# VIRULENCE GENES AND ANTIMICROBIAL RESISTANCE PATTERNS OF ENTEROPATHOGENIC *ESCHERICHIA COLI* FROM RABBITS IN SOUTHERN ITALY

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## ABSTRACT

A total of 56 *Escherichia coli* strains isolated from enteritis outbreaks in 28 rabbitries were biotyped and checked for the presence of the *eae*, AF/RI and AF/R2 genes. Antimicrobial resistance to gentamicin (GM10), amikacin (AN30), tetracycline (TE30), erythromycin (E15), spiramycin (SP100), enrofloxacin (ENR5), trimethoprim/ sulphametoxazole (SXT), flumequine (AR30), amoxicillin (AMX 25), apramycin (APR30), difloxacin (DFX10), marbofloxacin (MAR5), nalidixic acid (NA30), neomycin (N30), colistin (CL50), streptomycin (S10).on 55 *E. coli* isolates has been carried out . Either the virulence genes *eae* or AF/R2 were detected in 20 of the 56 isolates, belonging to 10 distinct farms (35,71% of the farms). The AF/R1 gene, encoding for the fimbrial adhesin, was not detected in any of the isolates. Ten biotypes were distinguished: 5 rhamnose (Rh) + (B16, B17, B24, B25, B28) and 5 rhamnose (Rh) - (B0, B1, B8, B9, B12) The *eae* gene was detected in 39,13% of the Rh- strains and 77,77% of such strains was also AF/R2+. The *eae* gene was not so significantly common among the Rh+ strains (21,21%). Nevertheless, the AF/R2 gene was detected in 42,85% of the Rh+ and eae+ isolates. The biotypes 8 and 24 were highly common in the investigated area. The *eae* and *AF/R2* genes were mostly identified in such *E. coli* byotypes. Evaluation of drug resistance showed that all the isolates (100%) were E15-resistant. High percentages of resistance were also found to SP100 (98,2%), SXT (92,8%), TE30 (87,5%), S10 (73,2%), GM10 (71,4%) and N30 (69,6%). A variety of multiple resistance patterns was observed in all the *E. coli* strains tested.

**Key words**: *Escherichia coli*, virulence genes, drug resistance.

## INTRODUCTION

In commercial fattening farms economical losses due to enteric diseases are often attributable to intestinal colonization by *Escherichia coli* (BLANCO *et al*., 1996; LICOIS, 1992). The enteric diseases due to EPEC strains are considered a major cause of weight loss, watery diarrhea and high mortality rates in rabbits. The disease affects weaned rabbits and occurs during the fattening period. The virulence properties of such strains seem to be related to the presence of the *eae* gene, encoding for the protein intimin that is required for the development of the Attaching-Effacing lesions, and to the presence of the

AF/R1 and AF/ R2 fimbriae (PENTEADO *et al*., 2002; PILLIEN *et al*.,1996). The existence of strains highly pathogenic for rabbits has consolidated the adoption, by farmers, of a therapeutic approach based on antibiotics instead of prevention with vaccines and observance of strict hygiene measures. However, empirical treatment with even the most efficacious antimicrobials (quinolones, polypeptides such as colistin and aminoglycosides such as apramycin), while decreasing the mortality rates caused by bad management, may allow for selection and spread of plasmid-born resistances among the strains (VILA *et al*.,1999). Of high concern, transmission of drug-resistant strains from animals to humans and stable introduction of novel drug-resistance genes in human pathogens represent a constant, major threat to humans.

In this study the presence in diarrheic rabbits of enteropathogenic *E.coli* possessing the *eae* and AF/R2 genes was investigated. In addition, a biotyping assay was performed to obtain epidemiological information on the relative distribution of the biotypes and to investigate the correlations between biotypes and pathogenicity of the strains. The most common antimicrobial resistance patterns of the strains causing diarrhoeal diseases were determined.

## MATERIALS AND METHODS

### *E.coli* isolates

A total of 56 *E.coli* strains isolated from fattening rabbits of 28 rabbitries located in Southern Italy, died of enteritis, was analysed. Samples were plated on Agar McConkey Agar (Oxoid) and incubated at 37°C for 24 h. Colonies were screened by the API 20E system (Biomerieux). Each isolate was suspended in Brucella Broth supplemented with glycerol 20% and stored in criovials at -80°C.

### Biotyping

The bacterial isolates were biotyped as previously described from CAMGUILHEM and MILON, (1989). Frozen colonies were thawed, plated on nutritive agar and then incubated at 37°C for 24 h. Biotyping was then performed by cultivating *as spot* the *E. coli* isolates on Red phenol agar (Difco) plates added with 1% of dulcitol, D-raffinose, sucrose, L-rhamnose and sorbose, respectively. Positive/negative results were read after 12-24 h of incubation at 37°C.
According to this biotyping system, each carbohydrate is given a number and the biotype is defined by the sum of numbers corresponding to the carbohydrates fermented (OKERMAN and DEVRIESE, 1985; CAMGUILHEM and MILON, 1989).

### PCR

Detection of the eae, *af/r1* and *af/r2* genes was tested by a PCR technique according to the protocol reported by BLANCO *et al*. (1996) and GANNON *et al*. (1993). *E.coli* strains were plated on nutritive Agar and after 24 h of growth at 37°C a single colony per strain was selected and re-suspended in 200 µl of sterile $H_2O$, boiled at 100°C for 10 min and

centrifuged at 10.000 *rpm* for 5 min. The supernatant was stored at -20°C as a template DNA stock. The primers used to detect the genes encoding for intimin, AF/R1 and AF/R2 are: ***eae*** (BLANCO *et al*., 1996) 5'- ACG TTG CAG CAT GGG TAA CTC -3; 5'- GAT CGG CAA CAG TTT CAC CTG -3'; **AF/R1** (PENTEADO *et al*., 2002) 5'- CGG GAT CAG CAA TTG CTG CTC -3'; 5'- ATC GCC ACT AAC TTC ACA TGG -3; **AF/R2** (PENTEADO *et al*., 2002) 5'- GTT TCG TTA CCG ATG AGG CAC C -3'; 5'- GAC AGA CGG CTA ACC ACC TCC -3'. PCR reaction was performed in a 50µl mixture with 5 µl of extracted DNA, PCR buffer 1 X, $MgCl_2$ 1,5 mM, dNTPs 0.2 mM, oligonucleotide primers 60 pmol, *Amplitaq Gold polymerase* (*Perkin Elmer Cetus Norwalk, USA*) 2,5 U and sterile $H_2O$. The amplification program consisted of 1 cycle at 94°C for 10 min, 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. The annealing temperature for the *eae*–specific fragment was decreased at 55°C.

**Antibiotic resistance**

Evaluation of drug resistance was performed using the method of Kirby-Bauer (Bauer *et al*.,1966) using Mueller-Hinton agar and antibiotic disks containing Gentamicin (GM10), Amikacin (AN30), Tetracycline (TE30), Erythromycin (E15), Spiramycin (SP100), Enrofloxacin (ENR5), Flumequine (AR30), Trimethoprim/Sulphametoxazole (SXT), Amoxicillin (AMX 25), Apramycin (APR30), Difloxacin (DFX10), Marbofloxacin (MAR5), Nalidixic acid (NA30), Neomycin (N30), Colistin (CL50), Streptomycin (S10).

## RESULTS AND DISCUSSION

Either the virulence genes *eae* or AF/R2 were detected in *E. coli* isolates (table 1). About 28,57% (16 of 56) of the *E.coli* strains, possessed the *eae* gene, and 23,21% (13 of 56) of the strains, collected from 9 (32,14%) distinct farms, exhibited the gene encoding for AF/R2. All the *E.coli* isolates were negative for gene encoding for AF/R1.

**Tabla.1 Relationship between biotypes and virulence genes (*eae* and AF/R2) of *E. coli* isolated from diarrheic rabbits**

| | Biotypes | n. of | E. coli eae | E. coli | E. coli eae + and |
|---|---|---|---|---|---|
| Rhamnose - | **0** | 3 | 1 | 2 | 1 |
| | **1** | 2 | 0 | 0 | 0 |
| | **8** | 11 | 7 | 5 | 5 |
| | **9** | 6 | 0 | 1 | 0 |
| | **12** | 1 | 1 | 1 | 1 |
| | **Total Rh -** | 23 | 9 (39,13%) | 9 (39,13%) | 7 (77,77%)* |
| Rhamnose | **16** | 4 | 0 | 0 | 0 |
| | **17** | 1 | 0 | 0 | 0 |
| | **24** | 16 | 7 | 3 | 3 |
| | **25** | 9 | 0 | 1 | 0 |
| | **28** | 3 | 0 | 0 | 0 |
| | **Total Rh +** | 33 | 7 (21,21%) | 4 (12,12%) | 3 (42,85%)* |
| Rh – and | | 56 | 16 | 13 (23,21%) | 10 (76,9%) |
| * calculated on eae + strains | | | | | |

A total of 10 different biotypes were distinguished (Table 1). Five biotypes were rhamnose (Rh) - (B0, B1, B8, B9, B12) and five were rhamnose (Rh) + (B16, B17, B24, B25, B28). Our data suggest that different biotypes may be contemporarily spreading in the same farm. B8 (Rh-) and B24 (Rh+) were the most common biotypes. The majority of the *eae*+ (14 of 16 isolates eae+- 87,5%) and AF/R2+ (8 of 13 isolates AF/R2 - 61,5%) *E. coli* fell into such biotypes.

The *eae* gene was detected in 39,13% of the Rh- strains, with 77,77% of such strains (Rh- and *eae*+) being also AF/R2+. In contrast, only 21,21% of the Rh+ isolates were *eae*+ and 42,85% of such strains (Rh+ and *eae*+) were also AF/R2+ (Table 1).

By evaluation of the antimicrobial resistance, all the isolates (100%) were found to be E15 resistant, as expected. High percentages of resistance were also found to SP100 (98,2%), SXT (92,8%), TE30 (87,5%), S10 (73,2%), GM10 (71,4%), N30 (69,3%). About 91% of the *E. coli* strains were susceptible to MAR5 and CL50. Susceptibility to DFX10, ENR5, AR30, NA30, was exhibited by 80,3%, 78,5%, 75%, 71,4%, of the strains, respectively. Susceptibility to AMX25 was quite high (76,7%). Only few isolates were found to be resistant to quinolones ENR5 (8,9%), AR30 (12,5%), DFX10 (12,5%), MAR5 (1,7%) and NA30 (23,2%). Multiple antibiotic resistance was expressed by all the *E. coli* tested. The most common resistotypes were TE30-E15-SP100-SXT, that was detected in 47 strains (83,93%), TE30-E15-SP100-SXT-S10, that was identified in 60,71% of the strains and TE30-E15-SP100-SXT-GM10, that was detected in 58,92% of isolates (table 2).

*E.coli* is a normal component of rabbit digestive flora and it does not always exert direct pathogenic activity in rabbits. Stress or other pathogens may trigger its overgrowth in the gut environment, which can result in diarrhoea or death (MILON, 1996). In addition, pathogenicity of some strains may be enhanced by the presence of virulence genes, such as *eae*, AF/R1 and AF/R2, and a precise evaluation of the distribution of these genes in *E. coli* population is required to comprehend the attitude to induce severe forms of enteric disease. For instance, the *eae* gene, that encodes for intimin, is required to allow the development of *Attaching-Effacing* lesions (AGIN *et al*., 1996; LEROY *et al*., 1994). In the present study, rabbits with a history of enteritis and deaths were screened. *E. coli* strains displaying the *eae* gene were identified in 32,14% of the farms. Furthermore, 77.77% of the *eae*+ isolates were shown to possess the fimbrial adhesin AF/R2. The contemporary presence of the *eae* and AF/R2 genes might account for increased virulence and/or direct pathogenic activity of such strains, thus explaining the severity of the clinical signs and lesions observed in the herds (MILON, 1996; FIEDERLING *et al*., 1997). In agreement with the results of analogous studies on rabbits (POHL *et al*. 1993), the AF/R1 gene, specific for strain RDEC-1, was not detected in the *E. coli* strains tested in this study, suggesting that such gene is rare in the field.

Biotyping surveys have demonstrated that different biotypes are variously distributed. In this study the prevalence of biotypes B24 (Rh+) and B8 (Rh-) has been found. The finding that some biotypes may be predominant in restricted geographical settings represents an epidemiological pattern that has already been described in literature (PENTEADO *et al*., 2002; CAMGUILHEM and MILON, 1989).

Of note, there was no remarkable correlation between enteropathogenic *E. coli* (*eae*+ and AF/R2+) and the patterns of antimicrobial resistance. All the *E. coli* strains exhibited antimicrobial resistance. Nearly 75% of the *E. coli* strains were susceptible to quinolones, and, in particular, the highest rate of susceptibility was found to marbofloxacin (91%).

## CONCLUSIONS

The findings of the present study give support to the hypothesis that Rh- strains possess higher pathogenicity properties, as they are more frequently associated with the virulence determinants *eae* and AF/R2. However, as the presence of virulence genes can be also demonstrated in Rh+ isolates, it may not be excluded that Rh+ strains displaying the *eae* and AF/R2 genes may be responsible for diarrhoeic diseases in rabbits. During our survey, 90% of the Rh+ strains carrying eae and AF/R2 genes belonged to the B24 biotype. Biotyping is considered a fast and efficient approach to identify highly pathogenic strains but precise evaluation of the pathogenic properties of *E.coli* needs to be supported and correlated with the presence of the virulence-associated genes. Interestingly, LEROY *et al*. (1994) and PENTEADO *et al*. (2002) reported the presence of *eae* gene in slightly or non-pathogenic strains. These findings may indicate that the *eae* gene, although being present in the *LEE* region, may not be constantly expressed as a protein and that expression of the gene might be involved in regulation of the pathogenic activity of *E.coli*.

In this report a correlation between enteropathogenic *E.coli* (*eae*+ and AF/R2+) and a specific pattern of antibiotic resistance was not found. Resistance to antibiotics was observable in all the isolates, and, moreover, there were high percentages of resistance to some of the drugs tested, with multiple resistance patterns being frequently observable. The fact that multiple antimicrobial resistance patterns are highly common in the bacterial population might have critical consequences on farm management and hinder the control of the disease. In addition, the onset and spread of antimicrobial resistance may represent a serious problem for public health, as animals could play a role of reservoirs of EPEC for humans and may transmit novel resistance-associated genes to human pathogens.

Of relevance, when evaluating antimicrobial susceptibility, quinolones were shown to have very good activity against *E. coli*, in particular marbofloxacin, a molecule adopted recently in veterinary medicine. Studies to assess the efficacy of this antimicrobial drug *in vivo* for treatment of enteropathies in rabbits are necessary.

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**Table 2. Relationships between resistotypes and virulence genes (*eae* and AF/R2) in *E. coli* isolated from diarrhoeic rabbits**

| | *Escherichia coli* isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Resistotypes | Total | | Eae + | | Eae - | | AF/R2+ | | AF/R2- | | AF/R2 + and EAE+ | |
| | n | % | n. | % | n | % | n. | % | n. | % | n. | % |
| TE30/E15/SP100/SXT | 47/56 | 83,93 | 14/16 | 87,50 | 33/40 | 82,50 | 13/13 | 100 | 34/43 | 79,06 | 9/9 | 100 |
| TE30/E15/SP100/SXT/S10 | 34/56 | 60,71 | 12/16 | 75,00 | 22/40 | 55,00 | 11/13 | 84,61 | 23/43 | 53,49 | 8/9 | 88,88 |
| TE30/E15/SP100/SXT/GM10 | 33/56 | 58,92 | 11/16 | 68,75 | 22/40 | 55,00 | 9/13 | 69,23 | 24/43 | 55,81 | 6/9 | 66,66 |
| TE30/E15/SP100/SXT/N30 | 32/56 | 57,14 | 10/16 | 62,50 | 22/40 | 55,00 | 7/13 | 53,84 | 25/43 | 58,14 | 6/9 | 66,66 |
| TE30/E15/SP100/SXT/S10/GM10 | 25/56 | 44,64 | 9/16 | 56,25 | 16/40 | 40,00 | 8/13 | 61,53 | 17/43 | 39,53 | 5/9 | 55,55 |
| E15/SP100/S10/GM10/N30 | 24/56 | 42,85 | 9/16 | 56,25 | 15/40 | 37,50 | 6/13 | 46,15 | 11/43 | 25,58 | 5/9 | 55,55 |
| TE30/E15/SP100/SXT/S10/GM10/N30 | 21/56 | 37,50 | 8/16 | 50,00 | 13/49 | 32,50 | 7/13 | 53,84 | 14/43 | 32,55 | 5/9 | 55,55 |
| TE30/E15/SP100/SXT/AR30 | 7/56 | 12,50 | 1/16 | 6,25 | 6/40 | 15,00 | 2/13 | 15,38 | 5/43 | 11,62 | 0/9 | 0,00 |
| TE30/E15/SP100/SXT/S10/GM10/N30 /APR30 | 6/56 | 10,71 | 2/16 | 12,50 | 4/40 | 10,00 | 2/13 | 15,38 | 4/43 | 9,30 | 1/9 | 11,11 |
| TE30/E15/SP100/SXT/S10/GM10/N30 /AMX25 | 5/56 | 8,93 | 1/16 | 6,25 | 4/40 | 10,00 | 1/13 | 7,69 | 4/43 | 9,30 | 1/9 | 11,11 |
| TE30/E15/SP100/SXT/S10/GM10/N30 /APR30/AN30 | 3/56 | 5.35 | 0/16 | 0,00 | 3/40 | 7,50 | 1/13 | 7,69 | 2/43 | 4,65 | 0/9 | 0,00 |