CHARACTERIZATION OF *E.COLI* STRAINS ISOLATED FROM RABBITS WITH ENTERITIS IN LOMBARDIA AND EMILIA ROMAGNA (NORTH ITALY) DURING THE PERIOD 2000-2003

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ABSTRACT

Colibacillosis are the most important cause of enteritis in rabbit breedings (MILON *et al.*,1999). In this paper we report the results of the serotyping of 711 *E. coli* strains, isolated during the period 2000-2003 in Lombardia and Emilia Romagna from European rabbits *(Oryctolagus cuniculus*) with enteritis. The aims of the study were first to investigate which selected O-antigens were mostly associated with rabbits enteric disease and then to detect other pathogenic factors like toxins (VT, LT, CNF) and *eae* genes. The results show that O2 and O103 are the most frequently identified serogroups, being respectively 25.9% and 25,2%, i.e. 79 and 77 out of 305 O-antigens recognized. The *eae* gene was identified in 185 of the 711 strains investigated (26%); toxins were found in 5 strains only (2 VT1, 2 VT2, 1 LT). The identification of O103 serogroup was associated with the presence of the *eae* gene in 60 out of the 77 (77.9%) strains isolated. Such result confirms the importance of the contemporaneous presence of both these pathogenic factors in *E. coli* strains responsible for outbreaks of enteritis in rabbits. Comparing these results with those previously obtained in the same geographic area during the triennium 1997-1999 (FINAZZI *et al.*, 2000), it is evident that we observe both a significant increase of the O2 and O157 serogroups' detection and an important decrease of the O103 detection. Results concerning the serotyping of other O-antigens are substantially superimposing.

Key words: Rabbit, *E. coli*, serotyping, toxins, *eae* gene

INTRODUCTION

E. coli is generally present in man's and animals' gut, where it contributes to the physiology of the digestive apparatus. However, in the case where virulence/pathogenic factors are associated with the occurrence of predisposing factors, it can easily induce disease (CAMGUILHEM *et al.*, 1986). Therefore, the diagnosis of Colibacillosis needs the contemporaneous observation of anatomo-pathological lesions, *E. coli* quantitative evaluation and data of characterization indicating the grade of virulence of the isolated strains (LICOIS, 1992).
The study consisted in the serotyping of *E. coli* strains, isolated during the period 2000-2003 in Lombardia and Emilia Romagna (North Italy) from European rabbits

(Oryctolagus cuniculus), reared both in rural and intensive units and all affected by enteric syndromes. These animals represented an heterogeneous picture of the rabbits population breeded in Lombardia and Emilia-Romagna. Samples were collected from rabbits showing at necropsy lesions of enteritis. *E. coli* strains were isolated from rabbits' gut contents and fecal specimens at Provincial Diagnostic Sections of the Institute using standard bacteriologic methods. Further examinations for the characterization of the isolated strains i.e. determination of serogroup, presence of pathogenic factors like toxins (VT, LT, CNF) and *eae* gene, were performed at the "Specialized Bacteriology Department" of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna - Brescia.

MATERIAL AND METHODS

Serotyping

The adopted serotyping technique was the one used in Lugo's *E. coli* Reference Laboratory – Spain (BLANCO and BLANCO, 1993), opportunely adapted according to our laboratory procedures. All the strains were in advance confirmed as *E. coli* by the Enterotube II Roche Test; then, cultivated on Trypticase Soy Agar (TSA), Mac Conkey Agar and Trypticase Soy Broth (TSB) by successive steps. Each broth culture, developed by only one strain of *E. coli*, was heated in autoclave for 1 hour at 100°C and doubled with 0,5% phenolic physiological solution (PPS). Serotyping was carried out using a battery of monospecific antisera towards 37 different somatic O antigens (O1, O2, O4, O6, O8, O9, O10, O11, O15, O18, O20, O21, O22, O26, O45, O49, O64, O73, O75, O78, O83, O86, O88, O101, O103, O109, O111, O115, O128, O132, O138, O139, O141, O147, O149, O153, O157). These antisera were selected according to national and international literature (BLANCO and BLANCO, 1993; FARINA *et al.*, 1996) and to the present diffusion of O serotypes among domestic animal species in Italy (FARINA *et al.* 1996; FINAZZI *et al.*, 2000).
A part of each broth culture (50 µl) was put in contact with the same volume of each antiserum in U bottom polystyrene microtitre plates and incubated for 24 hours at 37°C in a moist box to cause a slow sero-agglutination (SAL). The broth culture (50 µl) was also put in contact with the same volume of PPS to estimate the auto-agglutinating power of each strain. Negative reactions were indicated by a sharp point, whereas positive reactions by a carpet. The *E. coli* strain was considered non typeable when agglutination was observed in 4 or more microwells. In addition, titration was carried out if agglutination was observed towards 2 or 3 antisera. The titre was determined by SAL using 50 µl of the broth culture and 6 base dilutions of the positive antisera and corresponded to the highest positive dilution.

Toxins detection

The presence of toxins (verotoxins VT, heat-lable toxin LT and cytotoxic necrotizing factor CNF) was demonstrated by the appearance of a cytopathic effect on Vero cells mono-layer. Six millilitres of each *E. coli* broth culture were concentrated by centrifugation 20' at 5000 g at 4°C and inactivated for 30' in an ultra-sound bath in

presence of quartz powder; after a second centrifugation at 10000 g during 30' at 4°C and filtration through bacteriologic filters (2 µm), it was added to the tissue culture (CAPRIOLI *et al.*,1993).
A low amount (0,8 ml) of the *E. coli* broth cultures, inactivated by boiling, was used for molecular-genetic analysis (PCR) finalised to detect the genes which encode both for verotoxins VT1 and VT2 (RUSSMANN *et al.*, 1995) and the *eae* gene (SCHMIDT *et al.*, 1994). The primers used are indicated in table 1; the amplification conditions were those described by the authors.

Table 1. Primers used for PCR amplification

Primer	Gene	Direction	Nucleotide sequence	Bp	Reference
KS7	VT1	dir	atgaaaaaaacattattaatagc	265	RUSSMANN *et al.*, 1995
KS8	VT1	rev	agctattctgagtcaacg		
GK3	VT2	dir	atgaagaagatgtttatg	270	RUSSMANN *et al.*, 1995
GK4	VT2	rev	tcagtcattattaaacgt		
SK1	*eae*	dir	cccgaattcggcacaagcataagc	863	SCHMIDT *et al.*, 1994
SK2	*eae*	rev	cccggatccgtctcgccagtattcg		

RESULTS AND DISCUSSION

Table 2 schematically report the results of O antigens' detection carried out during last four years (2000-2003). O2 (25,9%) and O103 (25,2%) were the serogroups most frequently associated with enteritis outbreaks in the examined Italian regions, with a large prevalence over the other detected rabbit serogroups. These data are in agreement with the results of Blanco *et al.* (1996) obtained in France and Spain but different from those of the previous study carried out in Italy (FINAZZI *et al.*, 2000). In particular our serotyping data indicate an important increase of O2 and O157 identification and a decrease of O103 (Figure 1), whereas the frequencies of the other O serogroups are substantially superimposing.

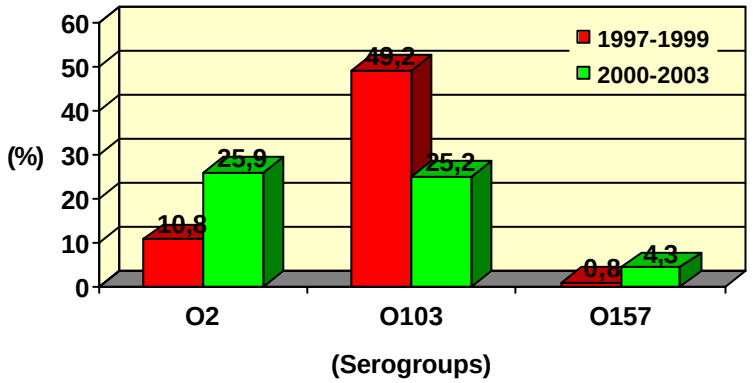

Figure 1. Comparison between O-antigens detection during 2000-2003 vs. 1997-1999 (FINAZZI *et al.*, 2000).

The comprehensive results of serotyping and toxins detection are reported in table 3. The VT, CNF and LT positivities were low (0,7%), thus suggesting their limited pathogenic role in rabbits as already affirmed by FINAZZI *et al.* (2000). This differs from what described for CNF factor in other species like dog, cat and cattle (POHL *et al.*, 1993) and for the LT in pigs (WILSON and FRANCIS, 1986).

The *eae* adherence factor (Table 4) was demonstrated in 185 of the 711 investigated strains (26%); within the serotyped strains, *eae* gene was mostly evidenced in O103 strains (77,9%). Moreover, the simoultaneous presence of O103 and *eae* gene confirms the importance of these factors in *E. coli* pathogenous capacity (LEROY *et al.*, 1994).

In spite of its high prevalence, the O2 antigen was never associated to the contemporaneous presence of *eae* gene.

The *eae* gene itself could be probably considered as an important pathogenic factor since it was detected in 104 of the non-typeable strains (25,6%).

Table 2. Somatic antigens (O) identified during the period 2000-2003

Antigen	2000	2001	2002	2003	Total	%
O1	0	1	1	0	2	0,6
O2	11	25	27	16	79	25,9
O4	1	0	1	0	2	0,6
O6	0	1	1	0	2	0,6
O8	4	1	8	2	15	4,9
O9	0	0	0	1	1	0,3
O10	1	1	0	0	2	0,6
O11	0	1	0	0	1	0,3
O15	0	1	0	2	3	0,9
O18	1	2	1	1	5	1,6
O20	1	1	0	0	2	0,6
O22	0	2	1	1	4	1,3
O45	0	1	0	0	1	0,3
O49	0	2	1	3	6	1,1
O64	0	0	0	1	1	0,3
O73	0	2	5	1	8	2,6
O75	2	2	1	4	9	2,9
O78	1	0	0	0	1	0,3
O83	0	1	0	1	2	0,6
O86	2	1	5	3	11	3,6
O88	0	1	0	0	1	0,3
O101	0	1	0	1	2	0,6
O103	16	23	17	21	77	25,2
O115	0	0	0	1	1	0,3
O132	0	1	2	0	3	0,9
O138	0	0	0	1	1	0,3
O139	2	7	4	5	18	5,9
O141	0	2	8	3	13	4,3
O147	1	0	1	3	5	1,6
O149	0	0	1	0	1	0,3
O153	1	2	4	6	13	4,3
O157	3	5	3	2	13	4,3
Total	47	87	92	79	305	

Table 3. Detection of *E. coli* toxins during the period 2000-2003

Year	Strains Examined	O antigen		Toxins producers			
		Typed	Non typeable	VT		CNF	LT
				VT1	VT2		
2000	147	47	100	1	0	0	0
2001	186	87	99	0	0	0	0
2002	207	92	115	0	1	0	0
2003	171	79	92	1	1	0	1
Total	711	305	406	2	2	0	1

Table 4: Results of *eae* gene detection in *E. coli* strains during 2000-2003

Year	Strains investigated			Presence of *eae* gene			
	Total	Typed	Non typeable	Typed	%	Non typeable	%
2000	147	47	100	12	25.5	21	21.0
2001	186	87	99	26	29.8	27	27.3
2002	207	92	115	17	18.4	28	24.3
2003	171	79	92	26	32.9	28	30.4
total	711	305	406	81	26.5	104	25.6

CONCLUSIONS

The aim of this work was to characterize the *E. coli* strains isolated from diarrhoeic rabbits, with particular reference to the presence of pathogenic factors such as toxins and *eae* genes, since is known the primary role of these *E. coli* strains in determining enteritis outbreaks in rabbitries. In fact, the *eae* gene presence is considered sign of pathogenous potentiality of these bacteria and the major characteristic to define the isolated strains as belonging to "Attaching Effacing *Escherichia coli*" (AEEC) (KENNY, 2002).

Consequently, the characterization of isolated strains, constitutes an indicative suggestion of *E. coli* serogroups and pathogenic factors mostly diffused in Lombardia and Emilia Romagna (North Italy) regions.

Our next purpose will be to compare the anamnestic, clinical, pathological and laboratory data for the presence of other pathogens, taken from each outbreaks of colibacillosis, with the results of the identification of pathogenous factors, in order to improve both therapeutical and laboratory approach to *E. coli* enteritis.

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