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First isolation of the enterohaemorrhagic *Escherichia coli* O145:H- from cattle in feedlot in Argentina

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Abstract

Background: Enterohaemorrhagic *Escherichia coli* (EHEC) is considered to be common cause of haemorrhagic colitis (HC), thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome (HUS) in humans. In a previous paper, we have demonstrated that EHEC are commonly found in the intestines of livestock. Infections in humans are, in part, a consequence of consumption of undercooked meat or raw milk. Argentina has one of the highest records of HUS (300–400 cases/year; 22/100,000 children under 4 years of age). The aim of this work is to communicate the first isolation of O145:H-from cattle in this country and characterize the virulence cassette, providing useful information to evaluate the risk of foodborne transmission of this emergent non-O157:H7 serotype.

Results: EHEC O145:H- was isolated from cattle in an Argentinian feedlot. Pheno- and genotype of nine strains were characterized, corresponding to several virulence cassettes: VT2$^+$*eaeA*$^+$ Mp$^+$ (n = 5), VT2$^+$*eaeA*$^+$ (n = 1), VT1$^+$*eaeA*$^+$ Mp$^+$ (n = 2), and VT1$^+$*eaeA*$^+$ (n = 1). Strains isolated from the same animal were considered only when they showed a different virulence pattern. The clonal relationship was studied by RAPD. Strains were distributed in two RAPD profiles, which corresponded to the presence of either, VT1$^+$ or VT2$^+$ genotype. No difference was detected by RAPD analysis between Mp$^+$ or Mp$^-$ strains.

Conclusions: This was the first isolation of EHEC O145:H- serotype in Argentina enlarging the list of non-O157:H7 serotypes isolated from cattle in this country by us. All O145:H-strains carried several virulence factors which allow us to predict their potential ability to develop haemolytic uraemic syndrome in humans.

Background

Enterohaemorrhagic *Escherichia coli* (EHEC) is considered to be common causes of haemorrhagic colitis (HC), thrombotic thrombocytopenic purpura, and haemolytic-uraemic syndrome (HUS) in humans [1,2]. In a previous paper, we have demonstrated that EHEC are commonly found in the intestines of livestock, specially young cattle; and infections in humans are, in part, a consequence of consumption of undercooked meat or raw milk [3–6]. Several outbreaks have occurred mainly in USA, Canada, United Kingdom and Japan during the last decade [7–10]. Argentina has one of the highest records of HUS (300–400 cases/year; 22/100.000 children under 4 years of age). This rate can be compared with the anual incidence of 2.6/100.000 in Oregon, 3.0 in King County-Washington, 3 to 5 in Canada, United Kingdom, Chile and Uruguay [11]. In spite of EHEC strain O157:H7 has been reported as a cause of HUS in this country [12], this strain does not seem to be so common in this country as in the USA [11]. We have recently published an extensive list of non-O157:H7 EHEC serotypes isolated from cattle and foods in Argentina [13], several of them involved as cause of HC and HUS in somewhere.

In Europe, eighty percent of the VTEC isolated from patients with diarrhoea corresponded to non-O157 serogroups such as O26, O91, O103, O111, O113, O128, O145 [14]. In 1984, Kudoh et al. [15] described the first outbreak of disease due to *E. coli* O145:H- in Japan, affecting to 100 children. Beutin et al. [16] have considered this serotype as a new emergen. Afterthere, several investigators notified the isolation of this serotype from cattle and humans in USA, Canada and Belgium [17–22]. Another serotype, O145:H25, was found associated to HUS cases in U.K. [19], meanwhile O145:H8 and O145:H28 were isolated from healthy cattle in Canada [22] and Germany [23], respectively.

Aside from known differences among the pathogenicity of VT1, VT2 and its variants, factors that may affect the virulence of EHEC are the ability to cause attaching and effacing lesions (*eae* gene) in the intestinal mucosa and possession of a 60 MDa megaplasmid (Mp) [1]. The aim of this work is to communicate the first isolation of O145:H- from cattle in this country and characterize the virulence cassette of several strains, providing useful information to evaluate the risk of foodborne transmission of this non-O157:H7 serotype.

Results and discussion

Nine Shiga toxin-producing *Escherichia coli* serogroups and eleven non-typable strains were identified from a feedlot in Argentina (data no shown). One of them (O145) corresponded to a serogroup never described before in this country. All O145 strains were characterized as H-. This non-O157:H7 serotype (O145:H-) had been previously involved in haemolytic uraemic syndrome outbreaks in Japan [15]. The genotype of those strains were characterized, corresponding to several virulence cassettes: VT2$^+$*eaeA*$^+$ Mp$^+$ (n = 5), VT2$^+$*eaeA*$^+$ (n = 1), VT1$^+$*eaeA*$^+$ Mp$^+$ (n = 2), and VT1$^+$*eaeA*$^+$ (n = 1). All these strains were able to ferment sorbitol within 18 h of incubation at 37°C. Strains isolated from the same animal were considered only when showed a different virulence pattern.

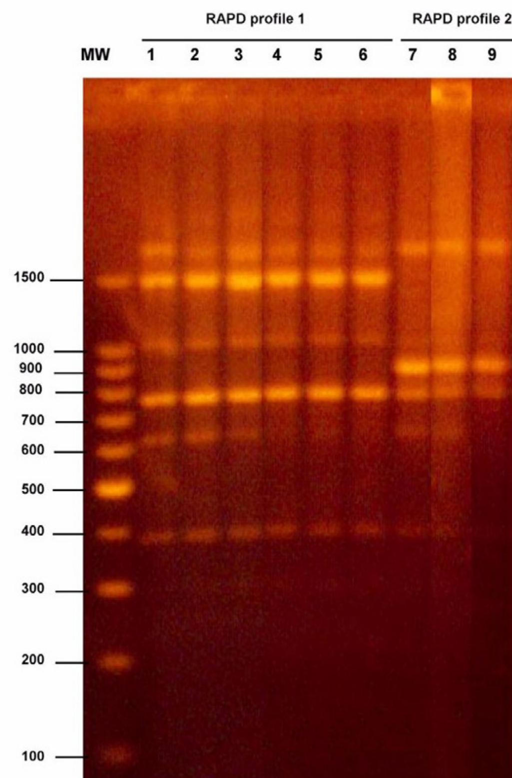

Figure 1
RAPD profiles generated using primer M13. Lane MW contains DNA molecular size marker (100 bp ladder, Promega, Madison, WI).

O145:H- strains were distributed in two RAPD profiles, which corresponded to the presence of either, VT1$^+$ or VT2$^+$ genotype (RAPD profiles 2 and 1, respectively). No difference was detected by RAPD analysis between Mp$^+$ or Mp$^-$ strains (Fig. 1). This fact suggests that would be necessary to carry on RAPD studies by using new primers or to apply pulse field electrophoresis technique in the study of clonal relationships.

The presence of the virulence genes codifying for verotoxin (1 or 2), the ability to develop attaching and effacing phenomenon on enterocyte surface (*eaeA* gene) and the expression of an EHEC haemolysin (Hly_{EHEC}), afford the potential of O145:H- as foodborne emergent human pathogen. This EHEC non-O157:H7 serotype, isolated from cattle in feedlot, was never found by us from grazing cattle in Argentina [13]. Our finding, and the high incidence rate of HUS in this country, put local health service authorities on guard.

Conclusions

This was the first isolation of EHEC O145:H- serotype from Argentinian cattle which enlarges the list of non-O157:H7 serotypes isolated from bovine and ground beef in this country by us. All O145:H- strains carried several virulence factors which allow to predict their potential ability to develop haemolytic uraemic syndrome in humans. The strains, which had been isolated from different animal in the same feedlot, conformed two RAPD profiles based upon their verotoxin subtype, without clonal differences.

Materials and methods

Sample collection from cattle

Samples were collected from an Argentinian farm. following the method described previously [6]. Briefly, 59 beef cattle, belonging to a feedlot of Pampeana region, were sampled fortnightly during six months by a single rectal swab from each animal. The swabs were placed in transport medium and processed immediately at the laboratory.

Bacterial colonies were grown on MacConkey agar incubating for 24 hs at 37°C. An aliquots of confluent growth, aproximately 100 colonies, was inoculated into Luria-Bertani broth for 3 h at 37°C and processed for DNA extraction [6,24]. One ml from each culture was frozen at -70°C with the addition of glycerol for further isolation of individual VT^+ colonies (50 to 100 colonies/sample) on a MacConkey agar plate. Microorganisms were confirmed as *E. coli* by means of biochemical test such as citrate, indole, urease and TSI profile.

Detection of VT1, VT2, eae and 60 MDa plasmid by PCR

Escherichia coli growth and DNA preparation were performed as described previously [6,24]. Primers *eae-1* and *eae-2* were designed to amplify a portion of the *eae* gene which is conserved between enteropathogenic and enterohaemorrhagic *E. coli* strains. Primer sequences for VT1, VT2 and *eae* were indicated in a previous paper [6]. Mp primer sequences were: MFS1F 5' ACGATGTGGTTTATTCTGGA 3' and MFS1R 5' CTTCACGTCACCATACATAT 3' (166 bp amplimer) [25]. T_m were calculated for each primer using Oligo 4.0 (Primer Analysis Software, National BioSciences). Amplification of bacterial DNA was performed in a total volume of 50 µl. A negative control (reagent blank) was included without addition of sample. Another control was designed by adding DNA from the strain lacking VT1, VT2, *eae* and Mp. Two reference strains O157:H7 were used as positive controls. The conditions for the PCR amplification of VT1, VT2, *eae* and Mp were as described previously [6,24,25]. Amplified products were analyzed by submarine gel electrophoresis and UV-transillumination (300 nm).

Fermentation of sorbitol

Sorbitol MacConkey agar was used to test the VT^+ strains for this condition.

Table 1: Feno-genotypic characteristics of O145:H- strains isolated from cattle.

Serotype	Animal & Sample	Virulence cassette				Virulence profile	Lane
		VT1	VT2	*eaeA*	Mp		
O145:H-	52(1)	-	+	+	+	1	1
O145:H-	18(1)	-	+	+	+	1	2
O145:H-	27(3)	-	+	+	+	1	3
O145:H-	46(3)	-	+	+	+	1	4
O145:H-	38(4)	-	+	+	+	1	5
O145:H-	27(3)	-	+	+	-	2	6
O145:H-	1(1)	+	-	+	+	3	7
O145:H-	17(11)	+	-	+	+	3	8
O145:H-	1(1)	+	-	+	-	4	9
n		3	6	9	7		

Lane column indicates positions of RAPD study for each strain in Fig. 1.

Serotyping

O and H antigens were determined by means of a micro-agglutination technique in tubes and plates described by Guinée *et al.*[26] and modified by Blanco *et. al.*[27] using all available O (O1 – O175) plus six putative new O antigens (OX176-through OX181) [28] and H (H1 – H56) antisera [29]. Non-specific agglutinins were removed by absorption with the corresponding cross-reacting antigens. All VTEC were processed for O serogroup determination. H serotyping was performed only on those strains which, having been isolated from the same sample, differed in either one virulence factor or the O serogroup.

Random amplified polymorphic DNA (RAPD) analysis

Bacteria were grown overnight at 37°C in LB broth with shaking. An aliquot of the culture was diluted 1/10 in water to determine the optical density at 600 nm [39]. For an optical density value of 0.5, a 500 μl aliquot of the stationary-phase culture was centrifuged (2 min at 12,000 × g) and suspended in 500 μl of bidestilled water. The suspension was then boiled for 10 min, centrifuged (2 min at 12,000 × g) and the supernatant was stored at -70°C. Five microliters were used as the template for PCR amplification. RAPD was performed in a final volume of 50 μl containing 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.1% (w/v) Tween 20, 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM primer, 2.5 U *Taq* DNA polymerase. Three different primers were evaluated: 1281 [31], 970–11 and M13 [32], but primers 1281 and 970–11 were not discriminatory.

Amplification was done in a Genius thermal cycler [Techne (Cambridge) Ltd.] as follows: initial denaturation at 94°C for 5 min (heating rate 60°C/min), followed by 40 cycles of denaturation at 94°C for 1 min (heating rate 29°C/min), annealing at 50°C for 1 $^1/_2$ min (cooling rate 26°C/min) and extension at 72°C for 1 $^1/_2$ min (heating rate 29°C/min). Reaction products (10 μl) were analysed in a 1.8% agarose gel stained with ethidium bromide.

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