

Automated Ribotyping Provides Rapid Phylogenetic Subgroup Affiliation of Clinical Extraintestinal Pathogenic *Escherichia coli* Strains

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Using the automated Riboprinter system, we have initiated the construction of an electronic Riboprint database composed of 72 ECOR reference strains and 15 archetypal virulent strains in order to provide a new simple molecular characterization method. More than 90% of the ECOR strains clustered in their original phylogenetic group. All but one of the archetypal virulent strains had a profile identical to that of one of the ECOR strains and could be easily affiliated with a phylogenetic group. This method appears to be an accurate and practical tool especially for investigating the genetic relationship between clinical extraintestinal pathogenic strains and B2 subgroup ECOR strains or archetypal pathotype strains.

Escherichia coli is both the most common commensal bacterium and the most frequent community-acquired pathogen in humans. *E. coli* belongs to the normal fecal flora but can cause various intestinal (gastroenteritis and colitis) and extraintestinal (urinary tract infection, septicemia, and neonatal meningitis) infections. The genetic structure of *E. coli* is considered clonal, and phylogenetic analyses have shown that strains of this species fall into four main phylogenetic groups (A, B1, B2, and D) (11, 24). Recent attempts to establish a link between phylogeny and virulence suggest that extraintestinal pathogenic *E. coli* strains are mostly derived from phylogenetic group B2 and, to a lesser extent, group D (3, 4, 18). In contrast, most human commensal strains originate from phylogenetic groups A and B1. Contrary to extraintestinal pathogenic strains, each pathotype of intestinal pathogenic strains shows phylogenetic diversity: enteropathogenic *E. coli*, enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffuse adherent *E. coli* (DAEC), and enteroinvasive *E. coli* are distributed among all the phylogenetic groups (8, 20, 22). Studies based on molecular characterization and genetic relatedness of pathogenic and commensal strains have improved our understanding of the pathogenicity and acquisition of virulence traits in *E. coli*.

The reference techniques for phylogenetic grouping are multilocus enzyme electrophoresis (MLEE) (11, 23) and ribotyping (3, 9, 16, 19), but both techniques are complex and time-consuming. Moreover, neither method is standardized. Thus, to determine the phylogenetic group of a given strain, a collection of typed reference strains, such as the ECOR collection, must be tested in parallel (3, 9, 12, 20). The ECOR collection is a set of 72 reference *E. coli* strains isolated between 1973 and 1983 from a variety of animal hosts and geo-

graphic locations. Those strains are well characterized and represent the entire range of genotypic variation in the species as a whole (17).

Being automated and standardized, the Riboprinter microbial characterization system is suited to rapid and high-throughput typing of bacterial strains. The Riboprinter can yield 32 ribotypes a day directly from fresh colonies. Given their worldwide distribution and interconnection, Riboprinters permit immediate comparisons of ribotypes through connection of their databases. The purpose of the present study was to initiate the construction of an electronic Riboprint database of archetypal virulent strains and the ECOR collection, in order to provide the scientific community with a rapid tool for investigating genetic relationships between clinical and reference *E. coli* strains.

Bacterial isolates. The 72 strains of the ECOR collection (17) were kindly provided by R. Selander (Department of Biology, University of Rochester, Rochester, N.Y.). Of these, 68 belong to the four main phylogenetic groups (A, B1, B2, and D) and 4 are unclassified (group E) (11, 24). Information concerning these strains can be obtained at the T. Whittam laboratory web site (<http://foodsafemsu.edu/whittam/ecor/index.html>). We also used several archetypal strains representing different *E. coli* pathotypes, including neonatal meningitis strains RS218 (kindly provided by K. Kim, Johns Hopkins University School of Medicine, Baltimore, Md.), C5 and RS176 (obtained from R. Bortolussi, Dhalousie University, Halifax, Nova Scotia, Canada), uropathogenic strains J96 and E536 (provided by J. Hacker, Institut für Molekulare Infektionsbiologie, Würzburg, Germany), uropathogenic strain CFT073 (obtained from H. Mobley, University of Maryland, Baltimore), enteropathogenic strain E2348/69, EHEC strain EDL933, ETEC strains EDL1493, E2539-C1, and TX-1, EAggEC strains O42 and JM221, and DAEC A30 and C1845. All the diarrheagenic strains were kindly provided by C. Le Bouguenec (Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris, France). *E. coli* laboratory K-12 strain

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MG1655, which belongs to phylogenetic group A, was also studied (11).

Automated ribotyping. All *E. coli* isolates and ECOR strains were characterized by automated ribotyping with the Riboprinter (Qualicon Inc., Wilmington, Del.). Ribotyping was performed under the conditions recommended by the manufacturer (6, 25), with the following modifications. The validated *EcoRI* restriction enzyme was replaced by *HindIII* (New England BioLabs, Beverly, Mass.) at 100 U/ μ l in standardized reagents in 0.5-ml tubes (Sarstedt, Orsay, France). The other steps were unmodified and automated, and up to 32 isolates could be analyzed per day.

For each strain analyzed, one fresh colony was picked and resuspended in sample buffer and added to the processing module for a heat treatment step at 80°C for 10 min in order to inhibit endogenous DNA-degrading enzymes. The temperature was then reduced, and two lytic enzymes (lysozyme and *N*-acetylmuramidase) were added to the sample. The sample carrier was then loaded onto the Riboprinter system with the other commercial reagents. Restriction enzyme digestion, gel electrophoresis, and blotting steps were completely automated. Briefly, bacterial DNA was digested with the chosen restriction enzyme and loaded onto an agarose gel; restriction fragments were separated by electrophoresis and simultaneously transferred to a nylon membrane. After a denaturation step, the blotted nucleic acids were hybridized with a sulfonated DNA probe harboring the genes for the small and large rRNA subunits of *E. coli*. The hybridized probe was detected with alkaline phosphatase-labeled antibodies directed against sulfonated DNA. Bound labeled antibodies were then detected by capturing light emission from a chemiluminescent substrate with a charge-coupled device camera. The output consisted of a densitometric scan depicting the distribution of the restriction fragments and their molecular weights and was saved in the Riboprinter computer.

Ribotype analysis. For each batch of eight samples, ribotypes were normalized to the position of the molecular weight standards by using the Qualicon software. Computerized ribotypes were exported for analysis in .txt files, converted to .int files with GelConvert 1.01 software (Qualicon), and imported into Gel Compar software, version 4.1 (Applied Maths, Ghent, Belgium). Clustering analysis was performed with the unweighted pair group method using arithmetic averages (UPGMA) based on the Dice coefficient for the band matching (10), with a position tolerance setting of 0.8% and an optimization setting of 0.25% (default values are 1% for position tolerance and 0.5% for optimization). Bands for analysis with the Dice coefficient were assigned manually, according to densitometric curves and the accompanying hard-copy photograph. As previously reported, *HindIII* generates a few bands of very low intensity, especially under 1 kb (15). One third of the ECOR reference strains, which were ribotyped twice,

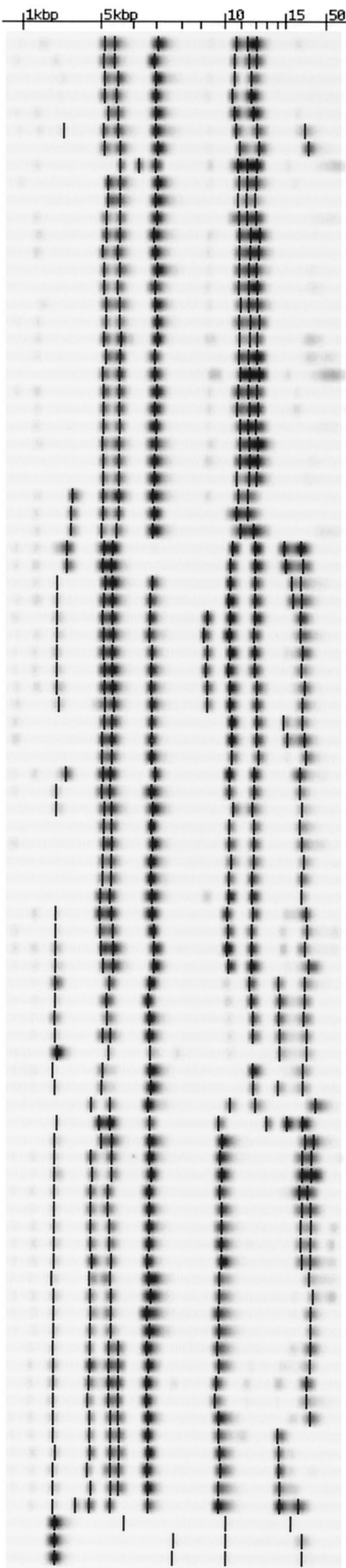
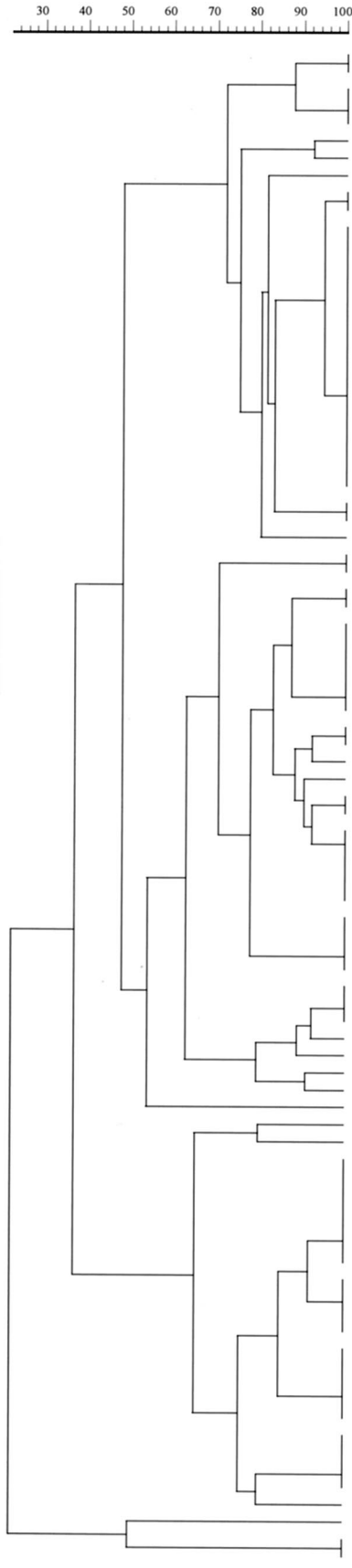
showed that these faint bands were nonreproducible (data not shown). Thus, these bands were not taken into account in the band-based cluster analysis. On the other hand, major bands were always reproducible and were perfectly in accordance with manual ribotypes patterns previously obtained (3) (data not shown).

Access to the Riboprinter database for downloading ECOR ribotypes will be made available upon request (edouard.bingen@rdb.ap-hop-paris.fr or marc.lange@pasteur-lille.fr). Furthermore, all data (including the dendrogram) are available at the Molecular Typing Center website (<http://www.pasteur-lille.fr/english/techno/ctm/ecor.html>).

Ribotyping of the 72 ECOR strains using the *HindIII* enzyme yielded 32 ribotypes after exclusion of the faint bands generated by this enzyme (see Materials and Methods). The number of strains presenting a given ribotype ranged from 1 to 12. All strains with the same pattern belonged to the same phylogenetic group. Only one exception was noted: the patterns for ECOR 24, 26, 31, and 37 belonged to groups A, B1, E, and E, respectively. A phylogenetic tree of the 72 ECOR strains, 15 pathogenic reference strains, and K-12 strain MG1655 was obtained by UPGMA (Fig. 1). Three major clusters containing 69 ECOR strains were clearly distinguished on the tree. Strains belonging to groups A and B2 were clearly separated from each other and from a cluster containing the B1 and D strains. Three strains (ECOR 35, 36, and 66, belonging to groups D, D, and B2, respectively) displayed an atypical ribotype and were separated from the three major clusters. Group B1 and D strains clustered very close together but remained distinguishable from each other. In subcluster B1, one pattern was yielded by strains belonging to group A and the group of unclassified strains (Fig. 1). Thus, strains yielding this pattern could not be unambiguously grouped. Apart from this exception, each of the other 31 patterns always contained strains belonging to a given phylogenetic group. Thus, a clinical strain yielding one of these 31 patterns can be unambiguously categorized in one of the four phylogenetic groups.

To evaluate this grouping method, we tested 15 pathogenic *E. coli* strains. The automated ribotyping technique readily grouped these strains, all but one of which yielded patterns identical to one of those obtained with the ECOR collection. As expected, the six extraintestinal pathogenic strains were classified in groups B2 and D. Interestingly, the neonatal meningitis strains (RS218 and C5) and the three uropathogenic strains (J96, E536, and CFT073) belonged to four different subgroups within group B2. The intestinal pathogenic strains were distributed among all the phylogenetic groups, and strains belonging to one pathotype were distributed among different phylogenetic groups. For example, EAggEC strains JM221 and O42 belonged to groups A and D, respectively, while ETEC strains belonged to groups A (E2536-1 and EDL1493) and B1 (TX-1). However, EHEC strain EDL933

FIG. 1. Comparative analysis of *HindIII* ribotypes obtained with the Riboprinter for the ECOR collection and a subset of *E. coli* pathogenic reference strains. Clustering was performed by UPGMA, and similarity analysis was based on the use of the Dice coefficient (see Materials and Methods). For each strain, name, serotype, phylogenetic group or pathovar, and source is indicated. O:H serotypes are as listed at the T. Whittam laboratory website. ON, HN, nontypeable with standard antisera; NM, nonmotile strain; EPEC, enteropathogenic *E. coli*; NMEC, neonatal meningitis *E. coli*; DAEC, diffuse adherent *E. coli* EAggEc, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; UPEC, uropathogenic *E. coli*; ABU, asymptomatic bacteriuria; UTI, symptomatic urinary tract infection.



ECOR 4 ON:HN
 ECOR 15 O25:NM
 ECOR 22 ON:HN
 ECOR 16 ON:H10
 ECOR 23 O86:H43
 ECOR 17 O106:NM
 ECOR 13 ON:HN
 ECOR 20 O89:HN
 ECOR 6 ON:NM
 ECOR 1 ON:HN
 ECOR 21 O121:HN
 ECOR 12 O7:H32
 ECOR 14 OM:HN
 ECOR 3 O1:NM
 ECOR 25 ON:HN
 ECOR 5 O79:NM
 ECOR 7 O85:HN
JM221 O92:H33
 ECOR 2 ON:H32
 ECOR 9 ON:NM
E2539-C1 O25:HN
EDL1493 O6:H6
 ECOR 8 O86:NM
 ECOR 18 O5:NM
 ECOR 11 O6:H10
K12-MG1655
 ECOR 10 O6:H10
 ECOR 19 O5:HN
 ECOR 43 ON:HN
TX-1 O78:H12
 ECOR 69 ON:NM
 ECOR 71 O78:NM
 ECOR 68 ON:NM
 ECOR 32 O7:H21
 ECOR 67 O4:H43
 ECOR 34 O88:NM
 ECOR 33 O7:H21
 ECOR 30 O113:H21
 ECOR 29 O150:H21
 ECOR 45 ON:HM
 ECOR 28 O104:NM
 ECOR 27 O104:NM
 ECOR 72 O144:H8
 ECOR 58 O112:H8
 ECOR 70 O78:NM
 ECOR 26 O104:H21
 ECOR 24 O15:NM
 ECOR 37 ON:HN
 ECOR 31 O79:H43
EDL933 O157:H7
 ECOR 46 O1:H6
 ECOR 47 OM:H18
 ECOR 48 ON:HM
O42 O44:H18
 ECOR 41 O7:NM
RS176 O7:K1
 ECOR 40 O7:NM
 ECOR 39 O7:NM
 ECOR 38 O7:NM
 ECOR 50 O2:HN
 ECOR 49 O2:NM
 ECOR 42 ON:H26
 ECOR 44 ON:HN
E2348/69 O127:H6
 ECOR 55 O25:H1
 ECOR 57 ON:NM
CFT073 O6:K2:H1
 ECOR 56 O6:H1
 ECOR 54 O25:H1
 ECOR 52 O25:H1
 ECOR 51 O25:HN
J96 O4:K6
 ECOR 53 O4:NM
 ECOR 59 O4:H40
 ECOR 60 O4:HN
 ECOR 64 O75:NM
E536 O6:K15:H31
C1845 O75:NM
A30
 ECOR 63 ON:NM
 ECOR 61 O2:NM
RS218 O18:K1:H7
C5 O18:K1:H7
 ECOR 62 O2:NM
 ECOR 65 ON:H10
 ECOR 66 O4:H40
 ECOR 35 O1:NM
 ECOR 36 O79:H25

Group A Human - healthy
 Group A Human - healthy
 Group A Steer - healthy
 Group A Leopard - healthy
 Group A Elephant - healthy
 Group A Pig - healthy
 Group A Human - healthy
 Group A Steer - healthy
 Group A Human - healthy
 Group A Human - healthy
 Group A Steer - healthy
 Group A Human - healthy
 Group A Human - UTI
 Group A Dog - healthy
 Group A Dog - healthy
 Group A Human - healthy
 Group A Orangutan - healthy
EAggEC
 Group A Human - healthy
 Group A Human - healthy
ETEC
ETEC
 Group A Human - healthy
 Group A Celebes ape - healthy
 Group A Human - UTI
 Group A Human - healthy
 Group A Celebes ape - healthy
 Group E Human - healthy
ETEC
 Group B1 Celebes ape - healthy
 Group B1 Human - ABU
 Group B1 Giraffe - healthy
 Group B1 Giraffe - healthy
 Group B1 Goat - healthy
 Group B1 Dog - healthy
 Group B1 Sheep - healthy
 Group B1 Bison - healthy
 Group B1 Kangaroo rat - healthy
 Group B1 Pig - healthy
 Group B1 Human - healthy
 Group B1 Giraffe - healthy
 Group B1 Human - UTI
 Group B1 Lion - healthy
 Group B1 Gorilla - healthy
 Group B1 Infant - healthy
 Group A Human - healthy
 Group E Marmoset - healthy
 Group E Leopard - healthy
EHEC
 Group D Ape - healthy
 Group D Sheep - healthy
 Group D Human - UTI
EAggEC
 Group D Human - healthy
NMEC
 Group D Human - UTI
 Group D Human - healthy
 Group D Human - healthy
 Group D Human - UTI
 Group D Human - healthy
 Group E Human - healthy
 Group D Cougar - healthy
EPEC
 Group B2 Human - UTI
 Group B2 Gorilla - healthy
UPEC
 Group B2 Human - healthy
 Group B2 Human - healthy
 Group B2 Orangutan - healthy
 Group B2 Infant - healthy
UPEC
 Group B2 Human - healthy
 Group B2 Human - healthy
 Group B2 Human - UTI
 Group B2 Human - UTI
UPEC
DAEC
DAEC
 Group B2 Human - healthy
 Group B2 Human - healthy
NMEC
NMEC
 Group B2 Human - UTI
 Group B2 Celebes ape - healthy
 Group B2 Celebes ape - healthy
 Group D Human - healthy
 Group D Human - healthy

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showed a ribotype associated with strains belonging to groups A and B1 and the group of unclassified strains and could not thus be affiliated with a given group. Nevertheless, this profile may be considered representative of the O157:H7 strains, since 10 other strains tested had an identical pattern (data not shown) (7).

Phylogenetic analysis is increasingly important in the analysis of bacterial virulence. Thus, for emerging pathogens for which virulence factors are partially known, determination of the genetic background could be of the utmost importance (5, 18, 21). New phylogenotyping methods have recently been developed, such as repetitive-element PCR fingerprinting (13), fluorescent amplified fragment length polymorphism (1), and multilocus sequence typing (14), but manual ribotyping and MLEE remain the gold standard in this field. However, these techniques are complex and time-consuming and are therefore unsuitable for studies of the relationship between virulence and genetic background. The availability of an automated, standardized ribotyping technique provides the opportunity to initiate a database of ECOR strains, making the Riboprinter a simple and rapid phylogenotyping tool.

We chose to ribotype the ECOR collection with *Hind*III, because of its discriminating power and the fact that the profiles it yields can easily be attributed to a given phylogenetic group (2, 3, 7). Most of the ECOR strains clustered in their original phylogenetic group (11), and the similarity level in the four major divisions (~70%) was not different from those found by Herzer et al. (11). The group B1 and D strains were the most difficult to discriminate. However, strains belonging to these two groups never yielded identical patterns. Despite a lesser discriminating power of ribotyping compared to MLEE typing, only one ribotype pattern, containing ECOR 24, 26, 31, and 37, carried a risk of erroneous phylogenetic group attribution. Considering that more than 90% of the pathogenic strains tested here yielded profiles identical to that of one of the ECOR strains, the phylogenetic group of each strain could be determined without recourse to a statistical clustering method. Indeed, analyzing retrospectively the ribotypes obtained from our collection of 69 *E. coli* neonatal meningitis strains described previously (3), we observed that 88% of these strains yielded a profile identical to that of one of the ECOR strains (data not shown). A recently published method determines the phylogenetic group of a strain using a rapid and simple PCR-based technique (7) but appears to be more suited to screening studies. Indeed, the automated ribotyping method has the advantage of attributing a strain to a phylogenetic subgroup defined by one or a few ECOR strains. In this study we observed an excellent correlation between B2 subgroups described by Herzer et al. (11) and B2 ribotyping subgroups. Of particular interest, the five B2 archetypal extraintestinal pathogenic strains were distributed among the four major B2 subgroups (represented by more than one strain). Thus, this rapid method appears to be a more accurate tool for investigating the phylogenetic relationship of a strain, especially uropathogenic and neonatal meningitis strains, to the *E. coli* population as a whole. Moreover, the electronic ribopattern database that we have initiated provides rapid investigation of the genetic relationship between clinical extraintestinal and B2 subgroup ECOR or archetypal pathotype strains.

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