ANTIMICROBIAL RESISTANCE-RELATED GENE PROFILES OF RABBIT ENTEROPATHOGENIC *Escherichia coli* STRAINS ISOLATED FROM COLIBACILLOSIS OUTBREAKS IN NORTHERN ITALY

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ABSTRACT

A DNA microarray, designed to detect a large set of resistance genes to antimicrobials belonging to Aminoglycosides, β-lactams, Tetracyclines, Sulfonamides, Phenicols, Quinolones and Rifamicines families as well as Erythromycin, Trimetoprim, Olaquindox, Quaternary ammonium compound resistant determinants and mobile genetic elements such as Class 1, 2, 3 Integrons and transposon Tn21, was applied to genotyping twenty-six rabbit enteropathogenic *E. coli* strains coming from 14 rabbitries, located in Northern Italy. Each strain was positive at least for one family of antimicrobial resistance genes. The main frequent genes encoding for the Aminoglycoside resistance were *aac(3)-IV* (20 out of 26 strains), *aadA1* (24 strains), *strA* and *strB* (16 and 21 strains). At least one β-lactamase encoding gene was detected in 15 out of 26 strains. Within this family, the *blaCMY-2* plasmid-borne gene was the most frequent one. Relatively few antimicrobial resistance genes related to Phenicols, Erythromycin, Quinolones and Olaquindox antimicrobials were detected. Among mobile genetic elements, only the Class 1 integron genes were detected in 14 out of 26 strains, six of which have shown to be associated with the *tnpM* gene, confirming an important circulation of the transposon Tn21, never described in rabbit *E. coli*. The presence of mobile elements supporting the multidrug resistance to Aminoglycoside, Tetracycline and Sulfa-Trimethoprim antimicrobial families was discussed as well as the importance of the plasmid-carried *aac(3)-IV* gene as potential gene cassette in Apramicyn-resistant *E. coli* strains. The detection of *blaCMY-2* was reputed an indicator of the involvement of rabbit *E.coli* in the transfer of class C β-lactamases which hydrolyse broad and extended-spectrum cephalosporins (cephamycins).

Key words: Antimicrobial resistance, genotyping, *Escherichia coli*, Integron, Microarray.

INTRODUCTION

The spread of antimicrobial-resistance (AMR) organisms is found on horizontal transfer of genes encoding proteins (enzymes) among bacteria by mechanisms such as plasmid-carried genes, bacteriophage-transducted DNA and/or, mostly in the gram-negative bacteria, mobile genetic elements as transposons and integron-inserted gene cassettes (Hall and Collis, 1998; Van Hoek *et al.*, 2011).
Multiple AMR genes were found in pathogenic and non-pathogenic *Escherichia coli* of human, animal, food and environmental origin (Saenz *et al.*, 2004; Maynard *et al.*, 2003; Maynard *et al.*, 2005) and they have recently risen to health emergency situation with regard to extended-spectrum β-lactamase-producing *E. coli*, ESBL (Leverstein-van Hall *et al.*, 2011).

In a previous study on rabbit *E. coli* strains, a DNA microarray technology was applied and AMR genes were detected in enteropathogenic *E. coli* (EPEC) and non-EPEC strains, associated with Class 1 integron related genes (Tonelli *et al.*, 2008).

The present study aimed to test an upgraded version of the above mentioned microarray to characterize the genetic profiles of AMR determinants of a collection of rabbit enteropathogenic *E. coli* strains.

MATERIALS AND METHODS

Strain collection and microbiology

Twenty six *E. coli* strains were isolated from 7 to 87 days old rabbits, coming from 14 farms with diarrhoeic syndrome located in Veneto and Piemonte Regions (Northern Italy). An inoculum from caecum content of diseased animals was directly inoculated onto Eosin methylene blue agar (EMB, Oxoid Ltd, England). *E. coli* isolates were identified by API 20E® system (BioMérieux spa, France), re-isolated in a nutrient agar, suspended in phosphate buffer saline and their DNA was extracted with GenElute Bacterial Genomic DNA kit® (Sigma-Aldrich, USA) and selected on the basis of the presence of *eae* and *afr/2* genes, detected by classical polymerase chain reaction as previously described (Agnoletti *et al.*, 2004).

Microarray design and antimicrobial resistance genes

The microarray version used in the present study, was composed of 70-mer oligonucleotide probes targeting a large set of AMR genes belonging to the families of Aminoglycosides, β-lactams, Tetracyclines, Sulfonamides, Phenicols, Quinolones, Rifamicines, as well as Trimetoprim, Erythromycin, Olaquindox, Quaternary ammonium compound resistant determinants and mobile genetic elements such as Class 1, 2, 3 Integron and transposon Tn21.

E. coli DNA labeling, hybridization and array data acquisition

For each strain the extracted DNA was quantified, denatured, labeled with a Cyanine family's dye (Cy3) and hybridized as previously described (Salehi *et al.*, 2011). Three independent hybridizations could be carried out on each slide (sub array). Each DNA was tested in duplicate. In this way, six technical tests for each sample were subjected to analysis of the results. The data obtained was normalized as described previously (Maynard *et al*., 2005). At least five probes of the six gene probes had to be positive before a positive score was considered.

RESULTS AND DISCUSSION

Table 1 shows the distribution of 325 positive gene markers found in the examined strains. Each strain was positive at least for one family of AMR genes. The main representative genes encoding for the Aminoglycoside resistance were *aac(3)-IV* (detected in 20 out of 26 strains), *aadA1* (24 strains), *strA* and *strB* (16 and 21 strains). In particular, the plasmid-carried *aac(3)-IV* gene had been recently detected in Apramicyn-resistant *E. coli* strains (Zhang *et al.,* 2009).

Resistance genes to antimicrobials belonging to the Tetracycline, Sulfonamide and Trimetoprim families were detected in 24 out of 26 strains analyzed. Interestingly, 7 of 26 strains showed the simultaneous presence of AMR genes to these antimicrobial families. This multidrug resistance profile was previously highlighted in the rabbit *E. coli*, (Grilli, 2008; Tonelli *et al.*, 2008; Hassan and Abd Al-Azeem, 2009).

At least one β-lactamase encoding gene was detected in 15 out of 26 strains. Within the β-lactamase family, the *blaCMY-2* plasmid-borne gene, an ESBL encoding a cephamycinase, was the most frequent one (8 out 26 strains).

Relatively few AMR genes belonging to the Phenicols, Erythromycin, Quinolones and Olaquindox families were detected.

Table 1: Distribution of positive genes detected in examined strains.

Encoded enzyme/protein	Genes detected (in order to main detection)	Frequency of detection (n° of involved strains)
Aminoglycoside acetyltransferase	*aac(3)-IV, aacC2, aac(6)*	24 (20)
Aminoglycoside adeniltransferase	*aadA1, aadB*	26 (24)
Aminoglycoside phosphotransferase	*aphA(1), aph*	7 (7)
Streptomycin resistance protein	*strB, strA*	37 (21)
Tetracycline resistance protein	*tet30, tet(R), tet(A), tet(E), tet(C) , tetD), tet(B), tet(H), tet(M)*	75 (23)
Class A β-lactamase	*tem ; shv, blaSHV; blaKPC-3; blaCTX-M14, ctx143*	13 (8)
Class B β-lactamase	*blaIMP2; blaVIM-1*	6 (5)
Class C β-lactamase	*blaCMY-2; ampC; ampR; blaFOX-2*	12 (9)
Class D β-lactamase	*blaOXA-2,blaOXA26, blaOXA9*	7 (5)
Chloramphenicol acetyltransferase	*catII, catIII, catBe, cat; cmlA1*	5 (4)
Chloramphenicol/florfenicol exporter	*floR*	2 (2)
Sulphonamide dihydropteroate	*sulII, sulI, sulIII*	28 (20)
Dihydrofolate reductase	*dhfrI, dhfrVII, dhfrXIII, dhfrV, dhfrIX, dhfrVIII, dhfrXII*	19 (9)
Erytromycin esterase	*ereB, ereA, ereA2*	7 (5)
Fluoroquinolone resistance protein	*qnrB4, qnrB1, qnrA*	4 (3)
Plasmid-borne olaquindox resistance determinant	*oqxB, oqxA*	3 (3)
Quaternary ammonium compound resistance protein	*qac*	13 (13)
Integrase	*int1(2), int1(3), int1(1)*	28 (14)
Transposase	*tnpM*	8 (8)

Among mobile genetic elements, only the Class 1 integron genes were detected in 14 out of 26 strains. The integron-related genes, as the Quaternary ammonium compound resistance protein encoding gene (*qac*) and the transposase-encoding *tnpM,* were broadly detected.

The combination between plasmid conjugative machinery of gene movement and the integration of AMR gene into transposons is a powerful one which has been assisted by the strong selective pressure of antibiotic use. The integrons, which are themselves transposons or derivatives, encode an integrase which inserts small genetic units called gene cassettes. The role of gene cassettes as vehicle of AMR genes, mainly used by gram-negative bacteria, is well known (Hall and Collis, 1998).

Table 2 illustrates the profile of 14 strains containing the Class 1 integron and their association with the transposon Tn21 (whose marker is the *tnpM* gene). Six profiles show this association, confirming an important circulation of transposon Tn21 also in rabbit *E. coli*, as already found in avian *E. coli* strains (Bass *et al.*, 1999). These strains have a complete genetic makeup that may allow them to transfer the antibiotic resistance. In detail, the putative or already proven gene cassettes, *aac(3)-IV*, *aadA1*, and the set of *str*, *tet* and *sul* genes, are constantly associated with Class 1 integron and transposon Tn21. The *blaCMY-2* gene in 3 out of 6 strains containing the transposon Tn21, is reputed an indicator of the involvement of rabbit *E.coli* in the transfer of class C β-lactamases. These enzymes hydrolyze the broad and extended-spectrum cephalosporins (cephamycins), and they are not inhibited by β-lactamase inhibitors such as clavulanic acid.

Table 2: Class 1 integron profiles

Integron variable	*tnpM*	Multiresistence-associated gene profiles
int1(2)	no	*aphA1, aacC2, aac(3)-IV, aadA1, strB, tet30, blaTEM, dhfr, qac*
int1(2)	no	*aphA1, aac(3)-IV, aadA1, strA, strB, qac*
int1(2), int1(3)	no	*aacC2, aac(3)-IV, aadA1, tet(R), blaTEM, sulI, sulII*
int1(2), int1(3)	no	*aac(3)-IV, aadA1, strA, strB, tet30, tet(R), tet(A), blaTEM, blaCMY-2, blaKPC-3, blaVIM-1, blaIMP-2, dhfrV*
int1(2), int1(3)	no	*aadA1, strA, strB, tet30, tet(R), tet(A), blaTEM, blaOXA26, sulI, sulII, dhfrVII, ereB, qac*
int1(2), int1(3)	no	*aadA1, tet30, tet(R), tet(A), blaTEM, blaCMY-2*
int1(2), int1(3)	*no*	*aadA1 strA, strB, tet30, tet(R), tet(A), blaCMY-2, blaIMP-2, sulI, sulII, qac*
int1(1), int1(2), int1(3)	*no*	*strB, sulII, qac*
int1(2), int1(3)	*yes*	*aac(3)-IV, aadA1, strA, strB, tet30, tet(R), tet(A), sulI, sulII, qac*
int1(2), int1(3)	*yes*	*aphA1, aac(3)-IV, aadA1, strA, strB, tet30, tet(R), tet(A), tet(C), tet(D), tet(E), blaTEM, ampC, shv, blaCMY-2, blaCTX-M14, blaKPC-3, catII, floR, sulI, dhfrI, dhfrVIII, ereA, qnrB1, qnrB4, qac*
int1(2), int1(3)	*yes*	*aphA1, aac(3)-IV, aac(6), aadA1, aadB, strB, tet30, tet(R), tet(A), blaCMY-2, blaOXA-2, blaOXA26, blaIMP-2, sulI, sulII, dhfrI, qac*
int1(2), int1(3)	*yes*	*aphA1, aac(3)-IV, aadA1, strA, strB, tet30, tet(R), tet(A), tet(B), cat, sulII, dhfrI, dhfrV, dhfrVII, dhfrX, dhfrXIII, ereA, ereA2, ereB, oqxA, qac*
int1(2), int1(3)	*yes*	*aac(3)-IV, aadA1, strB, tet30, tet(R), tet(A), blaCMY-2, blaIMP-2, sulII*
int1(1), int1(2), int1(3)	*yes*	*aac(3)-IV, aadA1, strA, strB, tet30, tet(R), tet(A), sulI, sulII, dhfrI,dhfrXIII,qnrA, qac*

CONCLUSIONS

The DNA microarray technology has been proved to be a powerful tool for genetic characterization of the *E. coli* AMR, confirming the genetic basis of widespread multidrug resistance to Aminoglycoside, Tetracycline and Sulfa-Trimethoprim antimicrobial families. This multidrug resistance is supported by the combined presence of mobile genetic elements. It was underlined the broad presence of *aac(3)-IV* gene as potential gene cassette in Apramicyn-resistant *E. coli* strains, the detection of *blaCMY-2* as indicator of the involvement of rabbit *E.coli* in the transfer of class C β-lactamases and the circulation of transposon Tn21 in the tested strains, the latter never described in rabbit *E. coli*

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