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CHARACTERIZATION OF STRAINS OF *E. COLI* ISOLATED FROM RABBITS WITH ENTERITIS IN LOMBARDIA AND EMILIA-ROMAGNA DURING THE TRIENNIUM 1997-1999

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ABSTRACT

E.coli is certainly the most important microbial agent responsible for outbreaks of enteritis in rabbits. In this work the typing of *E.coli* isolated from rabbits with enteritis in Northern Italy in the triennium 1997-1999 is reported. The work was based on the serotyping of 500 strains to investigate which O-antigens were mostly associated with enteric disease of rabbits and if any other pathogenic factors, such as toxins (VT, LT, CNF) or the *eae* gene responsible for adherence, were involved. We observed that O103 is the serogroup most frequently isolated with a prevalence of 117 out 260 O-antigens identified (49%). We also found the adherence gene *eae* in 56 strains out of the 127 investigated (44%), while toxins were found in only 7 strains (1VT, 3 LT, 3 CNF). In O103 strains we observed the association of this serogroup with the presence of the *eae* gene in 35 out 40 (87%). This indicates the importance of the simultaneous presence of both these pathogenic factors in strains of *E.coli* responsible for outbreaks of enteritis in rabbits.

INTRODUCTION

E. coli is certainly the most important causal agent for bacterial enteritis in rabbit-breeding, thus, an understanding of which are the most spread serogroups of this micro-organism will allow researchers to have an idea of the pathogenicity of the various strains diffused within the territory of interest (ØRSKOV & ØRSKOV, 1984).

In this work the results of *E.coli* strains serotyping developed by the Department of "Specialized Bacteriology" of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna from 1997 to 1999 are reported.

The relative provincial Diagnostic Sections of the Institute isolated these strains by plating on MacConkey agar, caecal specimens taken from rabbits of different ages coming from different breeding categories, both rural or intensive, that presented wounds related to enteritis. These animals, however, represented a homogeneous sample of the rabbit population in Lombardia and Emilia-Romagna. Consequently the characterisation of isolated strains constitutes an indicative screening of *E.coli* serogroups mostly diffused in such a population.

Besides the serogroup, the presence of other indicators of pathogenicity, such as toxins or the presence of the *eae* gene were investigated.

MATERIALS AND METHODS

The method for the serotyping of *E.coli* based on that used at Lugo's Laboratory in Spain (BLANCO & BLANCO, 1993) was used, making any adjusting to fit the practical requirements of our Laboratory.

Such a method foresees a first cloning step during which all the strains, confirmed as *E.coli* by the Enterotube II Roche test, are brought to the same developmental stage by successive

passages on MacConkey agar, Tryptone soya agar (TSA) and Tryptone soya broth (TSB). The broth cultures developed by any strain of *E.coli* are than heated in an autoclave for an hour at 100°C, doubled with 0,5% phenolic physiological solution and kept in the refrigerator at 4°C until the following day. During the second step, typing is carried out using a battery of mono-specific antisera towards different somatic O antigens. An aliquot of the broth culture (50µl) thermically treated, is brought into contact with the same volume of each of the antiserum causing a slow sero-agglutination (SAL) in U bottomed microtiter plates which are then incubated for 24h at 37°C in a moist box. The thermically treated broth culture is also brought into contact with the same volume of physiological solution containing 0,5% phenolic to estimate the power of auto-agglutination of each strain.

Clear agglutination towards only one of the O antiserum establishes conclusive serotyping. If agglutination is observed towards 4 or more microwells, the strain is considered non typeable. Finally, if agglutination is observed with 2 or 3 antisera we proceed with titering, repeating a SAL in which 50µl of the thermically treated broth culture is brought into contact with 2 base dilutions of the positive antisera. If one antiserum shows a higher agglutination titre, this constitutes the final diagnosis of the serogroup, otherwise the strain is considered non typeable.

For this analyses a battery of 36 selected antisera was used (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, O115, O128, O132, O138, O139, O141, O147, O149, O153, O157).These antisera were selected for the routine typing of *E.coli* strains isolated from the main animals species, based on results obtained during the last decade and on predicted diffusion of the different serogroups from national and international literature.

The presence of toxins (verotoxins VT, heat-labile toxin LT and cytotoxic necrotizing factor CNF), is demonstrated by the appearance of a typical cytopatic effect on a mono-layer of Vero-cells; an aliquot of the *E.coli* broth cultures, concentrated by centrifugation at 5000g per 20' at 4°C, inactivated in an ultra-sound bath in presence of quartz powder, for 30', after a second centrifugation and filtration trough bacteriologic filters of 0,2µ it was added to the tissue culture (CAPRIOLI *et al*., 1993).

Starting from 1998 an aliquot of *E.coli* broth cultures inactivated by boiling was used for the investigation of genes that encode the various verotoxins (VT1, VT2, VTe) by molecular-genetic techniques (RÜSSMAN *et al*., 1995; FRANKE *et al*., 1995); subsequently the search for the *eae* gene that encodes an adherence factor for the bacterial strains, first only on typed strains of *E.coli* and than on all those analysed, was introduced (SCHMIDT *et al*., 1994). The presence of the VT genes and of the sequence for the *eae* gene were detected by the PCR amplification technique using the primers indicated in Table 1. PCR conditions were those described by the authors. Amplification products were analysed in a 1.8% agarose gel buffered with TBE and stained with ethidium bromide.

Table 1: Primers used for PCR amplification

Primer	Gene	Direction	Nucleotide Sequence	Bp	Reference
KS7	VT1	dir	atgaaaaaaacattattaatagc	265	RÜSSMAN *et al*., 1995
KS8	VT1	rev	agctattctgagtcaacg		
GK3	VT2	dir	atgaagaagatgtttatg	270	RÜSSMAN *et al*., 1995
GK4	VT2	rev	tcagtcattattaaactg		
FK1	VTe	dir	cccggatccaagaagatgtttatag	264	FRANKE *et al*., 1995
FK2	VTe	rev	cccgaattctcagttaaacttcacc		
SK1	*eae*	dir	cccgaattcggcacaagcataagc	863	SCHMIDT *et al*., 1994
SK2	*eae*	rev	cccggatccgtctcgccagtattcg		

RESULTS AND DISCUSSION

Table 2 presents the detail of the O antigens found during the triennium in *E.coli* isolated from rabbits. As one can deduce by this table, O 103 is the serogroup most frequently associated with outbreaks of enteritis in the region during the 3 years, with a large prevalence over others, according to the results of similar studies carried out in France and Spain (BLANCO *et al*., 1996).

Table 2: Somatic antigens (O) found in the period 1997-1999.

Antigen	1997	1998	1999	Total
O1			1	1
O2	9	6	11	26
O4				
O6	2			2
O8	2	3	1	6
O9	2			2
O10			5	5
O11	1			1
O15	4	3	2	9
O18		1	1	2
O20			2	2
O21				
O22	2	2	1	5
O26	Not tested	Not tested	since 5/99	
O45				
O49	1			1
O64	3	1		4
O73			1	1
O75	3	3	1	7
O78				
O83	1			1
O86		4	5	9
O88	1	2	1	4
O101				
O103	46	45	27	118
O109				
O115	Not tested	Not tested		
O128			1	1
O132			1	1
O138		Not tested	1	1
O139		2	1	3
O141	3	8	1	12
O147				
O149	4	1		5
O153	3	3	3	9
O157		1	1	2
NT*	103	74	83	260
TOTAL	**190**	**159**	**151**	**500**

*NT, non typeable strains

Table 3 reports the results of serotyping and of the search for toxins on Vero-cells during the monitored period.
Verotoxin was found in a not typeable strain and, by PCR analysis, was demonstrated to be VT2f. This strain is still being analysed in order to understand the pathogenic effect on rabbit of this factor, found in *E.coli* strains isolated from pigeons (SCHMIDT *et al*., 1999).

The positivity to CNF (observed in O2 strains) and LT (observed twice in untyped strains and once in an O138 strain) is low, and probably without great significance in rabbits although in other animals, such as dogs, cats and cattle (CNF) (POHL *et al.*, 1993) and pigs (LT) (WILSON & FRANCIS, 1986), the presence of these pathogenic factors is of great importance.

Table 3: results of serotyping of *E. coli* strains during the period 1997-1999.

YEAR	Strains examined	Typed (O antigen)	Toxins producers		
			VT	CNF	LT
1997	190	87			
1998	159	85		1	1
1999	151	68	1	2	2
Total	500	240	1	3	3

Table 4 are reports the results of the search for the adherence factor gene *eae* by the PCR method. This search was carried on 127 strains (93 typed) analysed in the second part of the triennium. The presence of *eae* gene was demonstrated in 56 strains: 35 typed as O103, 4 as O153, 2 as O20, 1 as O10, 1 as O1, 1 as O132 e 12 in non typeable strains. As one can see from those data, the presence of the *eae* gene was demonstrated in 44% of the strains responsible for episodes of enteritis, with a prevalence in typed strains (47%). The controlling of this pathogenic factor in a good percentage of non typeable strains (35%) confirms our decision to search for the *eae* gene in all the samples, independent of success of serotyping.

Table 4: results of the search for the *eae* gene in strains of *E. coli* during 1998-99.

YEAR	STRAINS INVESTIGATED		PRESENCE OF *eae* GENE
1998	27	TYPED : 27	16 (59%)
		NON TYPEABLE : 0	/
1999	100	TYPED : 66	28 (42%)
		NON TYPEABLE : 34	12 (35%)
TOTAL	127		56 (44%)

Table 5 reports the association between serotyping and the search for the adherence gene *eae*. As one can see, in only 5 cases out of 40 *E. coli* typed as O103, the presence of *eae* gene is not associated with the serogroup that in Italy, but also in other Mediterranean countries, characterises the strains with stronger pathogenicity in rabbit.

This confirms the importance of the simultaneous observation of these two pathogenic factors for epidemiological data, which is in accordance with the results of similar studies carried out in France and Spain (BLANCO *et al*., 1997; LEROY *et al*., 1994).

Table 5: Association between O serogroups and presence/absence of the *eae* gene

Serogroups	Strains investigated	Presence of *eae* gene
O1	1	1
O2	14	
O8	2	
O10	5	1
O15	1	
O18	1	
O20	2	2
O22	2	
O64	1	
O73	2	
O75	1	
O86	6	
O88	1	
O103	40	35
O128	1	
O132	1	1
O138	1	
O139	1	
O141	4	
O153	5	4
O157	1	
TOTAL	**93**	**44**

CONCLUSIONS

The purpose of this study was to screen all the strains that arrive to our laboratory for characterisation of pathogenic factors and to control for the presence of the *eae* gene, and to restrain as much as possible the number of un-typed strains which still represent almost 50% of those investigated, improving the method and the selection of antisera now in use, without making the diagnostic routine too laborious.
We also intend to control some commercial rabbit farms within the region and methodically characterise all the *E.coli* strains responsible for outbreaks of enteritis, correlating the results of serotyping with anatomo-pathologic observations and anamnestic data, such as the age of the infected subjects and the type of breeding environment.
By increasing the number of strains analysed more precise statistic analyses can be carried out, making the resulting data stronger.

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