2000). At present, in the genomic era, multilocus sequence typing (MLST) has been increasingly used to investigate *E. coli* population genetics (Tartof *et al.,* 2005; Wirth *et al.,* 2006; Lau *et al.,* 2008; Köhler and Dobrindt, 2011). However, the majority of the studies in the literature used MLST data to identify allelic profiles that define distinct sequence types (STs), but no in-depth phylogenetic reconstruction was carried out from the sequences. Moreover, a few investigations only compared the phylogeny of *E. coli* isolates from different hosts (Gordon and Cowling, 2003; Escobar-

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Páramo *et al.,* 2006; Baldy-Chudzik *et al.,* 2008; Tenaillon *et al.,* 2010).
Since poultry has been suggested as a reservoir for multidrug-resistant *E. coli* strains causing extraintestinal infections (Extraintestinal pathogenic *E. coli,* ExPEC) in humans, the phylogenetic analysis of strains circulating in both hosts can add useful data for the evaluation of the potential zoonotic risk (Johnson *et al.,* 2005; Collignon and Angulo, 2006; Vincent *et al.,* 2010; Bélanger *et al.,* 2011; Bergeron *et al.,* 2012; Nordstrom *et al.,* 2013). In a previous paper, we identified the major clonal groups associated with fluoroquinolone and multidrug resistance in *E. coli* of human and avian origin, but the phylogeny of this collection of strains was not investigated (Giufrè *et al.,* 2012). In the present study, we applied a Bayesian approach to reconstruct the phylogeny of the previously studied collection of human and avian *E. coli* strains, in order to improve our understanding of the origin and spread of ExPEC in humans.

MATERIAL AND METHODS

Bacterial strains and sequence datasets

A sample of 172 *E. coli* strains isolated from human extraintestinal infections (83 strains from UTI, of these 22 were susceptible and 61 were resistant to ciprofloxacin, and 46 strains from blood, of these 7 were susceptible and 39 were resistant to ciprofloxacin) and healthy avian species (43 strains, of these 19 were susceptible and 24 were resistant to ciprofloxacin) was chosen among a total of 378 *E. coli* strains, following criteria previously described (Giufrè *et al.,* 2012). All 172 strains were subjected to MLST analysis and 58 distinct STs were identified, irrespective of the ciprofloxacin susceptibility status (Giufrè *et al.,* 2012). Of these 58 STs, 28 STs derived exclusively from human strains (H-STs), 21 exclusively from avian strains (A-STs) and 9 were shared by human and avian strains (HA-STs). The assignment of each ST to one of the four major phylogenetic groups (A, B1, B2 and D) was carried out as previously described, according to the original triplex PCR method (Clermont *et al.,* 2000; Giufrè *et al.,* 2012).
For each ST, the seven MLST gene sequences were concatenated into a single sequence of 3423 bp. The overall dataset consisted of the 58 ST concatenated sequences. The date of collection (in months) was known for all STs and ranged from January 2009 to November 2010. To study the phylogeny of human and avian strains separately, two sub-datasets were built. The first sub-dataset included 37 STs recovered from human strains (28 H-STs and 9 HA-STs), of these 37STs, 22 were found associated with multidrug resistance; the second sub-dataset consisted of 30 STs from avian strains (21 A-STs and 9 HA-STs), of these 30 STs, 15 were found associated with multidrug resistance. ST was defined as associated with multidrug resistance when ≥60% of strains belonging to that ST exhibited a multidrug-resistant (MDR) phenotype (the strain was resistant to at least 3 antimicrobial agents of different classes, such as penicillins, 3rd generation cephalosporins, fluoroquinolones, folate pathway inhibitors, aminoglycosides).

Phylogenetic analysis

All the concatenated ST sequences were aligned using Clustal X software followed by manual editing using the Bioedit program, as already described (Ciccozzi *et al.,* 2011). All the phylogenetic trees were generated with the TN93 + I + G model of nucleotide substitution. The evolutionary model was chosen, for each sub-dataset, as the best-fitting nucleotide substitution model in accordance with the results of the hierarchical likelihood ratio test (HLRT) implemented in MODELTEST software (version 3.6) as already described (Swofford and Sullivan, 2009; Lo Presti *et al.,* 2012).
For the first and second sub-datasets, maximum likelihood phylogenetic trees were generated with Phyml 3.0 server (http://www.atgc-montpellier.fr/phyml/) (Zehender *et al.,* 2001). The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed with the bootstrap analysis. The evolutionary distances among different groups were calculated with MEGA4 software under the Logdet model (Tamura *et al.,* 2007).

Bayesian phylogenetic analysis

The dated tree for the overall dataset was estimated using a Bayesian MCMC approach (Beast v. 1.6.1, http://beast.bio.ed.ac.uk) implementing a TN93+ Invariant + Gamma model using both a

strict and an uncorrelated log-normal relaxed clock model (Drummond and Rambaut, 2007; Lo Presti *et al.,* 2012). As coalescent priors, we compared four parametric demographic models of population growth (constant size, exponential, logistic growth, expansional) and a Bayesian skyline plot (BSP, a non-parametric piecewise-constant model). Chains were conducted for at least 50 x10^6 generations, and sampled every 5,000 steps. Convergence was assessed on the basis of the effective sampling size (ESS). Only parameter estimates with ESSs >200 were accepted (Ciccozzi *et al.,* 2011). Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals and the best fitting models were selected by a Bayes factor (BF, using marginal likelihoods) implemented in Beast (Drummond and Rambaut, 2007). In accordance with Kass and Raftery (Kass and Raftery, 1995), the strength of the evidence against H_0 was evaluated as follows: 2lnBF <2 no evidence; 2-6 weak evidence; 6-10 strong evidence, and >10 very strong evidence. A negative 2lnBF indicates evidence in favour of H_0. Only values of ≥6 were considered significant. The trees were summarized in a target tree by the Tree Annotator program included in the Beast package by choosing the tree with the maximum product of posterior probabilities (maximum clade credibility) after a 10% burn-in (Ciccozzi *et al.,* 2011). TMRCA (the Time of the Most Recent Common Ancestor) estimates were expressed as mean and 95% HPD years before the most recent sampling dates, corresponding to 2010 in this study.

RESULTS

Maximum likelihood phylogenetic trees

Maximum likelihood (ML) phylogenetic trees of the first and second sub-datasets are shown in Figures 1 and 2, respectively. Phylogenetic relationships among the different *E. coli* STs were supported by the bootstrap analysis with values >70%.

Figure 1 shows the phylogenetic tree obtained from 37 concatenated MLST sequences from human *E. coli* strains. A main clade (clade 2) was observed, within which different statistically supported clusters could be identified. In this clade, STs belonging to phylogenetic groups B1 and A were intermixed, apart from a minor cluster of B2 STs (ST707, ST805) that segregated together with a B1 sequence (ST1086). Out of Clade 2, two statistically supported clusters (1 and 3) were identified. Cluster 1 exclusively included B2 sequences, while cluster 3 encompassed D group STs, only. Sequences associated with MDR isolates (marked in bold in the Figure) were intermixed with susceptible ones, but they predominated in clade 2, in particular among A and B1 STs (% Resistant STs =63.2), and cluster 3 (66.6%). To investigate whether STs associated with multidrug resistance exhibited a different tree topology, we constructed a new phylogenetic tree exclusively including such STs, but no significant modifications of the topology were observed (data not shown).

Figure 2 shows the phylogenetic tree obtained from 30 concatenated MLST sequences from avian *E. coli* strains. The presence of a main statistically supported clade (clade n. 6), which included A and D group STs, with the addition of a B2 sequence (ST1638) was detected. Out of the main clade, five statistically supported clusters (clusters from 1 to 5) were identified containing only pure subtypes. STs deriving from MDR isolates were dispersed among the different clade/clusters (50% in the clade 6). In agreement with such dispersion, neither substantial modifications of the tree topology nor an increase in clustering of ST sequences were detected exclusively using STs associated with multidrug resistance (data not shown).

Bayesian phylogenetic analysis

Figure 3 shows the Bayesian maximum clade credibility tree of the overall dataset including 58 MLST sequences from both human and avian strains, with the tMRCA estimates. BF analysis showed that the relaxed clock fitted the data significantly better than the strict clock (2lnBF between the strict and relaxed clock was 165.4 in favour of the relaxed clock). Under the relaxed clock, BF analysis showed that the exponential model fitted the data significantly better than the other models (2lnBF>100). Different statistically supported clades and clusters (posterior probability, p.p.≥98) were identified, but only clades/clusters with p.p.=1 were dated. Clade 1, that included most B2 phylogroup STs we analysed, dated back to the year 1993 (95% HPD:

1965-2007) with the sub-clade 1a dating back to the year 1999 (95% HPD 1979-2007). Looking at the single sequences, ST 117 (identified from an avian strain) was the most ancient sequence within clade 1. It appeared segregated from the other B2 STs comprised in the sub-clade 1a and all associated only with humans, including the well-known clone ST131 (Rogers *et al.,* 2011). Of note, ST131 was found to be the ancestor of other STs within clade 1a. Apart from ST131 (ciprofloxacin- and multidrug resistant clone) a minority of STs (22.2%) included in the sub-clade 1a was found associated with multidrug resistance. Cluster 2 and clade 8, which included only D phylogroup STs, dated back to 1995 (95% HPD: 1969-2008) and 1993 (95% HPD: 1963-2007), respectively. Cluster 2 included STs sequences deriving from both human and avian sources (all associated with multidrug resistance), while clade 8 comprised predominantly human STs belonging to phylogroup D. Similarly to that observed for B2 sequences, the most ancient ST of clade 8 (ST2200) was from poultry and appeared segregated from sub-clade 8a including mostly human sequences. Clusters 3, 4, 5 and 6 included STs belonging to phylogroups B1 and/or A that appear intermixed. In particular, clusters 3 and 5 (both consisting of B1 phylogroup STs) dated back to 2005 (1998-2009) and 2007 (2001-2009), respectively, while clusters 4 and 6 (both including A phylogroup STs) dated back to 2006 (95% HPD 2001-2009) and 2008 (95% HPD 2006-2009) re-

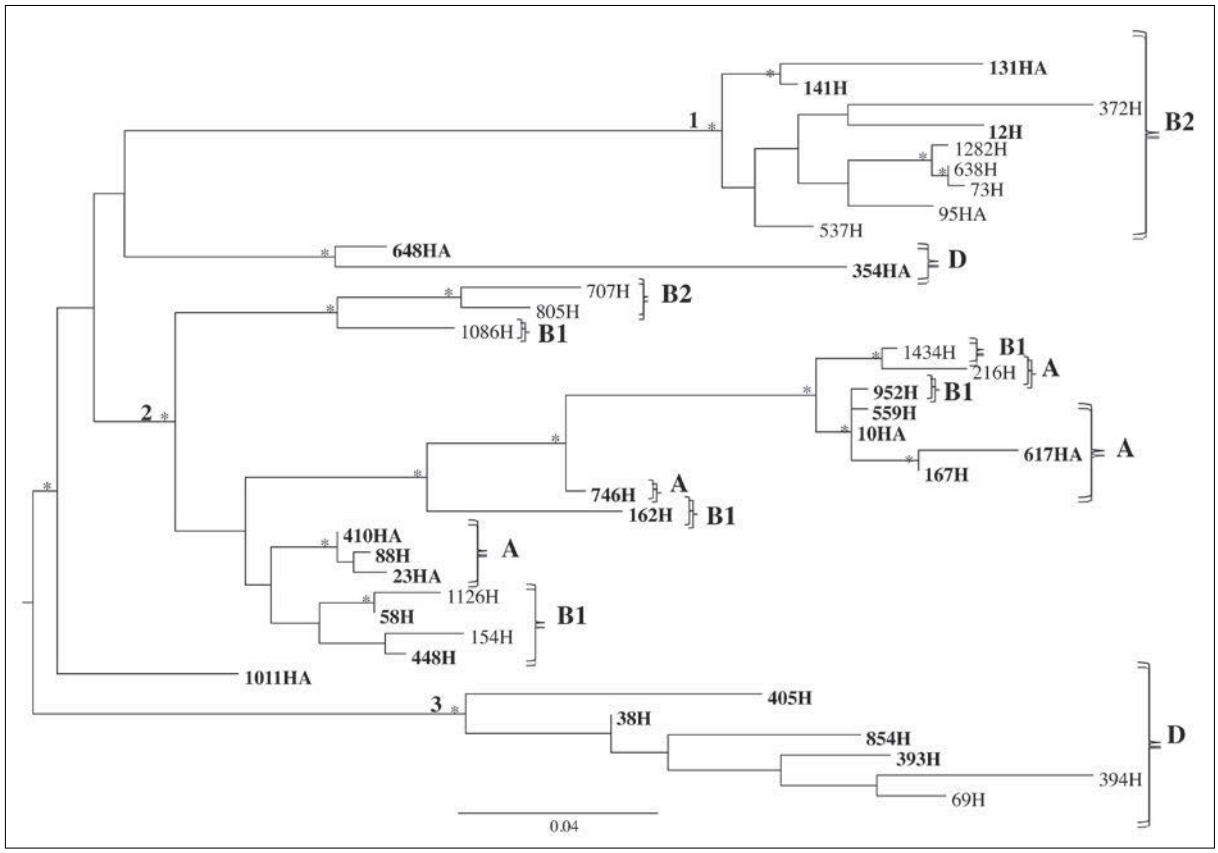

FIGURE 1 - *Maximum likelihood phylogenetic analysis of 37 concatenated MLST sequences obtained from human E. coli strains [28 exclusively from humans (H) and 9 shared by human and avian species (HA)]. The sequence type number is indicated at any branch. The tree was rooted using the midpoint rooting method. The main clades are numbered. Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test, and were drawn to scale with the bar at the bottom indicating 0.04 nucleotide substitutions per site. One * along the branches represents significant statistical support for the clade subtending that branch (bootstrap support >70%). The belonging of each ST to one of the four major phylogenetic groups (A, B1, B2 and D) is reported. STs associated with multidrug-resistant strains are marked in bold.*

spectively. Interestingly, STs of avian and human origin were intermixed within each of these four clusters and the majority of them were associated with multidrug resistance, especially in clusters 4, 5 and 6 (72.7% of all STs included in the three clusters). Cluster 7, which included a few B1 and B2 phylogroup STs, dated back to 2001 (95% HPD 1986-2008). Clade 9 encompassed STs mainly associated with avian species and belonging to all four phylogroups (A, B1, B2 and D); it dated back to the year 1996 (95% HPD 1972-2007). However, inside clade 9, sub-clade 9a included both avian and human sequences (almost one half associated with multidrug resistance) belonging to A, B1 and B2 phylogroups and dating back to 2001 (95% HPD 1987-2008).

Excluding STs shared by both human and avian species, comparison of the proportion of poultry-derived STs (Avian STs/Human STs +Avian STs) within each of the major clusters and/or clades showed the following rising scale: clade 1 (1/8, 12.5%), clade 8 (2/8, 25.0%), clusters 4, 5 and 6 (1/2, 50% in each cluster), sub-clade 9a (6/11, 54.5%), clade 9 (8/13, 61.5%) and cluster 3 (4/6, 66.6%).

Table 1 shows the mean genetic distances obtained on the overall dataset (human and avian sequences) stratifying sequences by phylogenetic group and using Mega 5 with LogDet model. The highest mean genetic distance (0.017) was found between phylogroups B2 and D, while the lowest mean genetic distance (0.008) was ob-

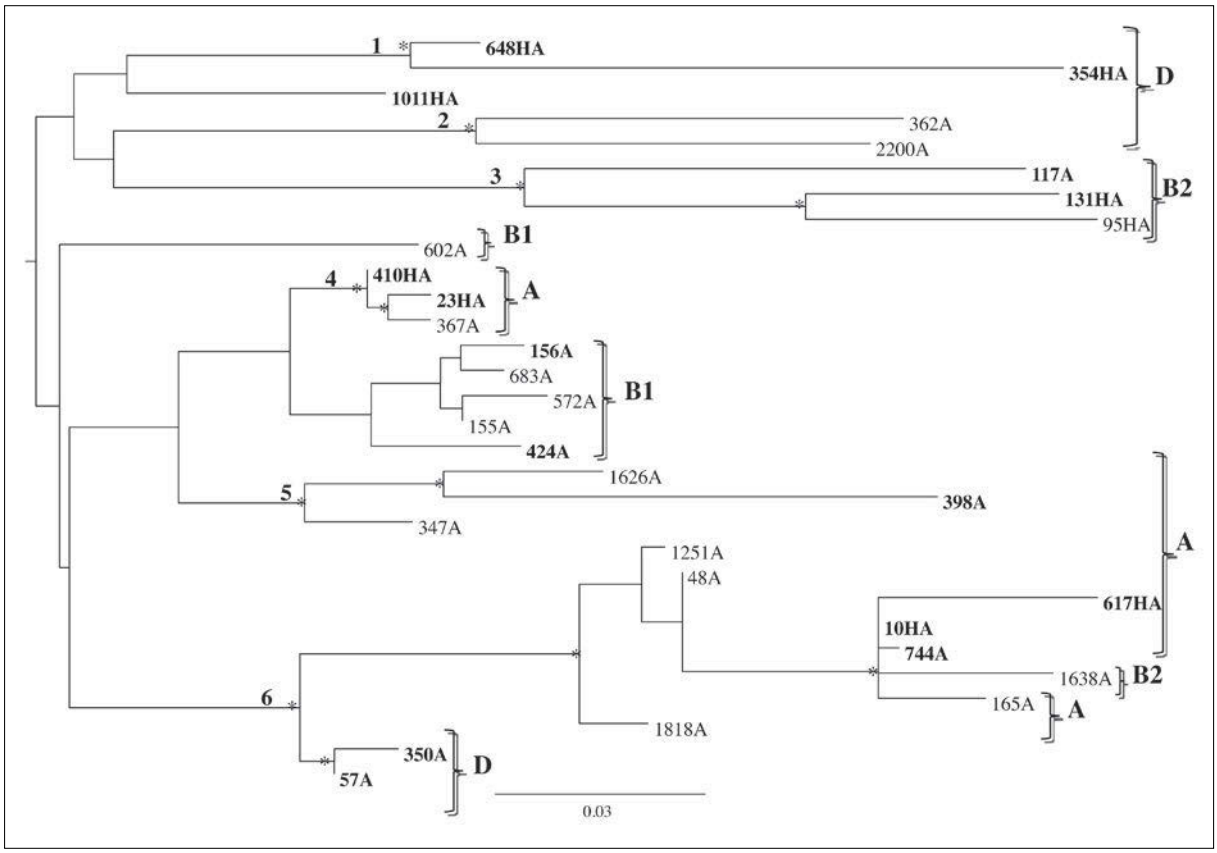

FIGURE 2 - *Maximum likelihood phylogenetic analysis of 30 concatenated MLST sequences obtained from avian E. coli strains [21 exclusively from avian species (A) and 9 shared by human and avian species (HA)]. The sequence type number is indicated at any branch. The tree was rooted using the midpoint rooting method. The main clades are numbered. Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test, and were drawn to scale with the bar at the bottom indicating 0.03 nucleotide substitutions per site. One * along the branches represents significant statistical support for the clade subtending that branch (bootstrap support >70%). The belonging of each ST to one of the four major phylogenetic groups (A, B1, B2 and D) is reported. STs associated with multidrug-resistant strains are marked in bold.*

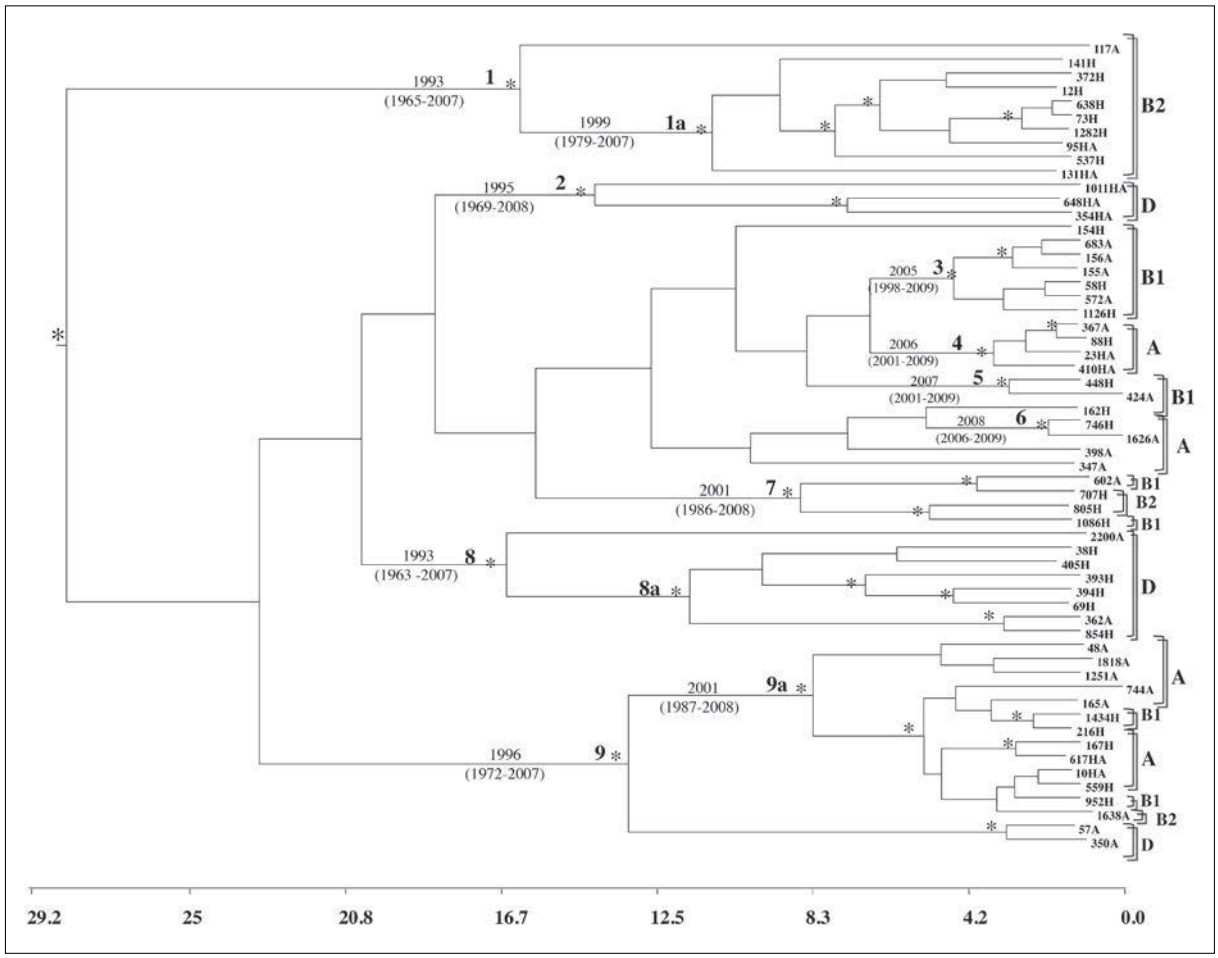

FIGURE 3 - *Bayesian maximum clade credibility tree of 58 concatenated MLST sequences obtained from human and avian E. coli strains [28 exclusively from human (H), 21 exclusively from avian species (A) and 9 shared by human and avian species (HA)]. The sequence type number is indicated at any branch. The main clades/clusters are numbered. One * along the branches represents significant statistical support for the clade/cluster subtending that branch (p.p.≥98%) Each clade/cluster with p.p. =1 is dated. The belonging to each ST to one of the four major phylogenetic groups (A, B1, B2 and D) is reported. Years are reported in the scale axis below.*

TABLE 1 - *Mean genetic distances among 58 sequence types (STs) deriving from both human and avian E. coli strains (overall data set), stratified by phylogenetic groups.*

	A	*B1*	*B2*	*D*
A	-	-	-	-
B1	0.008	-	-	-
B2	0.015	0.014	-	-
D	0.015	0.014	0.017	-

tained between phylogroups B1 and A. Comparing B1 with B2 and D, the mean genetic distance was identical (0.014). Between phylogroups A and B2 or D the mean genetic distance was comparable (0.015).

DISCUSSION

The debate on the origin and dissemination of *E. coli* MDR clones that infect humans is evergreen. Several recent studies have suggested that chicken products may be a source for potentially path-

ogenic resistant *E. coli* in humans (Johnson *et al.,* 2005; Collignon and Angulo, 2006; Vincent *et al.,* 2010; Bélanger *et al.,* 2011; Bergeron *et al.,* 2012; Nordstrom *et al.,* 2013). In view of these earlier studies, it is essential to assess genetic relatedness of clones circulating in both hosts. In the present study, some major differences in the topologies of the two (human/avian) phylogenetic trees were highlighted, irrespective of the multidrug resistance status. In the human tree, the majority of B2 strains segregated together and formed a significant cluster, as well as most D strains, while A and B1 strains were largely intermixed.

Conversely, the phylogenetic tree of avian *E. coli* was characterized by a greater heterogeneity: overall, avian strains appeared dispersed among different small clusters. These findings were in agreement with previous more comprehensive investigations on *E. coli* phylogenetic history, supporting the early emergence of the phylogenetic group B2 characterized by a high level of genetic diversification and reporting A and B1 as "sister groups", that branched more recently (Jaureguy *et al.,* 2008; Lescat *et al.,* 2009; Touchon *et al.,* 2009).

It is well known that the majority of "virulent" *E. coli* that are pathogenic to humans (ExPEC strains) belong to the B2 and D phylogroups and in this regard it is not surprising that they exhibit high levels of diversity. By contrast, phylogroup A followed by B1 are predominant among human commensal strains, although in this study all human sequences were derived from *E. coli* strains isolated from extraintestinal infections (Picard *et al.,* 1999; Johnson and Stell AL, 2000; Escobar-Páramo *et al.,* 2004). In poultry, pathogenic strains (avian pathogenic *E. coli*, APEC) predominantly belong to groups D and A, while the commensal population is rich in A and B1 strains, but variations in prevalence of the different phylogenetic groups has been reported depending on the studies (Rodriguez-Siek *et al.,* 2005; Sabaté *et al.,* 2008; Johnson *et al.*, 2009; Jakobsen *et al.,* 2010).

All avian sequences herein analysed were from commensal strains and this could explain the relatively high heterogeneity and the lack of divergent homogeneous clusters in their phylogeny.

A noteworthy finding of the present study derived from the observations of the Bayesian phylogenetic tree, in which all human and avian ST sequences were analysed together. In such a tree, most B2 and D human strains of our collection again segregated together according to the phylogenetic group and separated from avian strains (although in both cases the ancestor was an avian isolate) but the majority of B1 and A human strains were intermixed with avian strains belonging to the same phylogenetic group, in several clusters.

Notably, these clusters included most STs associated with multidrug resistance. Looking at estimated dates, the most ancient strains of our collection belonged to the phylogenetic groups B2 and D (human isolates and their avian ancestors), while the majority of B1 and A strains of both human and avian origin were found to have emerged more recently, in the second half of the 2000s. Based on these results, the hypothesis of the potential transmission of MDR *E. coli* clones from poultry to humans seems to involve phylogroups A and B1 strains rather than B2 and D strains.

In fact, phylogroups A and B1 strains from both sources (human/avian) were found to have a low level of genetic diversification in comparison with the B2 or D pathogens, were largely intermixed in the Bayesian tree regardless of the source and were dated recently, suggesting they may be more readily exchangeable between poultry and humans.

Finally, we would like to add a comment on the epidemic clone ST131 that has gained the attention of several researchers over the world (Nicolas-Chanoine *et al.,* 2008; Cerquetti *et al.,* 2010; Rogers *et al.,* 2011). Here, we did not find evidence supporting the hypothesis for the existence of a present avian reservoir for the ST131 clone, since it was clearly segregated together with other B2 strains from humans, although the occurrence of an avian ancestor suggests that its ancient origin from poultry may be possible. Additional studies using large datasets assembled over a long time period are needed to unambiguously clarify the origin of this important pathogenic clone.

A limitation of the present study could be the narrow time span of our dataset and an insufficient number of ST data. However, the dataset comprised all ST types identified in a wide collection of *E. coli* strains of human and avian origin iso-

lated in Italy (Giufrè *et al.,* 2012). Regarding the narrow time span of data, this should not significantly affect the estimated dates of emergence of ancestors, since we exclusively dated clades/clusters statistically supported with p.p.=1. Conversely, one value of the study is that the phylogeny of *E. coli* from different hosts (humans/avian species) was analysed together, although the lack of data deriving from *E. coli* strains isolated from faeces of healthy human subjects may be considered another limitation. Finally, since only recently an extended quadruplex PCR method for phylogroup assignment (able to detect the new minor phylogroups C, E and F) was developed and published, we cannot rule out the possibility that some strains may be differently classified using such a new method (Clermont *et al.,* 2013). However, when we re-tested some phylogroup D strains gathering out of the main D cluster by the new method they were found correctly assigned.

There is currently a wide debate on the possible avian origin of MDR *E. coli* strains causing extra-intestinal infections in humans. According to our phylogenetic reconstruction, a subgroup of *E. coli* clones belonging to phylogroups A and B1 and associated with multidrug resistance is potentially exchangeable between poultry and humans and may constitute a potential zoonotic risk. Conversely, *E. coli* clones included in phylogroups B2 and D are suggested to have a minor zoonotic potential. Further and more comprehensive studies are needed to elucidate such a complex issue, including whole-genome-based phylogeny reconstruction of single lineages within ST clones, but we would emphasize the usefulness of the phylogenetic analysis for a better understanding of the epidemiology of potential zoonotic pathogens.

ACKNOWLEDGMENTS

We are very grateful to Tonino Sofia for editorial assistance. This work was partially supported by Ministry of Health-Centro Controllo Malattie project "Surveillance on antibiotic resistance in the community and in food-borne and zoonotic diseases" (Grant number 9M06).

DISCLOSURE STATEMENT

No competing financial interests exist.

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