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O Serogroups, Biotypes, and *eae* Genes in *Escherichia coli* Strains Isolated from Diarrheic and Healthy Rabbits

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A total of 305 *Escherichia coli* strains isolated from diarrheic and healthy rabbits in 102 industrial fattening farms from different areas of Spain were serotyped, biotyped, and tested for the presence of the *eae* gene and toxin production. The characteristics found in strains isolated from healthy rabbits were generally different from those observed in *E. coli* strains associated with disease. Thus, strains with the *eae* gene (74% versus 22%); strains belonging to serogroups O26, O49, O92, O103, and O128 (64% versus 12%); rhamnose-negative strains (51% versus 5%); and rhamnose-negative O103 strains with the *eae* gene present (41% versus 1%) were significantly ($P < 0.001$ in all cases) more frequently detected in isolates from diarrheic animals than in those from healthy rabbits. Whereas a total of 35 serogroups and 17 biotypes were distinguished, the majority of the strains obtained from diarrheic rabbits belonged to only four serobiotypes, which in order of frequency were O103:B14 (72 strains), O103:B6 (16 strains), O26:B13 (12 strains), and O128:B30 (12 strains). These four serobiotypes accounted for 48% (112 of 231) and 5% (4 of 74) of the *E. coli* strains isolated from diarrheic and healthy rabbits, respectively. Only six strains were toxigenic (three CNF1$^+$, two CNF2$^+$, and one VT1$^+$). We conclude that enteropathogenic *E. coli* strains that possess the *eae* gene are a common cause of diarrhea in Spanish rabbit farms and that the rhamnose-negative highly pathogenic strains of serotype O103:K−:H2 and biotype B14 are especially predominant. Detection of the *eae* gene is a useful method for the identification of enteropathogenic *E. coli* strains from rabbits. However, a combination of serogrouping and biotyping may be sufficient to accurately identify the highly pathogenic strains for rabbits.

In industrial rabbit-fattening farms, enteritis caused by *Escherichia coli* is the main cause of morbidity and mortality in weaned rabbits (7, 22, 26, 29, 30). Because of the inhibitory influence of the cecal volatile fatty acids, the guts of healthy weaned rabbits contain low levels of *E. coli* (30, 37). Enteric disease is associated with colonization and proliferation of *E. coli* in distal ileum and cecum. Enteropathogenic *E. coli* (EPEC) strains which cause intestinal disease in rabbits do not produce heat-labile (LT) or heat-stable (STa) enterotoxins and are not enteroinvasive. Virulence seems to be associated with the ability of *E. coli* to adhere to intestinal epithelial cells and to colonize the digestive tract with removal (effacing) of the microvilli (10, 24, 32, 38). Moon et al. (25) termed such strains attaching and effacing *E. coli* (AEEC). Jerse et al. (16, 17) have identified a chromosomal gene, *eae*, required for the production of attaching and effacing (A/E) lesions in human EPEC strain E2348/69. The product of the *eae* gene is a 94-kDa outer membrane protein termed intimin. The gene has been cloned, and its nucleotide sequence has been determined. Hybridization trials with an *eae* probe showed that AEEC strains isolated from diarrheic rabbits possessed DNA sequences homologous to the *eae* sequence of the human EPEC strain E2348/69 (19, 36).

Experimental infections carried out mainly by Peeters et al. (31, 33, 34) have shown that *E. coli* strains involved in rabbit enteritis belong to different serotypes. Thus, serotype O109:K−:H2 is mainly associated with yellow diarrhea in suckling rabbits, whereas other serotypes (O2:K1:H6, O15:K−:H−, O20:K−:H7, O26:K−:H11, O103:K−:H2, O109:K−:H7, O128:K−:H2, O132:K−:H2, and O153:K−:H7) are associated with diarrhea in weaned rabbits (29, 30). Strains belonging to serotypes O15:K−:H−, O26:K−:H11, and O103:K−:H2 are highly pathogenic for weaned rabbits, and small numbers of cells belonging to these strains may cause a mortality of 50% or greater. Strains belonging to the other serotypes show moderate pathogenicity and require higher infective doses to cause clinical signs. Rabbit EPEC may also be differentiated by their fermentation patterns. Results obtained with the biotyping scheme developed by Okerman and Devriese (27) and modified by Camguilhem and Milon (9) indicate that O serogrouping, together with biotyping, permits rapid identification of highly pathogenic strains and leads to improved prognosis and treatment (9, 27, 33).

EPEC strains of serotype O15:K−:H− are frequently isolated in Belgium, The Netherlands, and the United States (33–35), whereas rhamnose-negative O103:K−:H2 strains are predominant in France and Spain (3, 4, 9, 20, 22, 23). However, the epidemiology of EPEC from rabbits remains incomplete, both because of the limited number of surveys performed and because most of the studies were carried out in only three countries (Belgium, France, and The Netherlands) (9, 22, 27, 29, 30, 33).

In the present study, we describe the serotypes and biotypes of EPEC strains that cause frequent epizootics in industrial rabbit-fattening farms in Spain. To our knowledge, this is the first report that describes and compares the prevalence of the *eae* gene in an important number of *E. coli* strains isolated

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TABLE 1. Primers used in PCR to amplify specific fragments from *eae*, VT1, VT2, CNF1, and CNF2 genes

Primer[a]	Oligonucleotide sequence (5′ to 3′)	Location (nt) within gene	Size of amplified product (bp)
eae-1	ACGTTGCAGCATGGGTAACTC	1054–1074	815
eae-2	GATCGGCAACAGTTTCACCTG	1869–1849	
VT1a	CAGTTAATGTGGTGGCGAAG	215–234	894
VT1b	CTGCTAATAGTTCTGCGCATC	1089–1109	
VT2a	CTTCGGTATCCTATTCCCGG	288–307	478
VT2b	GGATGCATCTCTGGTCATTG	747–766	
CNF1-A	GAACTTATTAAGGATAGT	1585–1602	
CNF1-B	CATTATTTATAACGCTG	2112–2128	543
CNF2-A	AATCTAATTAAAGAGAAC	841–858	
CNF2-B	CATGCTTTGTATATCTA	1368–1384	543

[a] References 13, 28, and 8 for *eae*, VT1 and VT2, and CNF1 and CNF2, respectively.

from diarrheic and healthy rabbits. In addition, this is the first study that makes use of PCR to amplify and detect the *eae* locus of human EPEC strain E2348/69 in *E. coli* strains isolated from diarrheic and healthy rabbits. The information obtained in our study may be used in the diagnosis and epidemiology investigation of EPEC infections in rabbits.

MATERIALS AND METHODS

***E. coli* strains.** A total of 305 *E. coli* strains isolated from the cecal contents of 191 diarrheic rabbits and 71 healthy controls between 1988 and 1994 were investigated in this study. Cecal specimens were plated on MacConkey agar, with a mean of three *E. coli* colonies selected from each rabbit. Only one strain was selected from rabbits which showed identical isolates regarding serogroup and biotype. When a rabbit yielded colonies with different serogroups or biotypes, one strain of each was selected. All selected strains were confirmed as *E. coli* by the API 20E system (bioMérieux, Marcy l'Etoile, France). The animals were gathered from 102 industrial fattening farms in seven (La Coruña, Lugo, Orense, Pontevedra, Zaragoza, Lérida, and Teruel) provinces of Spain. The majority of the strains (>90%) were obtained from weaned rabbits 4 to 8 weeks of age. Strains were stored at room temperature in nutrient broth with 0.75% agar.

Serotyping. The presence of O, K, and H antigens was determined by the method of Guinée et al. (15) with all available O (O1 to O171) and H (H1 to H56) antisera and O:K antisera specific for the K antigens usually associated with each O antigen. The antisera were obtained from the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). All antisera were absorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O antigen was established in all 305 *E. coli* strains investigated in this study, whereas the K and H antigens were established in only 85 representative *E. coli* strains.

Biotyping. Fermentation of carbohydrates was tested on phenol red agar base (Difco Laboratories, Detroit, Mich.), supplemented with 1% of the respective carbohydrate, in petri dishes. Strains were inoculated as spots on the medium, and the results were read after 24 and 48 h of incubation at 37°C. We have used the simplified biotyping scheme of Camguilhem and Milon (9). The following carbohydrates were tested: sorbose (test score 1), dulcitol (test score 2), D-raffinose (test score 4), sucrose (test score 8), and L-rhamnose (test score 16). Biotypes were defined by the sum of the test score numbers given by positive fermentative reactions. For instance, biotype 27 (B27) strains are as follows: 1, sorbose positive; 2, dulcitol positive; 0, raffinose negative; 8, sucrose positive; and 16, rhamnose positive.

Detection of *eae*, VT1, VT2, CNF1, and CNF2 sequences by PCR. DNA to be amplified was released from whole cells by boiling. Bacteria were harvested from Casomino Acid-yeast extract agar (CFA agar), suspended in 200 μl of sterile water, incubated at 100°C for 10 min, and centrifuged. The supernatant was used in the PCR mixture as described below. Base sequences, locations, and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. Oligonucleotide primers were synthesized by using a Gene Assembler Special (Pharmacia, LKB Biotechnology, Inc.) according to the protocol provided by the manufacturer. Amplification of bacterial DNA was performed with 50-μl volumes containing 10 μl of the prepared sample supernatant; the oligonucleotide primers (90 ng for *eae* and VT2 primers, 150 ng for VT1 primers, 450 ng for CNF1 primers, and 900 ng for CNF2 primers); 0.2 mM (each) dATP, dGTP, dCTP, and dTTP; 10 mM Tris HCl (pH 8.8); 1.5 mM $MgCl_2$; 50 mM KCl; and 1 U of DynaZyme DNA polymerase (Finnzymes OY). The reaction mixtures were overlaid with an equal volume of mineral oil. PCR was performed with a thermal cycler (Gene ATAQ Controller; Pharmacia, LKB Biotechnology, Inc.) at 94°C for 2 min for 1 cycle followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplified product was visualized by standard submarine gel electrophoresis of 10 μl of the final reaction mixture on a 2% agarose gel. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. Molecular size markers (*Hae*III digest of ϕX174 DNA) were included in each gel.

Production and detection of toxins. One loopful of each *E. coli* strain was inoculated in 50-ml Erlenmeyer flasks containing 5 ml of tryptone soy broth and incubated for 20 h at 37°C in an orbital shaker (200 rpm). Filtrates of cultures treated with mitomycin and extracellular fluids were obtained as previously described (2). Filtrates of cultures treated with mitomycin were inoculated on Vero and HeLa cells for the detection of heat-labile (LT) enterotoxin, verotoxins (VT1 and VT2), and cytotoxic necrotizing factors (CNF1 and CNF2). Extracellular fluids were assayed for heat-stable (STa) enterotoxin by the infant mouse test. All assays cited were comprehensively described in previous papers (1–4, 6). The variety of verotoxin or cytotoxic necrotizing factor produced by rabbit strains was established by PCR.

Statistical methods. Results were compared by the χ^2 test with Yates' correction for continuity.

RESULTS

O serogroups. Serogroups found in the 305 *E. coli* strains investigated are shown in Table 2. The 231 strains isolated from diarrheic rabbits belonged to 28 groups, and 169 (74%) were from one of seven serogroups (O2, O26, O49, O92, O103, O128, and O153). The serogroups found in the strains isolated from healthy rabbits were generally different from those found in *E. coli* strains associated with disease. Thus, five serogroups (O26, O49, O92, O103, and O128) accounted for 64% (147 of 231) and 12% (9 of 74) of *E. coli* strains isolated from diarrheic and healthy rabbits, respectively ($P < 0.001$). The most common serogroup (O103) observed among *E. coli* strains isolated from diarrheic rabbits (44%; 101 of 231) was found in only 6 (8%) of 74 strains from healthy rabbits ($P < 0.001$).

Biotypes. A total of 17 different biotypes were distinguished: five rhamnose negative (biotype codes below or equal to 14) and 12 rhamnose positive (Table 3). The majority (89%; 271 of 305) of rabbit strains were assigned to only six biotypes (B6, B13, B14, B27, B28, B30, and B31). Rhamnose-negative B6 (7% versus 0%), B13 (6% versus 1%), and B14 (34% versus 4%) biotypes were more frequently found among *E. coli* strains isolated from diarrheic rabbits, whereas rhamnose-positive B27 (26% versus 6%) and B30 (51% versus 21%) biotypes were more commonly detected among strains obtained from healthy controls. The correlation between biotypes and O serogroups is shown in Tables 2 and 3. Most O103 strains (73 of 107) fell into biotype B14 (sorbose and rhamnose negative; dulcitol, raffinose, and sucrose positive). Furthermore, 95 (89%) of 107 of the strains of serogroup O103 were rhamnose negative. In contrast, only 26 (12%) of 208 non-O103 strains were rhamnose negative ($P < 0.001$). It is interesting that 13 of 16 rhamnose-negative strains of biotype B13 belonged to serogroup O26.

O:B serobiotypes. The most common serobiotypes found among *E. coli* strains isolated from diarrheic rabbits in order of frequency were O103:B14 (72 strains), O103:B6 (16 strains), O26:B13 (12 strains), and O128:B30 (12 strains). They accounted for 48% (112 of 231) and 5% (4 of 74) of the *E. coli* strains isolated from diarrheic and healthy rabbits, respectively ($P < 0.001$). Among *E. coli* strains obtained from healthy rabbits, the serobiotypes O2:B27 (18 strains), O81:B30 (8 strains), and NT:B30 (7 strains) were predominant (Table 2).

O:K:H serotypes. The O:K:H serotypes were determined in 85 representative *E. coli* strains: 60 were isolated from diseased

TABLE 2. Relationship between serogroups and biotypes of *E. coli* strains isolated from diarrheic and healthy rabbits and the presence of the *eae* gene

Serogroup	Diarrheic rabbits			Healthy rabbits		
	No. of strains (n = 231)		Biotype(s) (no. of strains)	No. of strains (n = 74)		Biotype(s) (no. of strains)
	Total	*eae*$^+$		Total	*eae*$^+$	
O1	2	0	B27 (2)	0	0	
O2	11	1	B25 (1), B27 (9), B31 (1)	18	0	B27 (18)
O6	4	0	B20 (1), B27 (2), B31 (1)	0	0	
O7	2	0	B13 (1), B18 (1)	0	0	
O8	4	0	B18 (1), B31 (3)	6	0	B14 (1), B28 (1), B30 (3), B31 (1)
O10	1	0	B28 (1)	2	0	B30 (2)
O15	2	0	B31 (2)	0	0	
O21	2	0	B16 (2)	0	0	
O22	1	0	B30 (1)	0	0	
O26	12	12	B13 (12)	1	1	B13 (1)
O39	0	0		1	0	B30 (1)
O41	0	0		2	0	B30 (2)
O49	7	7	B22 (1), B28 (1), B30 (5)	0	0	
O50	1	0	B27 (1)	0	0	
O74	3	0	B30 (3)	3	0	B30 (3)
O81	0	0		9	0	B14 (1), B30 (8)
O86	1	0	B14 (1)	1	0	B30 (1)
O91	0	0		1	0	B30 (1)
O92	14	12	B13 (2), B14 (4), B22 (1), B28 (3), B30 (4)	1	0	B30 (1)
O101	1	0	B30 (1)	0	0	
O102	1	0	B30 (1)	3	0	B30 (2), B31 (1)
O103	101	100	B6 (16), B12 (6), B14 (72), B20 (4), B28 (1), B30 (2)	6	5	B14 (1), B20 (1), B22 (2), B30 (2)
O113	1	0	B28 (1)	1	0	B30 (1)
O119	2	2	B30 (2)	0	0	
O126	3	3	B28 (2), B30 (1)	0	0	
O128	13	12	B30 (12), B31 (1)	1	1	B30 (1)
O132	3	3	B30 (3)	1	1	B30 (1)
O139	0	0		1	0	B30 (1)
O148	2	0	B30 (2)	0	0	
O153	11	11	B28 (7), B30 (4)	5	5	B28 (4), B31 (1)
O157	1	0	B22 (1)	0	0	
O159	4	0	B16 (4)	0	0	
O165	0	0		1	1	B30 (1)
O166	2	0	B31 (2)	1	0	B27 (1)
O170	0	0		1	0	B18 (1)
NT[a]	19	9	B8 (2), B14 (1), B16 (1), B17 (1), B19 (1), B24 (2), B27 (1), B28 (2), B30 (7), B31 (1)	8	2	B30 (7), B31 (1)

[a] NT, not typeable strains.

rabbits, and 25 were obtained from healthy controls (Table 4). When the serotypes of the strains that showed the serogroups associated with diseased rabbits (O26, O49, O92, O103, and O128) were analyzed, it was observed that whereas all of the O49 and O103 strains showed a specific serotype, O:K:H, the O26, O92, and O128 strains showed different serotypes. It is important to emphasize the fact that strains with the same serotype can show different biotypes. This is the case of the strains of serotypes O2:K5:H6, O49:K?:H2, O92:K−:H2, O92:K?:H2, and O103:K−:H2. It is also relevant that most of the *E. coli* strains isolated from diseased rabbits showed the flagellar antigen H2. Whereas 53% (32 of 60) of strains isolated from diseased animals exhibited this antigen, it could be detected only in 16% (4 of 25) of strains isolated from healthy animals ($P < 0.001$). Flagellar antigen H2 was detected in strains belonging to serogroups O49, O92, O103, and O128.

***eae* gene.** Amplification of the *eae* gene by the PCR technique showed that 74% (172 of 231) and 22% (16 of 74) of the *E. coli* strains isolated from diarrheic and healthy rabbits, respectively, possessed this virulence determinant ($P < 0.001$) (Tables 5 and 6) (Fig. 1). The presence of the *eae* gene was also significantly more frequent (96%; 150 of 156) among strains belonging to serogroups O26 (13 of 13), O49 (7 of 7), O92 (12 of 15), O103 (105 of 107), O128 (13 of 14), and O153 (16 of 16) than among strains belonging to other serogroups (25%; 38 of 149) ($P < 0.001$) (Table 2). Interestingly, these six serogroups are included among the seven O serogroups which were more frequently detected in *E. coli* strains isolated from diseased rabbits. Furthermore, the *eae* gene was present in most of the rhamnose-negative strains (95%; 114 of 121) and most of the rhamnose-positive strains belonging to biotypes B20, B22, and B28 (82%; 28 of 34) (Table 3). The presence of the *eae* gene was also significantly more frequent among strains with H2 antigen (94%; 34 of 36) than among strains with other flagellar antigens (29%; 14 of 49) ($P < 0.001$) (Tables 4 and 5).

Toxic properties. From all of the *E. coli* strains isolated, only

TABLE 3. Relationship between biotypes of *E. coli* strains isolated from diarrheic and healthy rabbits and the presence of the *eae* gene and O serogroup

Biotype	No. of strains			Serogroup(s) (no. of strains)
	Diarrheic (n = 231)	Healthy (n = 74)	*eae*$^+$ (n = 188)	
Rhamnose negative				
B6	16	0	16	O103 (16)
B8	2	0	0	NT[a] (2)
B12	6	0	6	O103 (6)
B13	15	1	15	O7 (1), O26 (13), O92 (2)
B14	78	3	77	O8 (1), O81 (1), O86 (1), O92 (4), O103 (73), NT (1)
Rhamnose positive				
B16	7	0	0	NT (1), O21 (2), O159 (4)
B17	1	0	0	NT (1)
B18	2	1	0	O7 (1), O8 (1), O170 (1)
B19	1	0	0	NT (1)
B20	5	1	5	O6 (1), O103 (5)
B22	3	2	4	O49 (1), O92 (1), O103 (2), O157 (1)
B24	2	0	0	NT (2)
B25	1	0	0	O2 (1)
B27	15	19	1	O1 (2), O2 (27), O6 (2), O50 (1), O166 (1), NT (1)
B28	18	5	19	O8 (1), O10 (1), O49 (1), O92 (3), O103 (1), O113 (1), O126 (2), O153 (11), NT (2)
B30	48	38	44	O8 (3), O10 (2), O22 (1), O39 (1), O41 (2), O49 (5), O74 (6), O81 (8), O86 (1), O91 (1), O92 (5), O101 (1), O102 (3), O103 (4), O113 (1), O119 (2), O126 (1), O128 (13), O132 (4), O139 (1), O148 (2), O153 (4), O165 (1), NT (14)
B31	11	4	1	O2 (1), O6 (1), O8 (4), O15 (2), O102 (1), O128 (1), O153 (1), O166 (2), NT (2)

[a] NT, not typeable strains.

six were toxigenic: five necrotoxigenic *E. coli* strains (one CNF1$^+$ strain of serobiotype O2:K5:H6:B25, two CNF1$^+$ strains of serobiotype O6:K53:H−:B27, and two CNF2$^+$ strains of serobiotype O15:K−:H45:B31) isolated from diseased rabbits and one verotoxin-producing *E. coli* strain (VT1$^+$ strain of serobiotype O91:B30) obtained from a healthy control.

DISCUSSION

In Spain, as in other countries, epizootics of life-threatening enteric diseases occur frequently in industrial rabbit-fattening farms. These diseases occur in newborn and weaned animals mainly during the fattening period, and they have severe economic implications, derived from weight loss in diarrheic rabbits and from high mortality rates. However, the mortality varies from very low to very high, according to the strains involved. Strains of low pathogenicity mostly cause problems in rabbitries with poor hygiene that can easily be controlled by antibiotic treatment and hygienic measures. On the other hand, most antibiotics fail to overcome the disease caused by highly pathogenic strains, with the rabbits frequently being killed and with replacement of the whole rabbit stock. Early differentiation of the pathogenicity of the strains is therefore important for prognosis and treatment. Strains belonging to serotypes O15:K−:H−, O26:K−:H11, and O103:K−:H2 are highly pathogenic for weaned rabbits, and small numbers of these strains may cause a mortality of 50% or greater (29, 30, 33).

Whereas the 231 *E. coli* strains isolated from diarrheic rabbits that were investigated in the present study belonged to 28 different O serogroups, nearly half were of the O103 serogroup. Similar results were obtained by Camguilhem and Milon (9) in France. More than half of the 575 *E. coli* strains isolated from the cecal contents of weaned rabbits in French commercial farms belonged to serogroup O103, and 70% were of one of five serogroups (O2, O26, O103, O128, and O132). French O103 strains, like Spanish strains of the O103 serogroup, were rhamnose negative (majority were of biotype B14) (9) and presented the *eae* gene (19). In the experimental infections carried out by Camguilhem and Milon (9), rhamnose-negative O103 strains induced scouring in 39 (89%) of 44 rabbits and caused the death of 33 (75%) of 44 rabbits. This work shows that rhamnose-negative highly pathogenic O103 strains are widely spread not only among French rabbit farms but also among Spanish rabbit farms. This is not a surprising result, because during the last few years numerous breeding rabbits from genetic lines developed in France have been imported into Spain. In addition to Spain and France, strains of serogroup O103 were also detected in Belgium, Italy, Germany, Hungary, and the United States (29, 30, 33, 39). Epidemics of colibacillosis associated with O15 strains have been frequently reported in Belgium and The Netherlands (29, 30, 33). This class of *E. coli* strains, together with O103 strains, also appears to be frequently isolated from diarrheic rabbits in North America (35). In contrast, Camguilhem and Milon (9) found this serogroup in only 1 (0.2%) of 575 *E. coli* strains isolated in France, and we detected only 2 (0.9%) O15 strains in Spanish rabbits with diarrhea.

Not all *E. coli* strains that cause diarrhea in farm animals synthesize LT or STa enterotoxins. These newly recognized animal enteropathogenic *E. coli* strains attach to and efface the microvilli of gut epithelium. In rabbits, only this type of *E. coli* enteritis is known to be important, and it is caused by EPEC strains, also called AEEC (25). *E. coli* strains isolated from rabbits display a pathogenic mechanism similar to human EPEC strains (30). The *eae* gene was identified in human EPEC strain E2348/069 (O127:H6) as coding for an outer membrane protein that allows cells to attach to and efface the microvilli (16, 17). Sequences homologous to the *eae* gene have been detected in AEEC strains isolated from diarrheic rabbits (19, 36). In this study, we have used the PCR technique as an approach to detect the *eae* gene. Our results confirm those

TABLE 4. Relationship between O:K:H serotypes of *E. coli* strains isolated from diarrheic and healthy rabbits and the presence of the *eae* gene and biotype

Serotype	No. of strains		Biotype(s) (no. of strains)
	Total	*eae*$^+$	
Diarrhea			
O2:K5:H6	4	0	B27 (3), B25 (1)
O2:K5:H21	1	0	B27 (1)
O6:K53:H−	2	0	B27 (2)
O8:K?:H−	1	0	B18 (1)
O8:K?:H8	2	0	B31 (2)
O8:K?:H27	1	0	B31 (1)
O15:K−:H45	2	0	B31 (2)
O26:K−:H−	5	5	B13 (5)
O26:K−:H11	4	4	B13 (4)
O49:K?:H2	6	6	B22 (1), B28 (1), B30 (4)
O92:K−:H−	1	1	B13 (1)
O92:K−:H2	2	2	B14 (1), B30 (1)
O92:K?:H2	3	2	B14 (2), B28 (1)
O92:K?:H11	1	1	B13 (1)
O92:K?:H19	1	0	B30 (1)
O103:K−:H2	15	15	B6 (2), B14 (12), B20 (1)
O128:K?:H−	2	2	B30 (2)
O128:K?:H2	6	6	B30 (6)
O128:K?:H8	1	0	B31 (1)
Healthy			
O2:K5:H6	5	0	B27 (5)
O8:K202:H−	1	0	B31 (1)
O8:K87:H1	1	0	B28 (1)
O10:K5:H42	2	0	B30 (2)
O26:K−:H−	1	1	B13 (1)
O41:K−:H−	2	0	B30 (2)
O74:K?:H19	3	0	B30 (3)
O81:K?:H21	5	0	B30 (5)
O92:K?:H2	1	0	B30 (1)
O102:K?:H15	1	0	B31 (1)
O103:K−:H2	2	2	B20 (1), B22 (1)
O128:K?:H2	1	1	B30 (1)

TABLE 5. Prevalence of the *eae* gene in rabbit strains with different characteristics

Characteristic(s)[a]	No. of *eae*$^+$ strains/total no. assayed (% *eae*$^+$)
Diarrhea	172/231 (74)
Healthy	16/74 (22)
O26, O49, O92, O103, O128, O153	150/156 (96)
Other O serogroups	38/149 (25)
Rhamnose negative	114/121 (95)
Rhamnose positive	74/184 (40)
Antigen H2	34/36 (94)
Other H antigens	14/49 (29)

[a] In the four comparisons, the differences are statistically significant ($P < 0.001$).

previously reported (19, 36) and show that the *eae* gene can be used as a virulence marker associated with those *E. coli* strains that cause diarrhea in rabbits. Whereas we have not carried out experimental infections with strains isolated in Spain, we have found a good correlation between the isolation of *E. coli* strains with the *eae* gene and the production of A/E lesions in vivo. Fifty diarrheic rabbits, from which *E. coli* strains had been isolated, arrived alive at the laboratory and were carefully examined for the presence of A/E lesions in the small and large intestines. We could recover *E. coli* strains with the *eae* gene from 38 (86%) of 44 rabbits with A/E lesions but from none of 6 rabbits without A/E lesions. Furthermore, the *E. coli* strains harboring the *eae* gene were more frequently isolated from diarrheic rabbits (72%; 92 of 128) than from healthy rabbits (16%; 9 of 55) (data not shown in the Results section). Therefore, our results further support the role of the *eae* gene as an important virulence determinant of *E. coli* strains that cause diarrhea in rabbits.

Up to now, the definitive diagnosis of colibacillosis in rabbits has been based on the demonstration of the typical lesions of AEEC by histology. This makes diagnosis rather cumbersome, slow, and expensive. Colibacillosis may also be screened by semiquantitative evaluation of *E. coli* numbers in the gut, since *E. coli* cells are present in small numbers in healthy weaned rabbits. There is an 89% correlation between histological confirmation and confluent growth of *E. coli* from samples taken from the mid-small intestine. Medication of rabbits in outbreaks associated with these strains requires a different approach, depending on the strains involved. Therefore, early differentiation of the strains is necessary for prognosis and treatment, so determination of the serobiotypes may contribute to this differentiation (30). The results obtained in the present study confirm previous observations (9, 27, 33), and they indicate that O serogrouping, together with biotyping, leads to a rapid identification of highly pathogenic strains that cause diarrhea on Spanish farms. The O serogroups and biotypes found in the strains isolated from healthy rabbits were generally different from those observed in *E. coli* strains associated with disease. Although a total of 35 O serogroups and 17 biotypes were distinguished, an important proportion of strains from diarrheic rabbits were included in only four serobiotypes (O26:B13, O103:B6, O103:B14, and O128:B30). These four serobiotypes accounted for 48% and 5% of the *E. coli* strains isolated from diarrheic and healthy rabbits, respectively. It is relevant to emphasize that 100% of strains belonging to these four serobiotypes presented the *eae* gene. Thus, the PCR assay, used in this study for the detection of the *eae* gene responsible for A/E lesions, can be used as an alternative to the histological techniques used to confirm enteropathogenicity in laboratory diagnosis.

Because AEEC strains that cause diarrhea in rabbits apparently display a pathogenic mechanism similar to that displayed by human EPEC and enterohemorrhagic *E. coli* strains, some authors have suggested that AEEC strains can synthesize verotoxins (also designated Shiga-like toxins) or other types of related cytotoxins (22, 26). Our results indicate that *E. coli* strains from rabbits usually do not produce verotoxins (VT1 and VT2) nor the recently discovered cytotoxic necrotizing factors (CNF1 and CNF2) elaborated by clinical *E. coli* isolates

TABLE 6. Principal markers for EPEC strains from rabbits

Characteristic(s)[a]	No. (%) of strains from rabbits	
	Diarrheic (n = 231)	Healthy (n = 74)
eae$^+$	172 (74)	16 (22)
O26, O49, O92, O103, O128	147 (64)	9 (12)
Rhamnose negative	117 (51)	4 (5)
Rhamnose-negative O103 strains with *eae* gene	94 (41)	1 (1)

[a] In the four groups, the differences are statistically significant ($P < 0.001$).

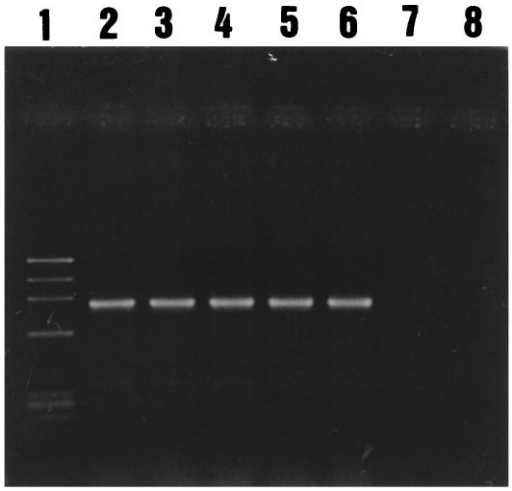

FIG. 1. Agarose gel electrophoresis of PCR-amplified DNA products. Lanes: 1, molecular size markers (*Hae*III digest of ϕX174 DNA; fragments with sizes of 1,353, 1,078, 872, 603, 310, and 281 bp); 2 to 6, *eae*-positive strains (815 bp); 7 and 8, *eae*-negative strains.

of human and bovine origin (1, 2, 5, 6, 11, 18). In previous studies, toxigenic *E. coli* strains were rarely identified among rabbit *E. coli* strains. Pohl et al. (36) examined a collection of 40 rabbit *E. coli* isolates and detected one O26:B13 strain which reacted with a VT1 probe. An O128:B30 strain has also been identified as a producer of cytolethal distending toxin (30). These strains produced light to moderate diarrhea after experimental infections (30). In this study, we detected six toxigenic strains: three $CNF1^+$, two $CNF2^+$, and one $VT1^+$. All of them belonged to serobiotypes not associated with rabbit diarrhea.

Interestingly, we have isolated nine *E. coli* strains of serotypes O26:K−:H11 and O26:K−:H− from rabbits with diarrhea. These two serotypes are included in the list of the classic EPEC serotypes and have recently been considered enterohemorrhagic *E. coli* rather than EPEC serotypes, because many *E. coli* O26:K−:H11 and O26:K−:H− strains that cause diarrhea in humans produce verotoxins (12, 14, 21). The nine *E. coli* strains isolated from rabbits in this study were negative for verotoxin production. However, like human EPEC strains, rabbit O26 strains presented the *eae* gene. Further studies are necessary to know if this class of rabbit strains is clonally related to O26 EPEC strains that cause diarrhea in humans.

We conclude that EPEC strains that possess the *eae* gene are a common cause of diarrhea in Spanish rabbit farms and that the rhamnose-negative highly pathogenic strains of serotype O103:K−:H2 and biotype B14 are especially predominant. Detection of the *eae* gene is a useful method for identification of EPEC strains from rabbits. However, a combination of serogrouping and biotyping may be sufficient to identify nearly all *E. coli* strains that are highly pathogenic for rabbits.

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