GENETIC CHARACTERIZATION OF RABBIT *ESCHERICHIA COLI* STRAINS WITH THE USE OF MICROARRAY TECHNOLOGY

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ABSTRACT

A DNA microarray able to detect most of the virulence and antimicrobial resistance genes in *Escherichia coli* was used to genetically characterize twenty three rabbit *E. coli* strains. These strains were isolated from enteric pathologies of rabbits observed in a problematic rabbitry during two years of health surveillance. Microarray data were analyzed with clustering software. Three different groups/clusters were identified. Common virulence, putative virulence genes or markers detected in all strains were *gad, ompA, ibeB, b1121, csgE, tspE4.C2*, *hlyE*, *mviN*, and *artJ* genes. Cluster 1 is composed of 10 strains included genes of the Locus of Enterocyte Effacement (LEE), like *eae*, *eae(beta)* and *espA-1*, which are typical of enteropathogenic *E. coli* (EPEC), *efa1*, *lpfA*, and the antimicrobial resistance gene *ant(3)-Ia* (*aadA1*). Other virulence genes, like *afr2* which encodes an adhesin characteristically found in rabbit *E. coli*, and *cif*, a gene encoding a toxin called cycle inhibiting factor, have been found in almost all the samples of cluster 1. Cluster 2 is composed of 12 strains, the strains prevalently have a more non EPEC gene profile with only the type 1 fimbriae encoding genes (*fimA* and *fimH*), *csgE*, *tet(A), int1(2)* and presence of integron class I. Cluster 3 is composed of only one strain and has a different gene profile like non EPEC, but with more virulence and antimicrobial genes. Main anatomo-pathological characters of diseased rabbits were also grouped according to the virulence gene clusters. The result has been an individualisation of a prevalently enterotyphlitis group, corresponding to cluster 1, and a prevalently constipating, mucoid enteropathies group corresponding to cluster 2.

Key words: *Escherichia coli*, Microarray, Rabbit, Virulence gene, Antimicrobial resistance gene.

INTRODUCTION

A DNA microarray used in this work has been developed and validated for the detection of an exhaustive list of virulence genes or markers and antimicrobial resistance genes in *Escherichia coli* strains (Bruant *et al*., 2006).

This microarray is composed of 348 70-mer oligonucleotides specific for 189 virulence genes or markers representative of *E. coli* pathotypes, and for 31 antimicrobial resistance genes representative of six antimicrobial family and the *class I integron*. It was performed to discriminate the different *E. coli* pathotypes on the basis of their set of virulence genes or markers: *eae, espA, espB, tir* genes and/or their variants from the Locus of Enterocyte Effacement (LEE), discriminating the enteropatogenic *E. coli* pathotype (EPEC); Shiga-like toxin encoding genes, genes from the LEE, *ehxA* discriminating the enterohemorrhagic *E. coli* pathotype (EHEC); heat-stable and heat-labile toxin encoding genes, F4 and F18 fimbria encoding genes discriminating the entorotoxigenic *E. coli* pathotype (ETEC). Also the virulence genes or markers of the enteroaggregative *E. coli* (EAEC), the diffusely adhering *E. coli* (DAEC) and the extraintestinal pathogenic *E. coli* (ExPEC) pathotypes are discriminated by this microarray (Bruant *et al*., 2006).

The aim of this work is to investigate the potential application of microarray technology for the genetic characterization of rabbit *E. coli* strains.

MATERIALS AND METHODS

Strain collection, microbiology

The twenty three *E. coli* strains characterized in this study are representatives of one hundred and five strains collection, isolated from small intestine, caecum or liver of 7 to 11 weeks-old rabbits coming from a rabbitry located in the province of Teramo (Abruzzi, Central Italy). This rabbitry was monitored for two years, a hundred rabbits were separately reared for fifteen production cycles, these rabbits were fed only with coccidiostatic supplement. A registration protocol of clinical symptoms and anatomopathological lesions was applied to the diseased rabbits.

Microbiological isolation of *E. coli* was obtained with selective agar medium for enterobacteriaceae (BPLS agar modified™, Merck, Darmstadt, Germany or Hectoen enteric agar™, Biolife italiana srl, Milano, Italy or Gassner medium™, Biolife italiana srl, Milano, Italy), biochemically identified with the API® 20E system (bioMerieux, Marcy l'Etoile, France) and kept at 4±1°C in nutritive agar medium (Tryptic soy agar™, Biolife italiana srl, Milano, Italy).

E. coli virulence and antimicrobial resistance microarray

Each oligonucleotide contained in microarray was printed in triplicate on Corning Ultra GAPS slides (Corning Canada, Whitby, Ontario) at the Biotechnology Research Institute in Montreal (National Research Council, Montreal, QC, Canada) as reported by Maynard *et al*. (2005). Microarrays were designed so that three arrays could be printed on the same slide. In this way, three independant hybridizations could be carried out on each slide using independent cover slips.

DNA extraction and labeling, and microarray experiments

DNA was extracted from selected *E. coli* strains with the Wizard Genomic DNA Purification Kit (Promega, Milano, Italy) according to the manufacturer protocol. Extracted DNAs were then quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Celbio Srl., Milan, Italy). An amount of DNA corresponding to 300 ng to 3 µg was brought to a total volume of 21 µl by desiccation (Savant SpeedVac®, ArrayIt, USA) and resuspension in water. DNA was then labelled with the Invitrogen's Bioprime DNA labelling system kit (Invitrogen Life Technologies, Milan, Italy). Labeling efficiency and the percentage of dye incorporation were determined by measuring the absorbance at OD_{260} for the nucleic acids and OD_{650} or OD_{550} for the dye, using the Nanodrop Spectrophotometer, and then incorporating the results in the following link: http://www.pangloss.com/seidel/Protocols/percent_inc.html.

Hybridizations were performed following a protocol derived from Bruant *et al.* (2006). Briefly, for each hybridization, 500 ng of labeled DNA were dried under vacuum in a rotary dessicator (Savant SpeedVac®, ArrayIt, USA). Desiccation was not complete and heat was not applied. Dried labelled DNA was resuspended in hybridisation buffer (Dig Ease Buffer, Roche Diagnostics spa, Milan, Italy). Before hybridization, microarrays were pre-hybridized during at least one hour at 42°C with a pre-heated pre-hybridization buffer containing 5X SSC, 0.1% SDS and 1.0% BSA. After pre-hybridization, the microarrays were hybridized with a solution that consisted of 25 µl of hybridisation buffer, 20 µl of Bakers tRNA (10 mg/ml) (Sigma Aldrich spa, Milan, Italy) and 20 µl of Sonicated Salmon Sperm DNA (10 mg/ml) (Sigma Aldrich spa, Milan, Italy), together mixed with the labeled DNA which has previously been denatured. Microarrays were hybridized overnight at 42°C in a SlideBooster (Advalytix, ABI, Milan, Italy). After hybridization, stringency washes were performed with Advawash (Advalytix, ABI, Milan, Italy) using 1XSSC, 0.02% SDS preheated to 42°C.

Microarrays were then scanned on ScanArray® with ScanArray Gx software (Perkin Elmer, Milan, Italy).

Statistical Analysis

Microarray data were first normalized as described previously (Maynard *et al.*, 2005). Briefly, after subtraction of local background intensity. The median value for each set of triplicate spotted probes was compared to the median value for the buffer or negative control spots. For each slide a cut-off for significant hybridization was established by calculating the mean and standard deviation of the signal-to-noise fluorescence ratio for positive control spots. The cut-off was established as being the difference standard deviation from the mean of the signal-to-noise fluorescence ratio.

RESULTS AND DISCUSSION

The clustogram in Figure 1 (A) shows positive virulence genes or markers for all the strains obtained for all genes questioned by microarray with Hierarchical Average Linkage Clustering (Eisen *et al*., 1998). Common genes to all strains are visualized, and two different groups (clusters 1 and 2) at 79.6% similarity and 0.41 distance, with 10 and 12 strains respectively (except for strain 25, named Group/Cluster 3), are identified. All of the *E. coli* strains have the following genes; *gad, artJ, ompA, ibeB, tspE.C2, b1121, mviN,* and *hlyE,* their function is described in Table 1. Figure 1 (B) and (C) show positive virulence genes or markers and antimicrobial resistance genes of the strains of the cluster 1. The similarity within the cluster 1 is 86.64% with a distance of 0.27. Gene profiles of cluster 1 are compatible with the EPEC pathotype. A complete LEE (*eae, espA, espB, tir* genes and/or their variants), attaching-effacing mechanism of *E. coli* (Jores *et al*., 2004; Kirsch *et al*., 2004), was found in strains 29, 15b, 10, 9, 6, 7, and 23 as may be observed in Figure 1 (D). The *afr2*, a gene encoding adhesin (Fiederling *et al*., 1997) and *cif,* a gene encoding toxin called cycle inhibiting factor (Marchés *et al*., 2003) are diffusely present within this cluster.

Figure 1 (E) and (F) show positive virulence genes or markers and antimicrobial resistance genes of the strains of the cluster 2. The similarity within cluster 2 is 83.09% and the distance is 0.34. Cluster 2 contain non-EPEC gene profiles, but include *fimA* and *fimH* genes encoding adhesin and fimbrial subunit of type 1 fimbriae (Hung *et al*., 2002). Antimicrobial resistance genes (*ant(3)-Ia (aadA1)* and *Tet(A)* genes involving on aminoglycosides resistance are present in almost all of the strains as may be seen in Figure 1 (C) and (F). The *integron class 1* that facilitate insertion of antibiotic resistance (Mercier *et al*., 1990) and *int1* (Scoulica *et al*., 2004), are prevalently in the cluster 2, Figure 1 (F).

Main anatomopathological characteristics of diseased rabbits were considered. Strains that clustered within cluster 1 (6, 7, 9, 10, 15, 23, 26, 28, 29, and 39), on the basis of their minimal common gene profile, came from diseased rabbits having prevalently (9/10 rabbits) liquid content of caecum and/or stomach and/or small intestine, with or without typhlitis and meteorism. On the contrary, strains 12, 17, 18, 19, 20, 21, 22, 24, 36, 37, 30 and 38, clustered within cluster 2, on the basis of their minimal common gene profile, came from diseased rabbits having prevalently (10/12 rabbits) liquid content of small intestine and/or stomach with meteorism and caecal impaction (6/10 rabbits) or mucoid content of colon (3/10 rabbits).

Table 1: List of major common genes present in strains

Gene	Function
gad	Glutamate decarboxylase A, isozyme (amino acid catabolism and metabolism)
artJ	L-arginine periplasmic binding protein, probably involved in virulence
ompA	Outer membrane protein (OMPA or OMPII)
ibeB	Invasion gene locus, putative resistance protein, putative outer membrane lipoprotein of copper ion antiporter
tspE4.C2	Neonatal meningitis strain-specific DNA sequence
b1121	Hypothetical protein ycfZ, homologous to virulence factor
mviN	Putative virulence factor
hlyE	Silent hemolysin, haemolytic phenotype when overexpressed, other name: sheA

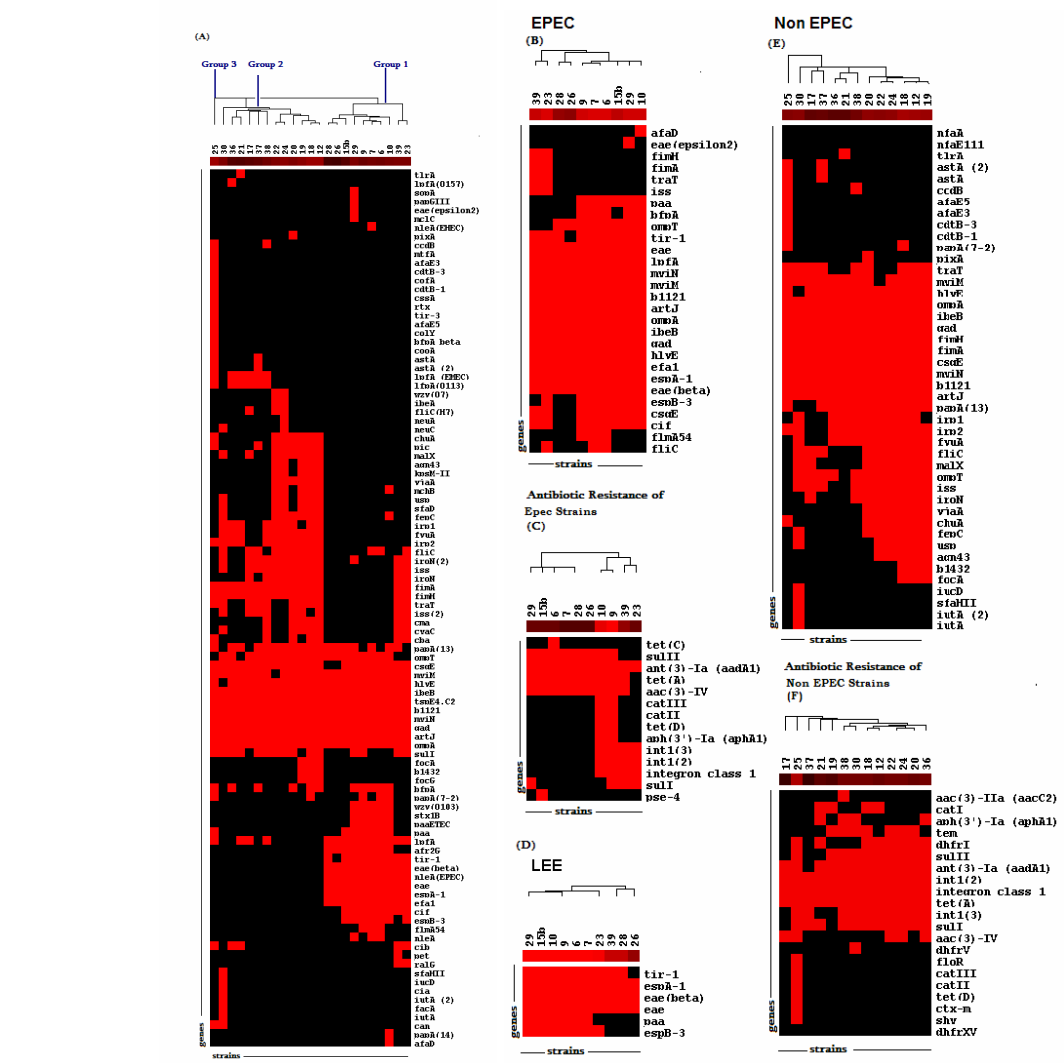

Figure 1: (A) Hierarchical Average Linkage Clustering of total strains to total virulence genes; (B) Clustogram of EPEC virulence genes observed to belong to the Cluster 1; (C) Clustogram of antimicrobial genes belonging to Cluster 1; (D) Clustogram of LEE genes present in Cluster 1; (E) Clustogram of non-EPEC virulence genes observed to belong to Cluster 2; (F) Clustogram of antimicrobial genes belonging to Cluster 2. The clustograms report only genes that are positive for strains, genes negative for all of the strains are not reported

CONCLUSIONS

The microarray technology is a very powerful tool of genetic characterization. Its application at rabbit enteropathogenic *E. coli* infections studies can contribute towards knowledge of genetic basis of pathogenetic mechanisms of enteric disorders. Also about the role of non-enteropathogenic *E. coli*, the microarray technique can help to circumstantiate their possible genetic contribution to rabbit enteropathy syndrome.

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