



## Characterization and antibiotics sensitivity of *E.coli* strains isolated from asymptomatic cotton-tail rabbits (*Sylvilagus floridanus*) in Umbria Region - Italy.

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**Abstract.** Three hundred and sixty free-ranging cotton-tail rabbits (*Sylvilagus floridanus*), hunted in Umbria region, Italy, were sampled over a 3-month period. Each animal was submitted to necropsy and examined in order to assess their healthy conditions. One hundred and ten animals were tested bacteriologically for the presence of coliforms in the caecum. One hundred and four strains of *Escherichia coli* were identified (94,5%) and subsequently biotyped, checked for antimicrobial sensitivity (SXT, flumequine, cephalixin, chloramphenicol, ceftriaxone, neomycin, enrofloxacin, apramicyn, amikacin, ampicillin, colistina, amoxicillin+clavulanic acid, streptomycin, oxytetracycline, nalidixic acid, kanamycin, gentamicyn) and investigated for the detection of the virulence genes coding for intimin and adhesive factors (*eae*, *af/r1*, *af/r2*). Fourteen different biotypes were identified: B9(n.1), B16(n.5), B17(n.5), B18(n.13), B19(n.16), B20(n.1), B24(n.1), B25(n.3), B26(n.3), B27(n.42), B28(n.2), B29(n.1), B30(n.4), B31(n.7). Most of the strains isolated showed large sensitivity to the great part of the antibiotics tested, except for neomycin (9,6%), cephalixin (42,3%) and kanamycin (74,4%). Bio-molecular investigations were performed by a multiplex PCR in order to detect the presence of the *eae*, *af/r1* and *af/r2* genes simultaneously. The results showed that only three isolates (B27) out of one hundred and four (2,8%) belonged to the pathotype EPEC thanks to the presence of the *eae* gene, but all of them lacked the adhesive factors AF/R1 and AF/R2. Since germs were collected from rabbits in healthy conditions without any signs of diarrhea, it seemed quite predictable to isolate only a few numbers of EPEC strains.

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### Introduction

Cotton-tail rabbit (*Sylvilagus floridanus*) is a small lagomorph of American origin introduced in Italy in the early Sixties principally for hunting reasons. Nowadays it's widely distributed throughout the Umbria region as well as in other Italian regions such as Lombardy, Piedmont, Tuscany, Emilia-Romagna, Veneto and Marche. Thanks to its territorial distribution, its limited home range and the possibility to be easily handled, it possesses most of the prerequisites of a good indicator species and it can be utilized as monitor for the detection of disease and prediction of epidemics, especially for those lagomorphs which are genetically correlated (*Lepus europaeus*, *Lepus timidus*, *Oryctolagus cuniculus*).

Enteritis is one of the most important cause of mortality in industrial rabbit farms and commercial hare breedings, both in suckling and weaned animals. Strains of *E.coli* belonging to the pathotype EPEC (Enteropathogenic *Escherichia coli*), which are considered a major cause of intestinal diseases in these species, are often isolated from diarrheic animals. Virulence in such germs is associated with the presence of a pathogenicity island (locus for enterocyte effacement or LEE) containing the *eae* gene, which encodes intimin, an outer membrane protein required for production of the typical A/E lesions. The intestinal colonization and adhesion aptitude of EPEC is also

correlated to the presence of two fimbrial adhesins, called AF/R1 and AF/R2, which confer to *E.coli* major attribute of virulence. Since many authors have already conducted works on the characterization and antibiotics sensitivity of *E.coli* strains both in healthy and diarrheic rabbits and hares, we found interesting to extend these studies to cotton-tail rabbits too, because, as far as we know, there aren't any related articles in literature. Aims of this study were: 1) to verify the presence of a resident coliflora in the caecum of healthy cotton-tail rabbits; 2) to report the results of the characterization and antibiotics sensitivity of the *E.coli* strains isolated; 3) to make a comparison between our results and the ones previously published for other species genetically correlated.

## Materials and methods

### Animals and experimental design

Starting from March 2007 and for a 3-month period, three hundred and sixty cotton-tail rabbits (*Sylvilagus floridanus*) were culled in the framework of a remodulation program of carrying capacity in Montepetriolo, an area of Perugia Province. Each animal was sexed, aged, weighed, submitted to necropsy and parasitological examination in order to assess their healthy condition. Caecal swabs were collected from 110 animals and 104 *E.coli* strains were identified, biotyped, investigated for the presence of the typical virulence genes and checked for antibiotics sensitivity.

### Identification and Biotyping

A total of 104 *E.coli* strains isolated from 110 healthy cotton-tail rabbits were investigated. Caecal swabs were plated on MacConkey agar, incubated at 37°C for 24 hours, confirmed as *E.coli* by the API 20E system (bioMérieux, France) and conserved liofillized at -20°C. Fermentation of carbohydrates was tested on a fermentation broth containing bromocresol purple added as pH indicator. Strains were suspended in the medium and results were read after 24 hours of incubation at 37°C. We used the simplified biotyping scheme of Camguilhem and Milon. The following carbohydrates were tested: sorbose (test score 1), dulcitol (test score 2), D-raffinose (test score 4), sucrose (test score 8), L-rhamnose (test score 16). Biotypes were defined by the sum of the test score numbers given by positive fermentative reactions.

### Bio-molecular investigations

One hundred and four *E. coli* strains were characterized by a multiplex-PCR (m-PCR), set up to detect the presence of pathogenicity genes simultaneously. Amplification of bacterial DNA was performed with 25 µL volumes containing 5 µL of DNA sample, 1X Buffer, 0,6 µM (each) primer, 100 µM (each) dATP, dGTP, dCTP and dTTP, 1,5 mM MgCl<sub>2</sub>, and 0,75 U of Go Taq® flexi DNA Polymerase (Promega). The m-PCR primers and conditions were designed in 35 cycles, as follows: EAE-F (5'ACGTTGCAGCATGGGTAAGTTC 3') and EAE-R (5' GATCGGCAACAGTTTCACCTG 3'), AF/R1-F (5' CGGGATCAGCAATTGCTGCTC 3') and AF/R1-R (5' ATCGCCACTAACTTCACATGG 3'); AF/R2-F (5'GTTTCGTTACCGATGAGGCACC 3') and AFR2/R (5' GACAGACGGCTAACCACCTCC 3'), 95°C for 1 min, 62°C for 1 min and 72°C for 2 min.

Cycles were initiated by a denaturation time of 5 min at 95°C and were finished by an extension time of 5 min at 72°C. The amplified product was visualized by standard gel electrophoresis on a 2% agarose gel containing ethidium bromide.

### **Antibiotic susceptibility testing**

Antibiotics susceptibility was investigated by an agar diffusion method according to CLSI(ex NCCLS) M31-A2 procedures. Antibiotics tested were: sulf.+trimeth., flumequine, cephalixin, chloramphenicol, ceftriaxone, neomycin, enrofloxacin, apramicyn, amikacin, ampicillin, colistina, amoxicillin+clavulanic acid, streptomycin, tetracycline, nalidixic acid, kanamycin, gentamicyn.

### **Results and conclusions**

The digestive physiology of lagomorphs is characterized by an intense fermentative activity in the caecum. The microbial flora in this intestinal tract is typically represented by strictly anaerobes, predominantly belonging to the genus *Bacteroides*. Other genera such as *Bifidobacterium*, *Clostridium*, *Streptococcus* and *Enterobacter* complete the caecal population (Gouet and Fonty, 1973).

Rabbits usually have negligible amounts of coliforms in the gut, thanks to the inhibitory effect practiced by VFA, produced as a consequence of the microbial fermentations. Any perturbation of the caecal metabolism leads to a modification of the VFA production, and consequently to an increasing of the pH value, which may facilitate the proliferation of a coliform population, either pathogenic or not-pathogenic strains. Differently to other lagomorphs, in cotton-tail rabbits, *E.coli* seems to be a normal component of the alimentary flora.

This is substantiated by the evidence of large amounts of coliforms in nearly all the asymptomatic animals examined (104/110). This observation corresponds to what previously reported about the prevalence of the enteric bacteria in wild cotton-tail rabbits (Kozlowsky, Glantz, Anthony, 1976). The difference between commercial and wild species in the microbial composition of the intestinal tract may be attributed both to the lack of antibiotic treatments and to a different diet, which has an important influence on the composition of the intestinal flora.

As other authors have already pointed out, coliforms involved in enteric disorders of lagomorphs are in most cases EPEC. The characterization of the 104 *E.coli* strains collected showed that only three isolates out of one hundred and four (2,8%) belonged to this pathotype. Results of bio-molecular investigations have furthermore suggested that germs isolated should be considered part of the resident coliflora of cotton-tail rabbit.

<b>Table 1: Biotypes and Virulence genes</b>				
<b>Biotypes</b>	<b>N.</b>	<b>eae</b>	<b>afr1</b>	<b>afr2</b>
B9	1	-	-	-
B16	5	-	-	-
B17	5	-	-	-
B18	13	-	-	-
B19	16	-	-	-
B20	1	-	-	-
B24	1	-	-	-
B25	3	-	-	-
B26	3	-	-	-
B27	42	3	-	-
B28	2	-	-	-
B30	4	-	-	-
<b>Tot.</b>	<b>104</b>	<b>3</b>	<b>0</b>	<b>0</b>

All the isolates collected were biotyped. Twelve different biotypes were identified B9 (n.1), B16 (n.5), B17 (n.5), B18 (n.13) B19 (n.16), B20 (n.1), B24 (n.1), B25 (n.3), B26 (n.3), B27 (n.42), B28 (n.2), B30 (n.4). There is usually high correlation between the capability of *E.coli* to ferment selectively some carbohydrates and its pathogenic attitude. In commercial rabbit farms, many studies have already demonstrated that some biotypes such as B12, B14 and B28 are constantly associated to virulence factors (Agnoletti et al.,2003; Camarda et al., 2004; Pisoni et al., 2004). Unfortunately there are no data available on the prevalence neither of pathogenic nor not-pathogenic biotypes in cotton-tail rabbits. In this work, rhamnose-negative strains (B12, B14) haven't been detected, and B28 tested negative for the presence of *eae* gene.

Biotypes collected by Grilli et al. from asymptomatic wild rabbits (*Oryctolagus cuniculus*) showed a prevalence of rhamnose-negative strains (B2, B8, B10, B12, B14), while the same authors observed a majority of rhamnose-positive strains (B18, B28, B30, B31) in wild hares (*Lepus europaeus*). B27, the most predominant biotype occurred in this study (40,3%), was the only one which tested positive for virulence genes (n.3), but it has been rarely reported in other lagomorphs and never involved in enteric disorders. Also *E. coli* isolates belonging to biotypes B20 and B30, considered potentially pathogenic strains in rabbit breeding, lacked pathogenicity markers.

<b>Table 2: Antibiotics sensitivity results</b>			
<b>Antibiotics</b>	<b>Sensible%</b>	<b>Resistant%</b>	<b>Intermediate%</b>
SXT	100	-	-
Flumequine	100	-	-
Cephalexin	42,3	1	66,3
Chloramphenicol	91,3	-	8,6
Ceftriaxone	100	-	-
Neomicina	9,6	2,9	87,5
Enrofloxacin	97,1	-	2,9
Apramicina	76,9	1	22,1
Amikacin	90,4	1	8,6
Ampicillin	87,5	-	12,5
Colistina	77,9	1	21,1
Amoxicillin+clavulanic acid	100	-	-
Oxitetracycline	87,5	3,8	8,7
Nalidixic acid	99	-	1
Kanamycin	74,4	-	34,6
Gentamicina	99,1	-	0,9

Results of antimicrobial testing differs from what observed in commercial and wild rabbits. Most of the isolates showed large sensitivity to the great part of the antibiotics tested, except for neomycin (9,6%), cephalexin (42,3%) and kanamycin (74,4%).

This seemed quite predictable; sensitivity to molecules of older generation such as oxytetracycline, no longer used for the treatment of colibacillosis in industrial rabbit farms, is probably due to the poor antibiotic pressure exerted in the environment where these animals lived. As far as we know this preliminary study, conducted on the characterization and antimicrobial susceptibility of *E.coli* strains, should be considered unpublished in cotton-tail rabbits, especially for the detection of virulence genes. Further investigations will be necessary to give a fuller picture of the situation in these wild animals.

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