

Full Paper

Analysis of genetic relationships and antimicrobial susceptibility of *Escherichia coli* isolated from *Clethrionomys glareolus*

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Eleven strains of *Escherichia coli* were isolated from 54 bank voles living in the Łomża Landscape Park of the Narew River Valley, indicating that *E. coli* is not common in the alimentary tract of these mammals. On the basis of pulsed-field gel electrophoresis and computer-assisted analysis, the isolates were grouped into six genotypes at similarities of 39%. Chromosome length of *E. coli* under study differed by as much as 900 kb, ranging 2.7–3.6 Mb. All strains were susceptible to amikacin and ciprofloxacin, whereas, for tetracycline, streptomycin, ampicillin, and cefonicid, different results were noted. No differences were detected among the plasmid complements of eight strains (73%), for which plasmid profiles revealed the presence of two plasmidic bands. One, three and four plasmids were observed in a plasmid pattern of single isolates. The observation from the study indicated the high genetic polymorphism among the isolates recovered from the animals of one species living in the same environment.

Key Words——antibiotic susceptibility; *Clethrionomys glareolus*; *Escherichia coli*; PFGE; plasmid

Introduction

Escherichia coli is a very common member of the intestinal microflora of both humans and warm-blooded animals. It is a commensal or opportunist pathogen, implicated in acute infections e.g. of urinary tract, gastro-intestinal tract and lungs (Izumikawa et al., 1998; Kariuki et al., 1999; Krause et al., 1996; Tamura et al., 1996). Our understanding of *E. coli* has been largely derived from strains recovered from humans, zoo or domestic animals (Cobbold and Desmarchelier, 2001; Izumikawa et al., 1998; Krause et al., 1996; Tamura et al., 1996), and environments contaminated with human and animal waste (McLellan et al., 2001; Parveen et al., 1997; Rosas et al., 1997). However, lit-

tle is known about the occurrence and properties of *E. coli* in connection with free-living animals. Strains isolated from animals in zoos may not represent the species as found in natural habitats, because of the possibility of feedstuff having a large impact on intestinal bacterial composition (Jarvis et al., 2000), and of possible cross-contamination (Kariuki et al., 1999).

As deduced from the different genotypic and phenotypic studies, *E. coli* is a highly variable species. The study of Routman et al. (1985) and Pupo et al. (2000) on biotype diversity in collections of *E. coli* isolated from yellow baboons and Australian rats, respectively, suggest that *E. coli* isolates from non-human-associated hosts may be more diverse than those isolated from humans and domestic animals.

Recently, a number of studies indicated that genomic fingerprints from pulsed-field gel electrophoresis (PFGE), in which large DNA fragments are separated after digestion with cleaving restriction endonucleases, are an effective technique for discriminating bacteria at

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a genomic level. In addition, PFGE analysis is a powerful tool for evaluation of relationships among strains recovered from separate areas. Because of its typeability, reproducibility and discriminatory power, PFGE has been applied successfully and accepted widely for the molecular typing of many microorganisms (Bergthorsson and Ochman, 1995; Jarvis et al., 2000; Nauerby et al., 2000; Shimizu et al., 1997). Phenotypic differences like antibiotic resistance, have often been associated with the presence of extrachromosomal elements such as plasmids.

Several *E. coli* collections from different countries have been described. None of them included strains from north-eastern Poland. This region, called "The Green Lungs of Poland," contains a rich biodiversity. Therefore, a high diversity of *E. coli* strains can be expected. In this report, we assess genetic diversity as revealed by PFGE and plasmid profiling, and antibiotic susceptibility among *E. coli* isolated from bank voles living in the Łomża Landscape Park of the Narew River Valley (NE Poland). The study of *E. coli* isolated from free-living small mammals has not been undertaken in Poland.

Materials and Methods

Sample collection and *E. coli* strains isolation. Bank voles were caught in July 2000, during the investigation of the frequency of small mammals in the Łomża Landscape Park of the Narew River Valley (Fig. 1). Since 1994, this area (73.5 km²) has not been exposed to human activity; however, the territory around the park is agriculturally exploited.

The whole intestines of the bank voles (from stomach to anus) were recovered into sterile tubes and placed in the dark on ice (usually 6–8 h) before analysis. The intestines with content, were homogenized in a saline at the concentration of 10%. One hundred microliters of the inoculum were streaked adequately to produce single colonies onto McConkey's agar plates (Oxoid, Ltd., Basingstoke, England) and incubated at 37°C for 48 h. Single colonies indicating the typical growth for *E. coli*, were picked for further investigation and subjected to confirmatory phenotypic tests by the use of API 20E system (BioMerieux, Lyon, France).

Antibiotic susceptibility. The following antibiotics obtained from Becton Dickinson Microbiology Systems (Cockeysville, USA) were tested: amikacin (30 µg), ampicillin (10 µg), cefonicid (30 µg), ciprofloxacin

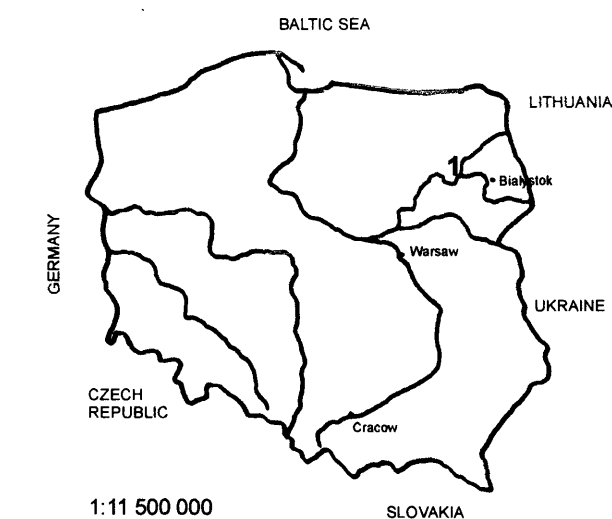


Fig. 1. Geographic location of the Łomża Landscape Park of the Narew River Valley.

(5 µg), streptomycin (16 µg), and tetracycline (30 µg). The procedure for testing and evaluation of the results followed the recommendations given in the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

Genome typing by PFGE. A single colony of each isolate was grown overnight at 37°C in LB broth (Sambrook and Russel, 2001). DNA for PFGE was isolated by the procedure of Jarvis et al. (2000). Slices of agarose blocks containing the DNA were digested for 3 h with 20 units of restriction enzyme *Xba*I (MBI Fermentas, Vilnius, Lithuania). PFGE was performed with the CHEF-DR II System (Bio-Rad Laboratories, Hercules, CA, USA). DNA fragments were separated in 1% (w/v) agarose gels, in 0.5× TBE buffer at 4°C. Pulse times were ramped from 5 to 50 s during a 22-h run at 6.0 V/cm. Lambda DNA concatemers (Sigma Chemical Co., St. Louis, MI, USA) was used as DNA size standards. After electrophoresis completion and staining with ethidium bromide solution (1 µg ml⁻¹), the gels were photographed in ultraviolet light using the Gel Doc 2000 System (Bio-Rad). Further analysis of the patterns was carried out with the Quantity One PC version 4.1 program (Bio-Rad). The molecular weights of the restriction fragments were calculated by comparison with the marker. The total molecular weight of the genomes of each organism was determined by adding the sizes of the fragments produced by restriction enzyme, as described by Bergthorsson and Ochman (1998).

Plasmid analysis. All strains were grown overnight

in LB broth at 37°C to an optical density at 620 nm (OD_{620}) of 0.7–0.8, after which 3 ml of culture was used for plasmid isolation by the alkaline lysis method (Sambrook and Russel, 2001). Samples were analyzed by electrophoresis in 1× TBE at 100 V for 6 h on 0.8% (w/v) agarose gels. A supercoil DNA ladder (Bio-Rad) was used to estimate plasmid sizes.

Numerical analysis. The similarities between strains was calculated on the basis of numerical analysis as described by Priest and Austin (1993). Each DNA profile was compared with every other using the Dice similarity coefficient. The simple matching coefficient was employed to calculate similarities between strains under biochemical properties. In both cases, dendrograms were formed to reflect these similarities by unweighted pair-group average linkage analysis (UPGMA), performed with the NTSys PC version 2.02 g program (Exeter Software, Setanket, NY, USA).

Results

In total, 96 strains of *Enterobacteriaceae* were isolated from the intestines of 54 bank voles. Eleven isolates were confirmed to be *E. coli*. Differences were detected among the isolates of *E. coli* subjected to biochemical properties determined by API 20E tests. All strains reacted positively to nine compounds (e.g. lysine utilization, sorbitol fermentation), negatively to six (e.g. acetoin production, inositol fermentation), while to six others different results were noted (e.g. arginine utilization, sucrose fermentation). Numerical analysis revealed 82% similarity for all isolates (data not presented).

PFGE analysis generated six unique patterns for 11 strains tested (Fig. 2). The three pairs of isolates (174 and 210, 556 and 563, 449 and 561) showed the same DNA patterns, so they can be considered genetically close or identical. Each profile included 12–17 DNA fragments, ranging from 41 to 668 kb (Table 1). Three bands of ca. 185, 112, and 53 kb, were present in five DNA profiles. Most patterns also possessed a fragment of ca. 277, 250, and 156 kb. However, only one fragment of ca. 72 kb was highly conserved and shared by all of the strains examined. Differences between DNA profiles were observed throughout the entire patterns, but principally in the major DNA bands with estimated molecular sizes ranging from 313 to 668 kb. In this partition a low number of restriction fragments were common to all strains. The differences

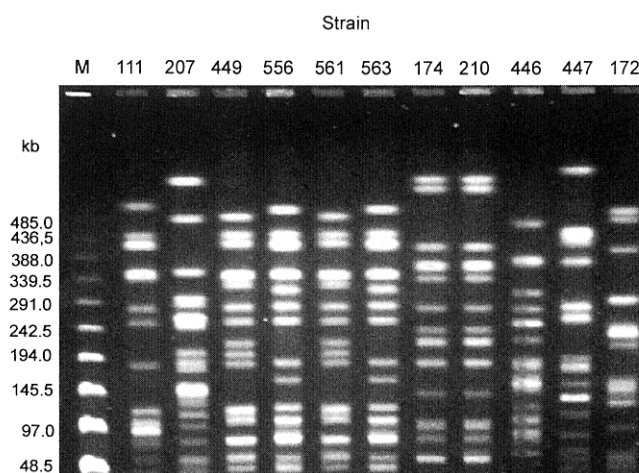


Fig. 2. Total DNA fingerprints of *Escherichia coli* isolates obtained after *Xba*I digestion.

Lane M, lambda 48.5-kb size marker (Sigma Chemical Co.). The values on the left are molecular weight markers in kilobases.

between DNA patterns of the strains under study were reflected in the numerical analysis, where six clusters were formed with a similarity of 39% (Fig. 3). Clusters I and VI contained five and two strains, respectively, while the remaining groups were formed by single isolates. The genome sizes of *E. coli* isolated from bank voles were estimated to be the largest (approximately 3.6 Mb) for isolates 174, 210, 449, 561, and the smallest (2.7 Mb) for strain 172 and 446 (Table 2).

All *E. coli* strains were susceptible to amikacin and ciprofloxacin, whereas for ampicillin, cefonicid, streptomycin, and tetracycline, different results were noted (Table 2). As antibiotic resistance is usually carried by plasmids, it was decided to study the plasmid content of *E. coli* isolates (Fig. 4). No differences were detected among the plasmid complements of eight strains (73%), for which the profiles revealed the presence of two plasmidic bands of about 12.2 and 27.8 kb. Three elements of 12.2, 26.4, and 28.5 kb were observed in a plasmid pattern of the isolate 111. A total of four plasmids (12.2, 26.4, 28.5, 39.6 kb) were noted for the isolate 446, while strain 447 contained a single plasmid of approximately 35.7 kb (Table 2).

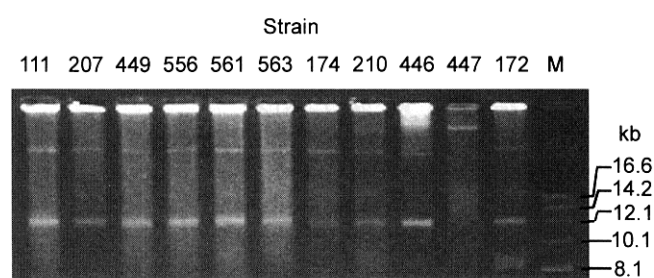
Discussion

Generally it is held that *E. coli* can be isolated from most warm-blooded animals. However in our study, the incidence of *E. coli* in 20.4% animals, indicated

Table 2. Characteristics of isolates of *Escherichia coli* studied.

Strain	DNA pattern	Genome size [Mb]	Plasmids [kb]	Antibiotic ^a					
				AM	CID	S	Te	AN	CIP
111	I	2.9	12.2; 26.4; 28.5			I	R		
449	I	3.6	12.2; 27.8					R	
561	I	3.6	12.2; 27.8		I				
556	I	3.3	12.2; 27.8	I					I
563	I	3.3	12.2; 27.8						I
446	II	2.7	12.2; 26.4; 28.5; 39.6				I	I	
447	III	3.3	35.7						
207	IV	3.1	12.2; 27.8	I				I	
172	V	2.7	12.2; 27.8			R			
174	VI	3.6	12.2; 27.8						
210	VI	3.6	12.2; 27.8	I					I

^aSusceptible results have been omitted for clarity. R, resistant; I, intermediate; AN, amikacin; AM, ampicillin; CID, cefonicid; CIP, ciprofloxacin; S, streptomycin; Te, tetracycline.

Fig. 4. Plasmid profiles of *Escherichia coli* isolates.

Lane M, supercoiled DNA ladder (Sigma Chemical Co.). The values on the right are molecular weight markers in kilobases.

types were unique for single isolates, indicating a very diverse genome for *E. coli* isolated from the same environment and the same species of mammals. These findings are consistent with those reported high levels of genetic diversity of natural populations of *E. coli* from wild hosts (Souza et al., 1999), domestic animals and humans (Cobbold and Desmarchelier, 2000; Kariuki et al., 1999), and from environments with fecal pollution (McLellan et al., 2001). However, in contrast to Souza et al. (1999), who suggested that the high genetic polymorphism is observed for the isolates from different hosts, our study showed that genomic diversity is not ecologically structured, and can characterize *E. coli* originating from one niche. Interestingly, the numbers of DNA fragments in the profiles of strains under study were similar to those found for *E. coli* isolated from dairy cattle (Cobbold and Desmarchelier, 2001) and from human and domestic birds (Kariuki et

al., 1999). Moreover, in our study the Dice coefficient for band pattern similarity (39–100%), was comparable with those for *E. coli* recovered from recreational water (McLellan et al., 2001) and cattle (Jarvis et al., 2000).

The *E. coli* under study differed significantly in the length of their chromosome. Generally, the value of the chromosome size of the isolates was smaller, when compared with those for natural strains of *E. coli* examined by Bergthorsson and Ochman (1998). However, the inner-strain variation of 900 kb for our isolates is similar, compared with 1 Mb differences in the length of *E. coli* chromosomes studied by these authors (Bergthorsson and Ochman 1998).

Most of the *E. coli* isolates presented similar antibiotic susceptibility. The differences were noted for four antibiotics out of six tested. This finding can suggest the lack of real isolation of bank voles from human environment, and may be due to the widespread presence of these antibiotics in human and domestic animal populations. In fact, the Łomża Landscape Park of the Narew River Valley was created in 1994 on an area which had been agriculturally exploited. The neighborhood of the park is still characterized by human activity. In our study a large proportion of isolates (55%) were resistant or intermediate to tetracycline. The high level of resistance to this antibiotic has been observed by Kariuki et al. (1999) for *E. coli* isolated from children and chickens living in the same area. In addition, a tendency of increased *E. coli* resistance to tetracycline, was noted by Kim et al. (1994).

Similarly to Souza et al. (1999), we did not observe that antibiotic resistance in our collection of *E. coli* is correlated with the numbers of plasmids.

To conclude, whole genome analysis using PFGE combined with plasmid profiling data was shown to be a useful tool to characterize natural isolates of *E. coli*. The observation from the study described here indicated that the isolates of *E. coli* from bank voles living in the Łomża Landscape Park of the Narew River Valley differ significantly in the DNA profiles and in the size of their entire genome, while being similar on the basis of numbers and sizes of plasmids and antibiotic susceptibility. The genotypic profiling indicated higher diversity between strains under study in comparison with phenotypic properties.

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