Enteropathogenic Escherichia coli (EPEC) strains are among the major causes of infantile diarrhea in developing countries (71) and can be classified as typical and atypical, depending on the presence or absence of the E. coli adherence factor plasmid (pEAF), respectively (39).

The pathogenesis of EPEC resides in the ability to cause the attaching and effacing (A/E) lesion in the gut mucosa of human or animal hosts, leading to diarrheal illness (40). The genes responsible for the A/E lesion formation are located in a chromosomal pathogenicity island of ~35 kb, known as the locus of enterocyte effacement (LEE) (23, 47). LEE encodes an adhesin called intimin (38), its translocated receptor (Tir) (42), components of a type III secretion system (36), and effector molecules, named E. coli-secreted proteins (Esp proteins) (41).

These virulence factors have a crucial role in the A/E lesion formation, and their detection in EPEC strains is an indicator of their potential to produce these lesions (19, 56).

Atypical EPEC strains have been associated with diarrhea outbreaks in developed countries (31, 73, 77) and with sporadic cases of diarrhea in developing and developed countries (1, 12, 26, 52, 55). At present, the prevalence of atypical EPEC is higher than that of typical EPEC in several countries (1, 12, 26, 52, 55, 65).

Different from the situation in developed countries, where atypical EPEC outbreaks and sporadic infections are associated with children and adults, atypical EPEC infection in Brazil is mainly associated with children’s illnesses (32, 71).

Typical EPEC strains are rarely isolated from animals, and humans are the major natural reservoir for these pathogens (14, 32, 53, 71). In contrast, atypical EPEC strains are present in both healthy and diseased animals (dog, monkey, cat, and bovines) and humans (4, 6, 18, 28, 71). Some studies have associated pets and farm and wild animals as reservoirs and infection sources of atypical EPEC strains for humans (32). However, these studies did not compare atypical EPEC strains isolated from humans and animals by gold-standard molecular methods like multilocus sequence typing (MLST) or pulsed-field gel electrophoresis (PFGE) (15, 35, 43, 53). For this reason, there are some doubts about whether atypical EPEC strains isolated from animals represent risks for human health and whether animals really play the role of reservoirs of atypical EPEC.

The aim of this study was to compare atypical EPEC strains isolated from humans and different animals, including pets (cats and dogs), farm animals (bovines, ovines, and rabbits), and wild animals (monkeys), by molecular phylogenetic techniques to verify the role of animals as reservoirs of and sources of infection with atypical EPEC in humans.

MATERIALS AND METHODS

Bacterial strains. This study was based on the analysis of 42 atypical EPEC strains isolated from humans (n = 20) and from pets and farm and wild animals (cats, dogs, rabbits, bovines, ovines, and monkeys; n = 22) described in previous studies (3, 13, 14, 19, 21, 29, 44, 46, 51, 53, 59, 60, 66, 72). Seven typical EPEC strains isolated from humans and animals, belonging to serotypes O127:H40 and O142:H6 (14, 46), were also included to be compared with atypical EPEC strains. All selected isolates belong to classical EPEC serotypes that commonly cause disease in both humans and animals and were isolated in unrelated epidemiological studies from diseased or healthy humans and animals between 1954 and 2003 in Brazil and other countries (Table 1).

Serotyping. The somatic (O) and flagellar (H) antigen typing was performed as described by Ørskov and Ørskov (57). Strains classified as nonmotile were analyzed by using a fliC PCR-restriction fragment length polymorphism (RFLP) method as previously described (24).

Detection of virulence factors. PCR tests were carried out as previously described (45). DNA to be amplified was obtained from whole cells by boiling. The

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† In memoriam.

† Published ahead of print on 2 October 2009.
PCRs mix consisted of a 25-μmol/l of each primer and the following reagents (Invitrogen, Carlsbad, CA): Taq DNA polymerase (1.5 U); 10× PCR buffer (5.0 μl); dATP, dCTP, dGTP, and dTTP (0.1 mM each); MgCl2 (2.0 mM); and 1 μl of cell lysate containing template DNA. Sequences of primers, annealing temperatures, and amplicon sizes were described previously, as follows: eae (58); astA (76); bfaA (30); enterohemorrhagic E. coli (EHEC) hhaA (69); espA, espB, espD, espF, and sepL (46); PEAF (25); stx1 and stx2 (10); stx2m (70); and tir-α, tir-β, and tir-γ (17) (Table 2). The PCR products were separated by electrophore-

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a | b | c | d

a, b, c, d: PCR positive; +: PCR positive with primers based on EPEC strain E2348/69 sequence (GenBank accession no. AF022236); †: PCR positive with primers based on EHEC EDL933 sequence (GenBank accession no. Y13859).

PCR negative; -: PCR negative; +: PCR positive; = PCR positive with primers based on EPEC E2348/69 sequence (GenBank accession no. AF022236).
sis in 1.5% agarose gels, stained with 0.5 µg/ml ethidium bromide, and visualized under UV light.

eae gene subtyping. Intimin subtyping was performed based on the eae RFLP protocol described by Jenkins et al. (37). The intimin genes classified as α, β, and γ were also classified as the α1, α2, β1, β2, γ1, and γ2 types, respectively, by the PCR-RFLP protocol, as described by Oswald et al. (58).

EHEC hemolysin production. EHEC hemolysin production was detected by a method previously described by Beutin et al. (5), using sheep blood agar containing 10% CaCl₂. These assays were performed only with the EHEC positive strains.

Phylogenetic analysis. Phylogeny was based on a superflegene constructed by concatenating the individual gene sequences in the following order: arcA, cydA, fasD, icdA, lysP, rpoS, and rpsO. The phylogenetic trees were rooted with homologous sequences from E. coli strains EDL933 and E2348/69 and Salmonella enterica strain Ty2 as an outgroup, extracted from the GenBank database. Phylogenetic trees were inferred by distance, maximum-likelihood (ML), and Bayesian methods. The phylogenetic tree inferred by distance was constructed using the neighbor-joining (NJ) algorithm, the Kimura 2-parameter algorithm of nucleotide substitution, and bootstrapping of 1,000 replications, using MEGA4 software (http://www.megasoftware.net).

ML analysis was performed with bootstrapping of 1,000 replications using PAUP*, version 4.0b10, software (75). The best tree search was realized by heuristic methods, using tree bisection and reconnection as a branch-swapping method. Bayesian analysis was performed with MrBayes software, version 3.1 (68), for 10 million generations, sampling every 1,000 generations, with the final tree being constructed from generations 2.5 to 10 million. The Bayesian tree was inferred to validate the bootstrapping values found on NJ and ML analyses. The

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</table>

* Where indicated, primers were based on the expB and expD sequences of a particular strain or serotype.
RESULTS

Serotypes and fliC RFLP typing. Most O and H types of atypical EPEC strains studied in this study were determined by conventional serological methods. Nine strains classified as nonmotile had their flagellar antigen determined by fliC RFLP typing. The strains belonged to nine different serotypes, previously characterized as atypical EPEC (Table 1).

Detection of LEE, bfpA, and toxin-encoding genes. All strains presented positive PCRs for the eae gene, and none was positive for genes encoding Shiga-toxins (stx1, stx2, and stx2L), the enteroaggregative heat-stable toxin (astA), or for the pEAF plasmid. The presence of bfpA was detected only in the seven strains previously classified as typical EPEC. The remaining 42 strains were classified as atypical EPEC strains, i.e., having the genetic profile eae positive, pEAF positive, and bfpA negative (Table 1).

Intimin typing resulted in six eae genotypes including α1 (5 strains), α2 (4 strains), β1 (25 strains), γ1 (3 strains), γ2/0 (10 strains), and ξtw (2 strains). The tir subtyping identified tir-β as the most common intimin receptor subtype. Specifically, tir-β was detected in 23 of 49 (46.9%) strains, followed by tir-α in 18 (36.7%) and tir-γ in 4 (8.2%) strains. Four (8.2%) strains showed negative PCRs for all tir subtypes studied (Table 1).

Genes encoded by LEE4 were detected with different frequencies. The presence of espB, espD, and sepL genes was detected in all strains, whereas espA was detected in 40 (81.6%) strains. espF was detected in only 22 (42.8%) strains. Amplified espF fragments of 11 strains were distinct in size compared to espF fragments of strain E2348/69 (520 bp). These fragments ranged from 400 to 600 bp.

Detection and expression of EHEC hlyA. The presence of the EHEC hlyA gene was detected in five strains of serotype O26:H11 (Table 1). The expression of this gene was detected in these strains by the formation of a hemolytic zone around the spots of inoculation in blood agar plates after 24 h of incubation at 37°C.

MLST. In all strains, internal fragments of the seven housekeeping genes could be sequenced with acceptable raw traces. The analysis of multiple-sequence alignment of nucleotide sequences and of inferred amino acid sequences showed a total of 184 variable sites among 4,080 nucleotides of the supergene, 25 of which involved in amino acid replacements. SplitsTree analysis revealed several parallel paths, indicating phylogenetic incompatibility in the divergence of atypical EPEC clones, caused by recombination (Fig. 1). Using the $\Phi_w$ test, statistical
TABLE 3. Allelic profile and relative frequency of ST obtained by MLST analysis

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The allelic frequency of housekeeping genes ranged from 6 to 13 alleles per locus. The most variable loci were icdA and rpoS, with 10 and 13 alleles, respectively. The different alleles classified the strains in 29 STs (Tables 1 and 3). All STs found, with the exception of ST9, were represented by only one serotype. ST9 was represented by five strains of both O128:H2 (where brackets indicate antigens identified by fliC RFLP) and O119:H2 serotypes (Table 1 and Fig. 2).

The rooted trees inferred by NJ, ML, and Bayesian methods were consistent with the sequences of the seven housekeeping genes. All phylogenetic trees grouped the strains in well-defined clusters based on their serotypes, indicating that recombination events did not affect our analysis or that they occurred early in the divergence of the EPEC strains.

All three methods of phylogenetic inference yielded similar trees, with strains clustering into similar groups supported by high bootstrapping or Bayes credibility values. These high values obtained in different types of analyses validate the phylogenetic relationship of the strains analyzed (Fig. 2).

The ML and Bayesian trees divided the strains into five clusters (A to E). The Bayesian tree cluster A included strains of serotype O119:H2 and O128:H2 isolated from humans, dogs, rabbits, sheep, and monkeys. Cluster B comprised exclusively O26:H11 strains isolated from humans and bovines.

Cluster C comprised strains of O111:H9, O111:H25, and O127:H40 isolated from humans, cats, dogs, and monkeys. Although these strains shared the same cluster, they formed three distinct minor clusters (C1 to C3) separating these serotypes (Fig. 2).

Some strains showed close proximity to strains of different pathotypes. All O125:H6 strains belonged to the same cluster of typical EPEC strains, and O55:H7 strains were identified as belonging to the cluster of the EHEC EDL933 strain. In the ML tree shown in Fig. 2B, the strains are grouped as in the tree obtained in Bayesian analysis. The NJ tree also obtained similar results (data not shown).

PFGE. PFGE typing of 49 EPEC strains resulted in 15 pulsed-field patterns (PPs). The comparison of patterns revealed five clusters (A to E) with general similarity of 74% in the unweighted-pair group method using average linkages tree. Each cluster comprised strains belonging to different serotypes isolated from animals and humans (Fig. 3). One exception was cluster A, which consisted of four strains of serotype O119:H2. Among the 15 patterns found, PP14 and PP15 formed an isolated group with the EHEC strain EDL933. PP1 was the most divergent pattern, constituting a separated group from other strains analyzed. PP4, PP9, PP13, and PP14 were formed exclusively by strains isolated from humans, and PP5 and PP15 were recovered from monkey and bovine isolates, respectively. All other patterns were represented by human and animal isolates.

DISCUSSION

This study describes the role of some animals that act as reservoirs of and possible sources of infection with atypical EPEC in humans. In several countries, the frequency of atypical EPEC is increasing compared to typical EPEC, and it is currently considered an important emerging diarrheagenic pathogen for children (1, 12, 26, 32, 55, 65).

The LEE-encoded proteins are responsible for the A/E lesion formation. In some strains of our study, espA, espF, and tir were not detected by PCR analysis although this does not prevent these strains from causing A/E lesions, as indicated by their ability to cause A/E lesion in vitro (data not shown). For instance, the UIPA17 strain isolated from a cat was negative for espA but was positive in a fluorescent actin staining test, an indication of the ability to produce the A/E lesions in vitro (51). The unsuccessful detection of LEE genes in some strains may indicate the presence of gene subtypes not detected by the primers employed (46). In addition to PCR, DNA hybridization and DNA sequencing would be necessary either to confirm or to exclude the presence of the respective LEE genes in the PCR-negative isolates. Studies utilizing these techniques will be performed in forthcoming experiments.

Some reports have identified polymorphisms and different subtypes of LEE genes (2, 17, 54). As a matter of fact, some reports (20, 27) have detected molecular differences between espA genes according to the origin of the sequences used to design the primers for the PCRs. Neves et al. (54) showed also that an antisera against EspA of EPEC E2348/69 did not react with EspA filaments of EHEC O157:H7. Similarly, an antisera against EspA of EHEC 85-170 did not react with EspA filaments derived from typical and some atypical EPEC.
strains, with the exception of O55:H7 strains known to be the ancestors of the O157:H7 EHEC serotype. Interestingly, several strains belonging to different serotypes of typical and atypical EPEC strains, though able to show a positive fluorescent actin staining reaction and therefore harboring the espA gene and the respective filament, did not react with either antibody. Based upon these reports, we can assume that our negative PCR results for espA were due to the polymorphisms within these gene sequences.

The espB and espD genes were detected in all strains. To reach this frequency, we used a different set of primers based on different *E. coli* pathotypes and serotypes (Tables 1 and 2). These data indicate that atypical EPEC can acquire the entire LEE region or just single genes of this region from different origins, as previously suggested (2, 16). This hypothesis is feasible on the basis of the present study since strains harboring distinct subtypes of eae and tir were found (Table 1).

In contrast to some previous reports, the astA gene was not detected in the atypical EPEC strains studied (22, 77). The presence of the stx2f gene has been detected in diarrheagenic *E. coli* strains, previously classified as atypical EPEC strains by some authors (63). To exclude the presence of Shiga toxin-producing *E. coli* (STEC) strains in our analysis, PCR with specific primers for stx2f was conducted, and the presence of this gene was not detected.

The virulence factor profiles of strains isolated from humans and animals were similar, indicating the potential of animal strains to cause disease in humans.

MLST and PFGE were performed on all 49 atypical EPEC strains. In both techniques it was possible to identify strains isolated from humans and animals that shared similar clonal origins. MLST was more discriminative than PFGE since each PP was represented by more than one ST. The better discriminative power of MLST was restricted to detecting minor clones among strains of the same serotype. This indicates, as previously suggested, that each serotype has more than one clonal origin (13, 29, 59, 66, 74).

Some strains revealed a close relationship to typical EPEC or EHEC strains. In PFGE analysis, two groups of O26 strains (PP12 and PP13) were closely related to typical EPEC strains, and one O26 group (PP15) was close to the O55:H7 strains and to EDL933 (O157:H7). Based on these data, these strains and pathotypes may have evolved either by acquisition or loss of the stx or bfp genes (11). Some studies have demonstrated the

FIG. 2. Inferred phylogeny of 49 typical and atypical EPEC strains isolated from humans and different animal species. The strains were rooted with *E. coli* strains EDL933 and E2348/69; *S. enterica* strain Ty2 was included as an outgroup standard. (A) Phylogenetic tree inferred by Bayesian analysis. The serotypes and sources of the strains are listed. The nucleotide substitution model best fit was constructed using GTR +I +G (where GTR is general time reversible, I is the proportion of invariable sites, and G is gamma) where G = 0.6931 and I = 0.5112. In the majority of cases, the tree is supported by Bayes credibility values higher than 90%. ST9 was the only ST representing two serotypes. (B) ML-inferred tree with similar topology and groups found on Bayesian analysis. The serotypes of each group found are listed. The ML tree was constructed with the model TrN+I+G, where TrN is Tamura-Nei, G = 0.6876, and I = 0.5139. In the majority, the tree is supported by bootstrapping values higher than 80%.

FIG. 3. PFGE profiles and clusters of human and animal typical and atypical EPEC strains. The corresponding MLST sequence types, as well as the serotypes and sources of the strains for each PP, are listed.
loss of stx genes by EHEC strains associated with hemolytic-uremic syndrome during human infection, indicating that some atypical EPEC serotypes, like O26:H11, O119:H2, and O128: H2, may be EHEC strains that lost stx genes (8, 9, 48, 49, 50). Despite these reports, some atypical EPEC serotypes studied by us were phylogenetically unrelated to typical EPEC or EHEC strains, thus indicating that they are true atypical EPEC strains.

Strains of serogroups O111, O119, O125, and O128, unlike O26 serotypes, shared clonal relationships only with typical EPEC strains. Interestingly, O119:H2 strains were divided into two groups by PFGE. These results may indicate the acquisition of new virulence factors by these strains not detected in the MLST analysis.

The differences between MLST and PFGE may be the result of the type of analysis. While PFGE detects differences in the genome, MLST analyzes just small fragments of conserved metabolic genes. Therefore, events like recent acquisition of virulence factors cannot be detected by MLST.

In general, both in MLST or PFGE analysis, the animal and human strains showed close proximity. Therefore, animals can play a role as reservoirs and sources of infection of atypical EPEC for humans, as previously suggested by other authors (3, 15, 45, 51, 53, 72). This indicates that in contrast to typical EPEC strains, diarrhea caused by atypical EPEC can be considered zoonosis.

On the other hand, the possibility cannot be excluded that some animals, mainly pets, had contracted their atypical EPEC strains through contact with human feces. Atypical EPEC strains are known to play a role as pathogens in cats and other animals (14, 42, 62). Investigations of cycles of mutual transmission of pathogenic E. coli between humans and different animals have been performed mainly with dogs. EPEC has been isolated from a diarrheic child and a diarrheic dog living in the same house (67). Hence, fecal E. coli strains as potential agents of disease may be transmitted directly or indirectly between humans and animals (53, 67). Farm animals carrying atypical EPEC strains may represent indirect risk since E. coli pathotypes have been found in fresh animal-derived food (34).

Few studies are available on animals as possible sources of infection of diarrheagenic E. coli in humans. However, the presence of potentially human pathogenic EPEC types among EPEC strains from pets, farm animals, and wild animals indicates that transmission of pathogens between animals and humans can occur and have an impact on public health. The role of these animals as carriers of atypical EPEC should be considered in investigations of outbreaks.

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