Polymerase chain reaction typing of genes of the locus of enterocyte effacement of ruminant attaching and effacing *Escherichia coli*

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Abstract

The variability of the *tir*, *espA*, and *espD* genes of the locus of enterocyte effacement (LEE) in 185 attaching and effacing *Escherichia coli* (AEEC) strains isolated from healthy and diarrheic cattle, sheep, and goats was investigated by polymerase chain reaction. Nineteen of the strains were enterohemorrhagic *E. coli* (EHEC); the other 166 were enteropathogenic *E. coli* (EPEC). The combinations of the *tir* and *esp* genes were associated with the variants of the *eae* gene but not with a strain's belonging to the EPEC or EHEC group, animal species, or health status (healthy or diarrheic) of the animal. In addition, most of the strains showed the same combinations of LEE genes and serogroups as have been found in AEEC strains isolated from humans, which indicates that ruminants seem to be an EPEC reservoir for humans.

Résumé

La variabilité des gènes tir, espA *et* espD *du locus d'effacement des entérocytes (LEE) chez 185 souches d'*Escherichia coli *attachant et effaçant (AEEC) isolées de bovins, moutons et chèvres en santé et avec diarrhée, a été étudiée par réaction d'amplification en chaîne par la polymérase. Dix-neuf des souches étaient des* E. coli *entérohémorragiques (EHEC); les autres étaient des* E. coli *entéropathogènes (EPEC). Les combinaisons des gènes* tir *et* esp *étaient associées avec les variants du gène* eae *mais non avec une souche appartenant aux groupes EPEC ou EHEC, aux espèces animales, ou à l'état de santé (en santé ou diarrhéique) de l'animal. De plus, la plupart des souches présentaient les mêmes combinaisons de gènes LEE et de sérogroupes qui ont été trouvées chez les souches humaines d'AEEC, ce qui indique que les ruminants seraient un réservoir d'EPEC pour les humains.*

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Attaching and effacing *Escherichia coli* (AEEC) are a cause of diarrhea in humans and animals. These bacteria cause a characteristic attaching and effacing (AE) lesion in the gut mucosa because of the intimate bacterial adhesion to the enterocyte and effacement of the brush-border microvilli. Formation of the AE lesion is governed by the locus of enterocyte effacement (LEE) (1). Enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) cause AE lesions in the human intestinal mucosa. In contrast to EPEC, EHEC strains produce verotoxins (VTs) (1). The EPEC strains have been classified as typical (possessing the bfpA gene) or atypical (lacking the *bfpA* gene). Typical EPEC strains, a leading cause of infantile diarrhea in developing countries, are rare in industrialized countries, where the atypical EPEC strains seem to be a more important cause of diarrhea (1,2). By analogy with strains isolated from humans, AEEC strains isolated from animals are usually referred to as EPEC or EHEC preceded by the animal species from which the strain was isolated, for example, bovine EPEC. Ruminants are recognized as the main natural reservoir of EHEC strains that infect humans. However, the role of ruminants as a reservoir of EPEC for humans is not known (1).

The LEE genes are separated into 3 functional domains: a region encoding intimate adherence (Tir and intimin), a region encoding the EPEC-secreted proteins (EspA, EspB, and EspD) and the region encoding a type III secretion system (3). Tir, EspA, EspB, and EspD are essential for the subversion of host-cell signal transduction pathways, the delivery of proteins into the host cell, and the formation of AE lesion (3).

On the basis of antigenic variation, polymerase chain reaction (PCR) analysis, and sequencing, at least 16 variants of the *eae* gene, which encodes intimin, have been identified (4). Variants of the *tir*, *espA*, *espB*, and *espD* genes have also been described (4–11). Differentiation of *eae*, *tir*, and *esp* alleles is an important tool for EHEC and EPEC typing in routine diagnostics as well as in epidemiological and clonal studies (4). In contrast with the *eae* and *espB* genes, little is known about diversity in the *tir*, *espA*, and *espD* genes in AEEC from ruminants. The variants of these genes have been studied in a limited number of AEEC strains from cattle (4,5,7,8,10) and in only 1 AEEC strain from sheep (4). Moreover, to our knowledge, typing of the *tir*, *espA*, and *espD* genes of AEEC from goats has not been performed.

The aim of this study was to investigate the variability of the *tir*, *espA*, and *espD* genes in a large collection of AEEC strains isolated from diarrheic and healthy cattle, sheep, and goats.

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Table I. Serogroup, variants of genes of the locus of enterocyte effacement (LEE), and type of verotoxin (VT) of strains of attaching and effacing *Escherichia coli* (AEEC) isolated from cattle

Source and serogroup	Number of strains	Gene and variant					Type of VT[b]
		eae[b]	*tir*	*espA*	*espB*[b]	*espD*	
Healthy cattle							
05	1	β1	β1	β1	β	β1	1
05[a]	1	γ2/θ	γ2/θ	NT	α	NT	
010[a]	3	γ2/θ	γ2/θ	NT	α	NT	
020	1	γ2/θ	γ2/θ	NT	α	NT	
026[a]	6	β1	β1	β1	β	β1	
049	1	ι	α1	β2	α	NT	
051[a]	1	β1	β1	β1	β	β1	
071	1	ι	β1	β1	β	β1	
071	1	ξ	β1	β1	β	β1	
084	1	ζ	α1	NT	α	NT	1
085	1	β1	β1	β1	β	β1	
098	1	ζ	α1	NT	α	NT	1
098	1	ι	γ1	NT	γ	γ1	
0103	1	β1	β1	β1	β	β1	1
0111	2	γ2/θ	γ2/θ	NT	α	NT	1, 2
0145[a]	2	γ1	γ1	NT	γ	γ1	
0146	1	γ2/θ	γ2/θ	NT	α	NT	
0156	4	γ2/θ	γ2/θ	NT	α	NT	
0172	1	β1	β1	β1	β	β1	2
0177[a]	2	β1	β1	β1	β	β1	
NT	1	γ1	γ1	NT	γ	γ1	
NT	2	ζ	α1	NT	α	NT	
NT	1	ι	α1	β2	α	NT	
NT	1	ι	β1	β1	β	β1	
Diarrheic calves							
04	3	ι	β1	β1	β	β1	
04	2	ξ	β1	β1	β	β1	
05	1	β1	β1	β1	β	β1	1
014	2	β1	β1	β1	β	β1	
017	1	γ2/θ	γ2/θ	NT	α	NT	
026	5	β1	β1	β1	β	β1	1
026[a]	3	β1	β1	β1	β	β1	
0111	1	β1	β1	β1	β	β1	
0123	1	ι	β1	β1	β	β1	
0125	1	γ2/θ	γ2/θ	NT	α	NT	
0128	1	β1	β1	β1	β	β1	1
0145	1	γ1	γ1	NT	γ	γ1	1
0153[a]	1	β1	β1	β1	β	β1	
0180	1	ρ	NT	NT	β	NT	
NT	1	ζ	α1	NT	α	NT	

NT — Not typable.

[a] Serogroups and variants of *eae* previously found in atypical enteropathogenic *E. coli* (EPEC) strains from humans with diarrhea or other gastrointestinal alterations (14).

[b] Orden et al (9) and Yuste et al (13).

A total of 185 AEEC strains isolated from diarrheic calves, lambs, and goat kids (25, 17, and 7, respectively) and from healthy cattle, sheep, and goats (38, 64, and 34, respectively) were used in this study. Nineteen of the strains were EHEC (*eae*$^+$, VT$^+$), and 166 were atypical EPEC (*eae*$^+$, VT$^-$, *bfpA*$^-$). Of the 91 strains that had previously been tested in the rabbit ileal loop assay, 84 (92%) were able to induce AE lesions (12). These strains have been described previously (6,9,13) (Tables I to III).

Table II. Serogroup, variants of LEE genes, and VT type in AEEC strains isolated from sheep

Source and serogroup	Number of strains	Gene and variant					Type of VT[c]
		eae[c]	*tir*	*espA*	*espB*[c]	*espD*	
Healthy sheep							
O2[a]	13	γ2/θ	γ2/θ	NT	α	NT	
O4[a]	2	β1	β1	β1	β	β1	
O20	1	ζ	α1	NT	α	NT	
O26	2	β1	β1	β1	β	β1	1, 2
O55	1	γ2/θ	γ2/θ	NT	α	NT	
O55, 91, 103[b]	4	β1	β1	β1	β	β1	
O55, 91, 103	2	γ2/θ	γ2/θ	NT	α	NT	
O71[a]	1	γ2/θ	γ2/θ	NT	α	NT	
O76	1	ε	β1	NT	β	β1	
O103[a]	4	γ2/θ	γ2/θ	NT	α	NT	
O103[a]	13	ε	β1	NT	β	β1	
O109	2	β1	β1	β1	β	β1	
O153[a]	12	β1	β1	β1	β	β1	
O153[a]	2	γ2/θ	γ2/θ	NT	α	NT	
O177[a]	3	β1	β1	β1	β	β1	
NT	1	β1	β1	β1	β	β1	
Diarrheic lambs							
O2[a]	1	γ2/θ	γ2/θ	NT	α	NT	
O4[a]	1	β1	β1	β1	β	β1	
O26[a]	5	β1	β1	β1	β	β1	
O71	2	ι	β1	β1	β	β1	
O73	1	γ2/θ	γ2/θ	NT	α	NT	
O75	1	β1	γ2/θ	NT	β	NT	1, 2
O80[a]	1	β1	β1	β1	β	β1	
O103[a]	1	γ2/θ	γ2/θ	NT	α	NT	
O153[a]	3	β1	β1	β1	β	β1	
NT	1	β1	β1	β1	β	β1	

NT — Not typable.

[a] Serogroups and variants of *eae* previously found in atypical EPEC strains from humans with diarrhea or other gastrointestinal alterations (14).

[b] Strains that had an identical response to all 3 monovalent sera.

[c] Cid et al (6), Orden et al (9), Yuste et al (13).

The AEEC strains were tested for the different variants of the *tir*, *espA*, and *espD* genes with a PCR scheme that has previously been described (4). The *E. coli* strains E2348/69 (O127:H6, *tirα1*, *espAα1*, *espDα1*), RDEC-1 (O15:H-, *tirβ1*, *espAβ1*, *espDβ1*), EPEC-359 (O119:H6, *tirα1*, *espAβ2*), EDL933 (O157:H7, *tirγ1*, *espAγ1*, *espDγ1*), and 95NR1 (O111:H-, *tir*γ2/θ) were used as positive controls.

Four variants of *tir* (α1, β1, γ1, and γ2/θ), 3 of *espA* (β1, β2, and γ1), and 2 of *espD* (β1 and γ1) were identified in the 185 ruminant AEEC strains analyzed. Tables I to III show the PCR results according to source of isolation, serogroup, and type of VT. Listed in Table IV are the 13 combinations found for the *eae*, *tir*, *espA*, *espB*, and *espD* genes.

In previous studies (5,7,8,10), 4 variants of *tir* (α, β, γ, and θ), 4 variants of *espA* (α, β, βv, and γ), 3 variants of *espD* (α, β, and γ), and between 4 and 7 LEE profiles were identified. Later, Garrido et al (4) studied 25 AEEC strains with the PCR scheme used in this work and found 4 variants of *tir* (α1, β1, γ1, and γ2/θ), 4 variants of *espA* (α1, β1, β2, and γ1), 3 variants of *espB* (α1, β1, and γ1), 3 variants of *espD* (α1, β1, and γ1), and 12 combinations of these LEE genes.

As in the previous studies (4,5,7,8,10), we found that most of the AEEC strains with a specific *eae* variant, except for types γ1 and ι, showed the same combination of *tir* and *esp* genes. Our results also show that there is no correlation between combinations of the *tir* and *esp* genes and belonging to the EPEC or EHEC group, animal species, or health status (healthy or diarrheic) of the animal and that there is great genetic diversity among the LEE genes of the ruminant AEEC strains. In contrast to EHEC, the role of ruminants as a reservoir of EPEC for humans is not known (1). Most of the EHEC and EPEC strains studied in this work showed the same combinations of LEE genes as were previously found in AEEC strains isolated from humans (4). In addition, 59% (98 of 166) of the atypical EPEC strains from the ruminants presented *eae* variants and serogroups previously found in atypical EPEC strains from humans with diarrhea or other gastrointestinal alterations (Tables I to III). Thus, our results show

Table III. Serogroup, variants of LEE genes, and VT type of AEEC strains isolated from goats

Source and serogroup	Number of strains	Gene and variant					Type of VT[b]
		eae[b]	*tir*	*espA*	*espB*[b]	*espD*	
Healthy goats							
O2[a]	1	γ2/θ	γ2/θ	NT	α	NT	
O3	4	β1	β1	β1	β	β1	
O4[a]	3	β1	β1	β1	β	β1	
O15[a]	1	β1	β1	β1	β	β1	
O35	1	γ2/θ	γ2/θ	NT	α	NT	
O55[a]	2	γ1	γ1	γ1	γ	γ1	
O56[a]	1	β2	α1	β2	α	NT	
O76	1	ε	β1	NT	β	β1	
O91	1	γ2/θ	γ2/θ	NT	α	NT	
O121	5	ε	β1	NT	β	β1	
O127	1	γ2/θ	γ2/θ	NT	α	NT	
O128	1	γ2/θ	γ2/θ	NT	α	NT	
O145[a]	2	γ1	γ1	NT	γ	γ1	
O149	2	ρ	NT	NT	β	NT	
O153[a]	3	β1	β1	β1	β	β1	
O153[a]	2	γ2/θ	γ2/θ	NT	α	NT	
O153	1	ε	β1	NT	β	β1	
O156	1	ζ	α1	NT	α	NT	1
NT	1	β1	β1	β1	β	β1	
Diarrheic goat kids							
O3	2	β1	β1	β1	β	β1	
O153[a]	2	β1	β1	β1	β	β1	
O163	2	β1	β1	β1	β	β1	
NT	1	β1	β1	β1	β	β1	

NT — Not typable.

[a] Serogroups and variants of *eae* previously found in atypical EPEC strains from humans with diarrhea or other gastrointestinal alterations (14).

[b] Cid et al (6), Orden et al (9), Yuste et al (13).

that ruminants seem to be a reservoir of atypical EPEC for humans. However, further studies are necessary to support this hypothesis.

The results herein are in accordance with the findings of Garrido et al (4) concerning the pathotypes of the AEEC strains with the variants β1, β2, ε, ζ, and ξ of the *eae* gene. However, our results with strains with other *eae* variants do not fully agree with those obtained by these authors. Thus, Garrido et al (4), who found that the *eae*γ*1* strains possessed the variant γ1 of *espA* and that the *espB* gene of the *eae*γ2/θ strains was not typable. We found that only 2 of the 8 *eae*γ*1* strains (both of serogroup O55) had the variant γ1 of *espA* [like the AEEC O157 strains described by Garrido et al (4)], whereas the *espA* gene of the other 6 *eae*γ*1* strains (5 O145 strains; O was not typable in the remaining strain) could not be typed. It is possible that this difference may be associated with the serogroups of the *eae*γ*1* strains and that the *espA* variant of the AEEC O55 and O157 strains with the *eae*γ*1* gene is different from that of the AEEC strains with the same *eae* variant but of other serogroups (such as O145). The EHEC O157 strains are believed to have evolved from EPEC O55 (15). The different results for the *espB* variant of the *eae*γ2/θ strains in the 2 studies may be due to the difference in primers used to amplify the *espB* gene. Our results with this variant are in accordance with those obtained by Bertin et al (10).

None of the 3 pathotypes that were found for the 11 *eae*ι strains was identical to the pathotype of the only AEEC strain with this *eae* variant described by Garrido et al (4). These results show the high variability of associations between the *eae*ι gene and the variants of the other LEE genes.

In contrast, only 1 of the 84 *eae*β*1* strains (isolated from a diarrheic lamb and of serogroup O75) showed a pathotype different from that of most of the AEEC strains having that *eae* variant.

Variants of *tir*, *espA*, and *espD*, of the *eaep* strains, a variant of *eae* recently described by our group (13), were not typable (Table IV).

Interestingly, we observed a relationship between the combination of *tir* and *esp* genes and the *eae* clusters described by Ito et al (16). That association was closer with the variants of *tir* than with the variants of *esp*. Ito et al (16) categorized the *eae* variants into 5 clusters (a, b, c, d, and x) by analysis of 5′-terminal nucleotide sequences of different intimin types and heteroduplex mobility assay. Thus, *eae*β2 and *eae*ζ were included in cluster a, *eae*β*1*, *eae*ε, and *eae*ξ in cluster b, *eae*γ*1* in cluster c, *eae*γ2/θ in cluster d, and *eae*ρ in cluster x. In

Table IV. Combinations (pathotypes) of LEE genes of the AEEC strains isolated from ruminants

Pathotype	Number of strains	Gene and variant				
		eae	*tir*	*espA*	*espB*	*espD*
1	83	β1	β1	β1	β	β1
2	1	β1	γ2/θ	NT	β	NT
3	1	β2	α1	β2	α	NT
4	2	γ1	γ1	γ1	γ	γ1
5	6	γ1	γ1	NT	γ	γ1
6	47	γ2/θ	γ2/θ	NT	α	NT
7	21	ε	β1	NT	β	β1
8	7	ζ	α1	NT	α	NT
9	8	ι	β1	β1	β	β1
10	2	ι	α1	β2	α	NT
11	1	ι	γ1	NT	γ	γ1
12	3	ξ	β1	β1	β	β1
13	3	ρ	NT	NT	β	NT

NT — Not typable.

contrast, we did not find a relationship between the combination of *tir* and *esp* genes and the 6 groups of closely related intimins described by Blanco et al (14,17), which were determined by analysis of the complete nucleotide and amino acid sequences.

In conclusion, combinations of the *tir* and *esp* genes of ruminant AEEC strains are associated with variants of the *eae* gene but not with the origin of the strains. In addition, our results show that ruminants seem to be a reservoir of atypical EPEC for humans. However, further studies are necessary to support this hypothesis.

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