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ORIGINAL ARTICLE

Isolation and selection of coliphages as potential biocontrol agents of enterohemorrhagic and Shiga toxin-producing *E. coli* (EHEC and STEC) in cattle

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Abstract

Aims: To isolate, characterize and select phages as potential biocontrol agents of enterohemorrhagic and Shiga toxin-producing *Escherichia coli* (EHEC and STEC) in cattle.

Methods and Results: Sixteen STEC and EHEC coliphages were isolated from bovine minced meat and stool samples and characterized with respect to their host range against STEC, EHEC and other Gram-negative pathogens; their morphology by electron microscopy; the presence of the *stx1*, *stx2* and *cI* genes by means of PCR; RAPD and rep-PCR profiles; plaque formation; and acid resistance. Six isolates belonged to the Myoviridae and 10 to the Podoviridae families. The phages negative for *stx* and *cI* that formed large, well-defined plaques were all isolated using EHEC O157:H7 as host. Among them, only CA911 was a myophage and, together with CA933P, had the broadest host range for STEC and EHEC; the latter phage also infected *Shigella* and *Pseudomonas*. Isolates CA911, MFA933P and MFA45D differed in particle morphology and amplification patterns by RAPD and rep-PCR and showed the highest acidity tolerance.

Conclusions: Myophage CA911 and podophages CA933P, MFA933P and MFA45D were chosen as the best candidates for biocontrol of STEC and EHEC in cattle.

Significance and Impact of the Study: This work employs steps for a rational selection and characterization of bacteriophages as therapeutic agents. This report constitutes the first documentation of STEC and EHEC phages isolated in Argentina and proposes for the first time the use of rep-PCR as a complement of RAPD on DNA fingerprinting of phages.

Introduction

Since the discovery of bacteriophages, the use of specific bacterial pathogen phages in the treatment of infectious diseases has been a highly attractive proposition. Nonetheless, the appearance of antibiotics during the 1940s caused that approach to be essentially abandoned for decades in the Western countries (Summers 2001; Wang *et al.* 2006). Nowadays, however, an increasing number of antibiotic-resistant bacterial pathogens have revived the interest in phage therapy as a relevant biocontrol strategy. Phages selected as biocontrol agents should be highly infective on a wide variety of strains of the target pathogen (Brussow 2005; Denou *et al.* 2009; Donlan 2009), they must furthermore be obligate lytic infectants (and thus unable to recombine with the bacterial chromosome), and they should be devoid of virulence factors or antibiotic resistance genes that could become incorporated into the host bacterial genome (Matsuzaki *et al.* 2005). Finally, the success of the treatment depends on choosing phages with the most suitable characteristics for the desired application. For example, when used *in vivo*,

they should be innocuous with respect to the commensal bacteria, and in oral applications they would have to tolerate gastric passage so as to maintain a sufficient concentration at the pathogen's location to enable infection and lysis (Tanji *et al.* 2005; Callaway *et al.* 2008; Denou *et al.* 2009).

Previous authors have proposed phage therapy in cattle as a possible prophylactic method against the O157:H7-Shiga toxin-producing *Escherichia coli* (STEC) (Johnson *et al.* 2008). Shiga toxins are endotoxins encoded by temperate lamboid phages (*stx*-phages) of STEC (Brussow *et al.* 2004; Robinson *et al.* 2006). The enterohemorrhagic *E. coli* (EHEC) is a subgroup among the STEC able to attach to the human intestinal wall and produce an effacing lesion by a type III secretion system. EHEC is considered the principal cause of haemolytic-uremic syndrome (HUS) (Rivero *et al.* 2004; Johnson and Taylor 2008). Although the STEC serotype most frequently isolated from persons with diarrhoea is O157:H7; other serotypes – such as O26:H11, O103:H2, O111:NM and O145:NM – have also been linked to foodborne disease outbreaks worldwide (Allerberger *et al.* 2003; Meichtri *et al.* 2004; Wickham *et al.* 2006).

Argentina is among the countries with the greatest incidence of HUS in the world (Rivero *et al.* 2004; Rivas *et al.* 2008), so that the establishment of precautionary measures to avoid the spread of this pathogen constitutes a major public health concern in this country. STEC and EHEC are innocuous to ruminants and are transient members of their gastrointestinal flora. Cattle are the main reservoir of these bacterial pathogens (Nastasijevic *et al.* 2008), and faecal contamination of meat during slaughter is the most common route for transmission to human beings (Kudva *et al.* 1999; Chase-Topping *et al.* 2008). Thus, elimination of STEC and EHEC from the bovine gastrointestinal tracts before the butchering would constitute the first barrier needed to prevent the introduction of these bacteria into the food chain.

The aim of this work was therefore to isolate, characterize, and select phages as potential biocontrol agents of STEC and EHEC for oral administration to bovines. The sources used for phage isolation were bovine minced meat and bovine faecal samples to look for phages whose natural environment was already the bovine intestine, and pork sausage, as this meat has also been reported to be contaminated with STEC and EHEC (Smith *et al.* 1991; Dontorou *et al.* 2003). Phage isolates that failed to infect nonpathogenic *E. coli* were further characterized with respect to their morphology and genomic-amplification patterns. Selection was performed by considering as best candidates those phages with a wide spectrum of STEC- and EHEC-host serotypes that were also lacking *stx1*, *stx2*, and *cI* gene amplification by PCR, because, for one, the gene *cI* encodes a protein that represses transcription from the promoters of the lytic cycle genes on bacteriophage Lambda and in this way maintains the lysogenic state (Oppenheim *et al.* 2005; Brussow 2006). This gene is present in the 933W prophage of the EHEC strain EDL 933 that also carries the *stx2* gene (Perna *et al.* 2001). Finally, large plaque size formation – as a measure of the ability of phages to spread in the intestinal mucosa of ruminants – and acid resistance were also considered as desirable characteristics of phages chosen for use in cattle phage therapy.

Materials and Methods

Bacterial strains

Phage isolation and host range determination were performed with strains EDL 933 (ATCC 700927) of EHEC, the nonpathogenic *E. coli* WG5 (ISO 10705-2:1999) and ATCC 25922, plus the additional STEC, EHEC, and pathogenic isolates listed in Table 1.

Phage isolation

The isolation of phages was attempted from two commercial minced meats, one pork sausage, and two bovine faecal samples, each collected from a different farm.

For faecal coliphage isolation, 10 g of each sample was mixed with 90 ml of *E. coli* broth modified with 0·02 g l^{-1} of novobiocin (mEC+n, Merck, Darmstadt, Germany) and homogenized in a model 400 Stomacher (Seward Medical, London, UK) for 2 min at maximum speed. For the minced meat and sausage samples, 10 g was mixed with 90 ml of SM buffer [100 mmol l^{-1} NaCl, 8 mmol l^{-1} $MgSO_4.7H_2O$, 50 mmol l^{-1} Tris–HCl (pH 7·5), gelatin 2% (w/v)] and homogenized in the Stomacher™ for 10 min at full speed before addition of 10 ml of concentrated mEC+n (10X) to the mixture.

In both isolations, the particulate material was removed by centrifugation at 4000 ***g*** for 15 min. The supernatants were poured into sterile flasks, $CaCl_2$ was added to a final concentration of 10 mmol l^{-1}, and enrichment was begun by adding a cocktail of nine isolates of EHEC and STEC – 300 μl of an overnight culture of each – as hosts, with these strains being highlighted in boldface in Table 1; 4 with the serotype O157:H7; four non-O157 clinical isolates with serotypes O111:NM, O113:H21, O103:H2 and O145:H25; and one foodborne isolate of serotype O26:H11. The samples were then incubated overnight at 37°C. After addition of chloroform to a final concentration of 4% (v/v), the enrichment was left for 30 min at room temperature and finally centrifuged at 4000 ***g*** for 15 min. This supernatant constitutes the first stage of enrichment.

Table 1 Bacterial isolates

Organism	Serotype	Isolate number	Source
EHEC/STEC	O157:H7	**933, 69160, 68911, 71945,** 474, 476, 537/448 466	1
STEC	O103:H2	**646/03** 118/05	2
STEC	O113:H21	**870/02** 889/06 370/02	2
STEC	O145:NM	002/02 1210/06	2
STEC	O26:H11	**CIDCA1** 395/06	2
STEC	O111:NM	**67/03** 207/02 534/05	2
STEC	O161:H2	154	2
STEC	O8:H19	763/02 215/06 209/03	2
STEC	O22:H16	412/03 238/04	2
STEC	O146:H28	656/04	2
STEC	O15:H27	189/01 356/02	2
STEC	O13:H6	654/04	2
STEC	O145:H25	14/00 **485/03**	2
STEC	O20:H19	805/03 32/05 537/04	2
STEC	O174:H28	257/03 439/04	2
STEC	ONT:H12	456	1
EIEC	–	202	1
Shigella flexneri	2	48	1
Sh. flexneri	3	2013 2019	1
Shigella sonnei	–	47-5035	1–3
Proteus spp.	–	6011	3
Citrobacter freundii	–	6293	3
Morganella morganii	–	364	3
Salmonella spp.	–	7452	3
Klebsiella pneumoniae	–	4482, 4336	3
Escherichia cloacae	–	6615, 6303	3
Serratia marescens	–	6606	3
Klebsiella spp.	–	6496	3
Acinetobacter spp.	–	4581	3
Pseudomonas aeruginosa	–	–	3

EHEC, enterohemorrhagic *E. coli*; STEC, Shiga toxin-producing *E. coli*; EIEC, enteroinvasive *E. coli*.

The bacterial isolates in bold were used for phage isolation from the following source: 1 – Hospital de Ninos 'Sor Maria Ludovica' La Plata, Buenos Aires, Argentina. 2 – Administracion Nacional de Laboratorios e Institutos de Salud (A.N.L.I.S.) 'Dr. Carlos G.Malbran', Ciudad autonoma de Buenos Aires, Argentina. 3 – Hospital de Pediatria 'Prof. Dr. Juan P. Garrahan', Ciudad autonoma de Buenos Aires, Argentina.

The presence of phages was screened by a spot test of serial dilutions of this initial enrichment mixture onto each individual strain of host bacterium used for phage propagation after the addition of 0·2 ml of an overnight culture of that host to 5 ml of molten soft tryptic-soy (TS; Biokar Diagnostics, Allone, France) agar (0·6% [w/v] agar-agar).

A second enrichment was performed with each individual host strain where lysis plaques were detected: 0·5 ml of an overnight bacterial culture of that host were added to 5 ml of TS broth along with 0·1 ml of the first enrichment supernatant; $CaCl_2$ was added to a final concentration of 10 mmol l^{-1}, and the mixture was incubated for 24 h at 37°C.

To isolate and purify phages from each second enrichment, individual plaques, obtained by the double-agar-layer method with the enrichment mixture and fresh host bacterium, were picked and placed in 5 ml of TS broth; 0·2 ml of an overnight culture of the host strain was added; and the suspension was incubated at 37°C until complete lysis of the culture. The resulting lysate was serially diluted and seeded onto a fresh layer of agar medium along with a suspension of fresh host bacteria for a second plaque isolation by the double-agar-layer method. A total of three such enrichments were performed. The isolated phages were finally filtered through a 0·22-μm membrane and kept at room temperature for further assays.

Host range

The susceptibility to each phage of *E. coli* strains EDL 933, WG5 and ATCC 25922, along with the Gram-negative

isolates listed in Table 1, was tested by a spot assay. Briefly, molten soft TS agar (5 ml) was mixed with 0·3 ml of an overnight host bacterium culture grown in TS broth supplemented with 10 mmol l^{-1} of $CaCl_2$ and then spread out on TS-agar plates. When the plates were dry, 10 μl drops of the filtered phage suspensions containing from 5×10^8 to 10^9 PFU ml^{-1} were spotted onto the bacterial lawn. Plates were incubated at 37°C for 20 h and then examined for lysis zones (Muniesa *et al.* 2003b).

Electron microscopy of bacteriophages

The morphology of phages was examined by transmission electron microscopy. A 10 μl drop of each filtered isolated phage was negatively stained with 2% (w/v) phosphotungstic acid and applied to the surface of a copper grid and examined in a JEOL 1200 EX II transmission electron microscope (Facultad de Veterinaria, UNLP, Argentina or INTA Castelar, Argentina). Phages were classified according to their respective families as stated in the guidelines of the International Committee on Taxonomy of Viruses (2000).

DNA isolation and quantification

DNA extraction was performed by the protocol described by Sambrook and Russell (2001). Of each phage lysate, 1 ml was incubated with 1 U of DNAase 1 (Invitrogen, Buenos Aires, Argentina) and 1 U of RNAase A (Biodynamics, Buenos Aires, Argentina) for 30 min at 37°C. After degradation of phage protein coats with EDTA (pH 8), proteinase K, and sodium dodecylsulfate, the proteins were removed by phenol–chloroform extraction, and the phage DNA was isopropanol-precipitated, washed with 70% (v/v) aqueous ethanol, and resuspended in 50 μl of Tris–EDTA buffer (pH 7·6).

Extraction of bacterial DNA from the EHEC strain EDL 933 was performed with a commercial kit (Illustra™ bacterial genomic prep mini spin, GE Healthcare, Little Chalfont, UK).

All phage and bacterial DNAs were quantified by optical density at 260/280 nm with a Model ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) and diluted in Tris–EDTA buffer to 30 μg ml^{-1} for PCR assays.

RAPD and rep-PCR

For RAPD fingerprinting, the primers RAPD6, RAPD9, and RAPD10 (Madueno *et al.* 2009) were used. The amplification was performed by means of a StrataGene Gradient Cycler™ under the following conditions: 4 low stringency cycles of 5 min at 94°C, 5 min at 40°C, and 5 min at 72°C; 30 high-stringency cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 70°C; and a final extension of 10 min at 72°C. The reaction products were stored at 4°C until needed.

For rep-PCR, the primers Eric1r and Boxa1r (Versalovic *et al.* 1994) were used. The amplification was performed by means of the StrataGene Gradient Cycler™ under the following conditions: 1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30 s, at 40°C for 45 s, and at 72°C for 2 min; and one final cycle at 72°C for 3 min. The reaction products were stored at 4°C until needed.

Both the RAPD and rep-PCRs were performed in a 20-μl volume containing 2 μl of (10X) PCR buffer (200 mmol l^{-1} Tris–HCl, 500 mmol l^{-1} KCl, pH 8·3; Invitrogen), 2 μl of 2 mmol l^{-1} dNTPs, 1 U of Taq DNA polymerase (Invitrogen), 30 ng of DNA template, 15 pmol of primer, with the final volume adjusted with DNAase-free miliQ water. The total volume of each PCR product was analysed by 1% (w/v) agarose gel electrophoresis and the bands visualized by ethidium bromide staining.

Screening for *stx1*, *stx2*, and *cI* genes by PCR

Screening for the presence of the *stx1* and *stx2* genes was performed by a multiplex PCR according to a standard protocol (Rivas *et al.* 2007) with the primers designed by Pollard *et al.* (1990). Stated in brief, the amplification conditions were 1 cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 1 final cycle at 72°C for 2 min.

Detection of the *cI* gene was performed by using the upper primer 5′-CATCTGCAGTTCTCCATCTCTGTCATAG-3′ and the lower primer 5′-CGGAATTCGTCTTGATGAACATGGTAG-3′.

Both of these primers were synthesized by Invitrogen Argentina and designed on the basis of the sequence for *cI* obtained from the genome sequence of the enterobacterium phage 933W (GenBank, accession number NC_000924). The amplification conditions were as follows: 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 45 s, 53°C for 45 s, and 72°C for 45 s; and a final cycle at 72°C for 1 min.

Both the *stx* and *cI* PCRs were performed in a 20-μl volume as described for RAPD and rep-PCR. PCR products were analysed by 1·5% (w/v) agarose gel electrophoresis. In both instances, the PCR bands obtained with the EDL 933 DNA template were used as positive controls.

Plaque morphology

The analysis of plaque morphology – obtained in Luria Bertrani (LB), TS, and Nutrient (N) agar with and without the addition of 10 mmol l^{-1} $CaCl_2$ – was performed

by adding 0·3 ml of an exponentially growing culture ($OD_{600\ nm}$, *c.* 0·3) of the EHEC strain used as host for phage isolation plus 0·1 ml of phage lysate diluted in SM buffer to 10^2 PFU ml^{-1} to 5 ml of molten soft agar in each medium. When $CaCl_2$ was to be present, 50 μl of a filtered 1 mol l^{-1} solution was added. The mixture was poured onto the corresponding agar medium plates and incubated for 24 h at 37°C. After incubation, the plaque sizes were analysed, and the plates kept at room temperature for another 72 h to determine whether the plaque morphology and/or size changed with time.

Resistance of the phages to acidity

Suspensions of each phage were exposed to a range of pH values from 1 to 7 over a 16-h period then checked for survival. A given phage suspension (0·1 ml, 10^9 PFU ml^{-1}) was added to 0·9 ml of saline adjusted to each specific pH with HCl (1·5 mol l^{-1}), and the mixture incubated at 37°C for 16 h (Hazem 2002). A mixture of 0·1 ml of phage suspension and 0·9 ml of normal saline (pH 7·2) was also incubated at 37°C for 16 h as a control. After incubation, 0·1 ml of the phage suspension, previously neutralized with NaOH (1 mol l^{-1}), was serially diluted 10-fold, mixed with 0·2 ml of a suspension of the host bacterium (10^9 CFU ml^{-1}), and incubated for 20 min at room temperature. The mixture was then added to 5 ml of molten soft LB agar (0·6% [w/v] agar-agar) and spread over a plate of LB agar. Titres of the surviving phages were determined by plating 10-fold dilutions according to the soft agar–overlay method.

Statistical analysis

Comparisons of the mean plaque sizes and of the mean titres after incubation at pH 4 were evaluated by the one-way analysis of variance (ANOVA) with a significance level of 5% ($P < 0·05$) followed by Fisher's least significant difference test at a $P < 0·05$.

Results

Phage isolation and host range

A total of 20 phage isolates was obtained and designated according to their original source (C for minced meat, MF for faecal samples and CH for pork sausage plus A, B, or C for sample identification and a final number indicating the bacterial strain used as host for phage isolation). For phages isolated from the same agar plate, a letter indicating a characteristic of its plaque morphology comes at the end of the name. For example, phage CA933D was isolated from meat sample A with EHEC 933 as host and formed diffuse plaques. The phages isolated are listed in Table 2.

Table 2 Isolated phages

Phage isolate	Propagation host
CHA60, CB60P, CB60H, MFA60D, MFA60N	Clinical isolate 69160 (O157:H7)
CA911, MFA911	Clinical isolate 68911 (O157:H7)
MFA45D, MFA45N	Clinical isolate 71945 (O157:H7)
CA933N, CA933P, CA933D, MFA933H, MFA933P	Clinical isolate 933 (O157:H7)
CBO103, CCO103	Clinical isolate 646/03 (O103:H2)
CBO113, CCO113	Clinical isolate 870/02 (O113:H21)
CCO26, MFCO26	Food-borne isolate CIDCA1 (O26:H11)

For fast screening of the phages that targeted non-pathogenic *E. coli*, strains WG5 and ATCC 25922 were first tested as hosts to discard those phages as possible biocontrol agents for phage therapy in cattle as phages infecting nonpathogenic *E. coli* could perturb the bovine commensal flora.

None of the isolates infected *E. coli* ATCC 25922; but the isolates CHA60, CCO26, MFCO26 and MFA911 did infect WG5 and were thus excluded. The other phage isolates, after testing against all the Gram-negative pathogens listed in Table 1, gave the host range results summarized in Table 3. None of the remaining phage isolates were able to infect the STEC serotypes O111:NM, O145:NM, O8:H19, O161:H2, O20:H19, O15:H27, O146:H28, or other Gram-negative pathogens (except for *Pseudomonas* and *Shigella*). Thus, those bacterial test strains are omitted in Table 3.

The host range among the phages isolated with EHEC O157:H7 as host (O157 phages) – except for CB60P, CA911, and CA933P – is almost identical, is quite narrow and differs only in EDL 933 sensitivity. The host ranges of non-O157 phages are furthermore similar to each other but include the serotypes O26:H11 and O113:H21, which are not infected by O157 phages. The widest host range for STEC and EHEC was observed for phage CA911 (Table 3). The isolate CA933P was the only O157 phage able to infect serotype O13:H6 but was also the only one capable of infecting *Shigella flexneri 2*, *Sh. flexneri 3*.

Of all the other Gram-negative pathogens tested, other than *Shigella*, only *Pseudomonas aeruginosa* was sensitive to any of the phages, and most of the latter gave clear lysis zones when spotted onto this strain (Table 3). Phages CBO103, CBO113, CCO103, CCO113 and CB60P exhibited extremely turbid lysis zones when inoculated into most of their bacterial hosts (indicated as T in Table 3).

Bacteriophage ultrastructure

Transmission electron micrographs of the different morphologies of the phage isolates and their approximate

Table 3 Bacteriophages host range

	O157 phages											Non-O157 phages				
Strains	CB60P	CA 933P	CA 911	MFA 933H	CA 933D	CA 933N	MFA 45D	CB 60H	MFA 60D	MFA 60N	MFA 45N	MFA 933P	CB O103	CB O113	CC O103	CC O113	
O157:H7	EDL 933	T	–	–	–	–	–	–	+	+	T	+	–	–	–	–	–
	69160	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
	933	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
	68911	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
	71945	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
	474	+	+	+	+	+	+	+	+	+	+	+	T	–	–	–	–
	476	–	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
	537	T	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
	448	T	+	+	+	+	+	+	+	+	+	+	+	T	T	–	–
	466	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O113:H21	870/02	–	–	–	–	–	–	–	–	–	–	–	–	T	T	+	+
	889/06	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	370/02	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O103:H2	646/03	–	–	–	–	–	–	–	–	–	–	–	–	T	T	+	+
	118/05	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
O145:H25	14/00	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	485/03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O26:H11	CIDCA1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	395/06	–	–	–	–	–	–	–	–	–	–	–	–	T	T	–	T
O13:H6	654/04	–	+	–	–	–	–	–	–	–	–	–	–	–	–	T	–
O174:H28	257/03	+	–	+	–	–	–	–	–	–	–	–	–	–	T	–	–
	439/04	T	–	+	–	–	–	–	–	–	–	–	–	–	–	T	–
O22:H16	412/03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	238/04	T	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
ONT:H12	456	T	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
Shigella flexneri 2	48	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sh. flexneri 3	2013	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2019	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Shigella sonnei	47	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EIEC	202	–	+	–	+	–	–	–	–	–	–	–	–	+	–	–	–
Pseudomonas aeruginosa	–	–	T	–	T	+	+	+	+	+	+	+	T	–	–	+	+
n° serotypes infected		5	4	6	2	2	2	2	2	2	2	2	2	4	5	4	3
n° strains infected		12	11	14	9	9	9	9	10	10	10	10	9	2	3	4	3

Total lysis over the bacterial lawn is represented by (+), turbid lysis by (T), and no lysis by (–).

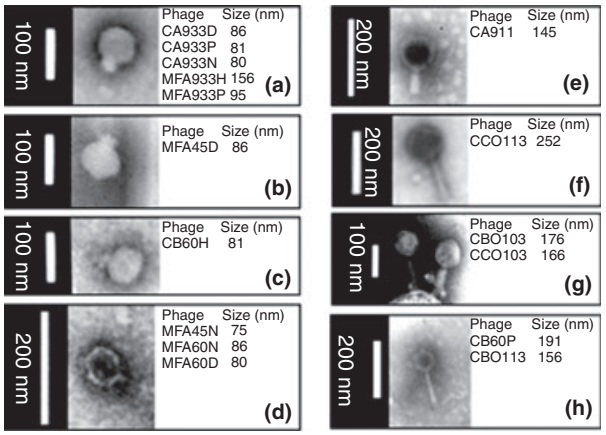

Figure 1 Bacteriophage electron microscopy. (a)–(h) show the different morphologies and particle length observed for each group of isolated bacteriophages.

particle sizes are shown in Fig. 1. All of the isolated phages were classified into two morphological families of tailed phages of the order Caudovirales: 10 isolates belonged to the Podoviridae family (head with icosahedral symmetry, straight and short noncontractile tail) morphotype C1 (isometric head) (Ackermann 2001) with four different morphologies (Fig. 1a–d) and the other six to the Myoviridae family (contractile sheath and icosahedral head) morphotype A1 (isometric head) (Ackermann 2001) with four different morphologies (Fig. 1e–h). All phages isolated from non-O157:H7 hosts belonged to the Myoviridae family.

Despite being isolated from different sources, the podophages infecting the O157:H7 strain 933 as host shared the same morphology, but with different particle sizes, as observed in Fig. 1a.

Screening of *cI*, *stx1* and *stx2* genes by PCR

The amplification of *stx1* and *stx2* was tested as these genes encode the virulence factors most frequently found among phages infecting STEC and EHEC (Muniesa and Jofre 1998, 2004; Muniesa *et al.* 2004b), as Shiga toxins are prophage encoded in the genome of these bacterial pathogens. A PCR amplification of the *cI* gene was also tested as evidence of the presence of phages capable of recombining with the host genome.

Myophage CB60P and podophage MFA60N, both isolated with O157:H7 strain 69160 as host, showed amplification of *stx2*. The Myoviridae phages CCO103 and CBO103, both isolated from the O103:H2 host strain 646/03, were positive for *stx1*. Only the non-O157 phages CBO103 and CCO113 were positive for *cI*, giving amplification of the 511-bp product expected according to the position of the internal primers on the *cI* gene of phage 933W (Table 4).

RAPD and REP-PCR profiles

Figure 2 shows the different patterns obtained by RAPD with the primers RAPD6, RAPD9, and RAPD10, and those produced by rep-PCR with primers BOXA1R and ERIC1R, while Table 4 lists the profiles corresponding to each phage. The phages were grouped according to the patterns arising from the different primers used. The members of group I are O157 phages whose set of

Table 4 Results of phages PCR characterization

Group	Phage	Stx	C1	RAPD6	RAPD9	RAPD10	ERIC1R	BOXA1R	Family
I	CB60P	Stx2+	–	F	C	F	D	B	M
	CA911	–	–	–	A	G	B	B	M
	MFA45N	–	–	A	B	A	A	C	P
	MFA45D	–	–	B	B	B	A	A	P
	MFA60D	Stx2+	–	C	A	C	B	B	P
II	MFA60N	Stx2+	–	G	B	D	C	C	P
	CB60H	–	–	G	B	D	C	A	P
	MFA933H	–	–	G	D	D	C	F	P
	MFA933P	–	–	G	B	D	C	F	P
	CA933N	–	–	G	D	D	C	F	P
	CA933P	–	–	G	B	D	C	F	P
	CA933D	–	–	G	B	D	C	F	P
III	CCO113	–	+	E	F	E	E	E	M
	CBO113	–	–	D	E	E	F	D	M
	CBO103	Stx1+	+	–	F	E	E	D	M
	CCO103	Stx1+	–	E	–	E	E	–	M

Results of PCR specific for *stx* and *cI* and the profiles obtained by RAPD and rep-PCR shown in Fig. 2. No amplification is represented by (–). Family (M) *Myoviridae*, (P) *Podoviridae*. Phages highlighted in grey have all the same morphology and differ only in RAPD9 profiles.

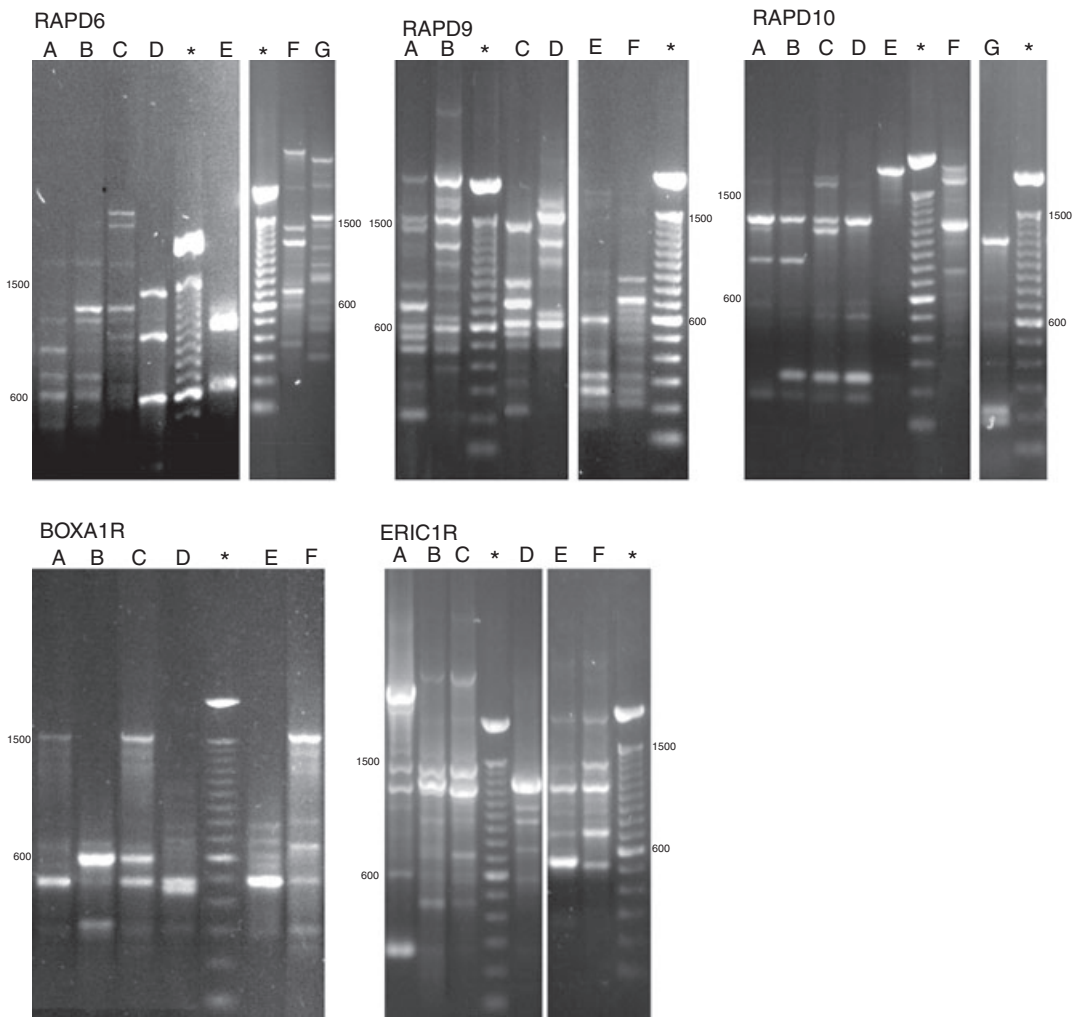

Figure 2 RAPD and rep-PCR profiles. Shown are the different RAPD and rep-PCR profiles. (a)–(h) correspond to the profiles obtained with each primer (Table 4) and * corresponds to the 100-bp ladder (Invitrogen).

profiles have either poor or no similarity to each other or to those of members of the other groups. Within this group, phage CB60P in particular has RAPD and ERIC1R profiles not only different from those of the other O157 phages but also unique among the profiles obtained from all the tested isolates. Members of group II are O157 phages that all produce identical amplification profiles with the primers RAPD6, RAPD10 and ERIC1R. Within this group, the phages isolated with strain 933 (highlighted in grey in Table 4) have identical BOXA1R profiles as well, with some of the members differing in only the RAPD9 profiles. This result agrees with the similarities observed with respect to morphology (Fig. 1a). Group III contains the phages isolated from the serotypes O103:H2 and O113:H21 (non-O157 phages). Their profiles are similar to each other, but all differ from those of the O157 phages. This distinction also pertains with respect to host range, as this group differs clearly from the phages of groups I and II with respect to the serotypes targeted. This group classification is furthermore reflected in the profiles obtained with the primer RAPD10, as groups II and III each have a characteristic RAPD10 profile, while group I has a completely different set of patterns.

Plaque morphology

Besides being exclusively lytic, the phages used in pathogen therapy should be highly effective as bactericidal agents. Abedon and co-workers have proposed that phage plaque growth in semisolid media be considered an approximation of phage population kinetics in natural spatially structured environments such as animal mucosal tissues (Abedon and Culler 2007a; Abedon

and Yin 2008), so that the formation of larger plaques *in vitro* (with host, bacterial growth conditions, and agar thickness remaining constant) could be compared to the more rapid spread of a phage population *in vivo* with faster access to an STEC and/or EHEC host attached to the bovine intestinal mucosa (Moxley 2004; Abedon and Culler 2007b). The plaque sizes and halo formations of the phages against their respective O157:H7 isolation host were analysed in TS, LB and N soft agars with and without the addition of 10 mmol l^{-1} $CaCl_2$. Table 5 shows the mean plaque sizes obtained for every phage on each medium, and significantly larger ($P < 0{\cdot}05$) plaque sizes among those of phages isolated from the same host are highlighted in bold in the table. The non-O157 phages CCO103, CCO113, CBO103 and CBO113 and the O157 phage CB60P are omitted in this table, because these phages formed only pinpoint plaques in all media. No differences in plaque sizes or morphologies were observed with the addition of $CaCl_2$. Statistical analysis of the plaque sizes indicated that the phages MFA60D and CB60H both attained significantly higher mean plaque sizes ($P < 0{\cdot}05$) than did MFA60N against strain 69160 in each of the three media. Among the phages tested against strain 933, phage CA933P had significantly larger plaques than all the other phages of the group in TS medium; whereas phages MFA933H and MFA933P, isolated from faecal samples, had plaque sizes significantly higher than the phages isolated from meat in LB medium. As phage CA911 was the only one isolated with strain 68911 as host, this phage was not compared with any other.

Plaques were also analysed for the appearance of a halo – the clear zone surrounding plaques caused by the diffusion of phage depolymerases that decapsulate bacteria to leave a translucent ring over the opaque bacterial lawn (Bessler *et al.* 1975; Kwiatkowski *et al.* 1982; Tomlinson and Taylor 1985; Hughes *et al.* 1998). Halo formation could be a desirable characteristic for phages chosen as biocontrol agents of STEC and EHEC, as the bacterial capsule is known to be a contributor to virulence in *E. coli* (Russo 2002).

Table 5 Mean plaque size in different media

Phage	Strain	Plaque size (mm)		
		N	TS	LB
CA911	68911	4·1*	3·9	3·5
MFA45N	71945	4·7*	3·5	3·8†
MFA45D		5·0*	2·9*	3·4†
MFA60D	69160	**4·9***	**4·0**	**4·6**
MFA60N		2·4†	2·4	3·0
CB60H		**4·6***	**4·1**	**4·8**
MFA933H	933	4·5*	4·3†	**4·2**
MFA933P		4·6*	4·1†	**4·1**
CA933N		4·7*	4·3†	3·7
CA933P		4·8*	**5·8†**	3·1
CA933D		5·5*	4·8†	3·6

Significantly larger mean plaque sizes ($P < 0{\cdot}05$) among those obtained with the same bacterial host are highlighted in bold.
*Great increase in halo diameter after 72 h.
†Moderate halo growth after 72 h.

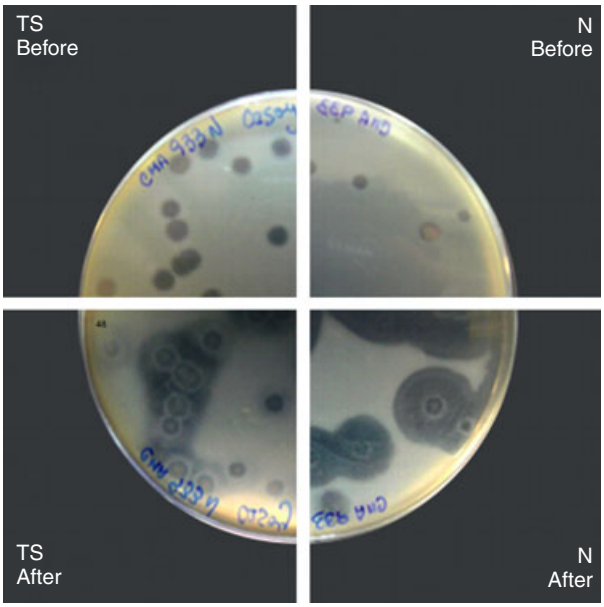

Figure 3 Plaques of phage CA933N in TS agar and N agar before and after 72 h at room temperature. TS show a moderate increase on halo size and N a great halo growth after 72 h (as described in Table 5). Each pair of 'before and after' quadrants is mirror image of the same agar plate.

All phages formed halos in N agar that greatly increased in size after 72 h at room temperature (Fig. 3), except for MFA60N, whose halo grew notably less. In TS agar, however, the plaques on the 933 host showed only a moderate increase in halo diameter (Fig. 3), with only those of phage MFA45D manifesting a similar substantial halo growth to that observed in N broth. In contrast, only phages from strain 71945 formed haloes with moderate increases in size in LB medium.

Acidity resistance

Phages selected for oral application in cattle must be able to resist the highly acidic gastric passage so as to enter the bovine intestine at a high enough concentration to be effective. To test for acid resistance, the phages were exposed to pH values ranging from 7 to 1 and titrated after a 16-h incubation at 37°C.

After incubation at pHs from 7 to 5, all phages conserved the same titres as the controls. At pH 4 the greatest difference in survival occurred among the phages, with the titres falling by 3–5 orders of magnitude. No viable particles were detected at pH 3 or lower. The phage CA911 had a titre decay of 3·1 orders of magnitude at pH 4 – a falling-off of viability equivalent to that seen with phages MFA933P, CA933N and MFA45D, but significantly lower ($P < 0·05$) than that observed for all the other phages. No significant difference in survival was observed among the phages of 71945, with titre drop-offs of 3·4 and 3·9 orders of magnitude. The three phages isolated from strain 69160 were the least resistant to acidity, with titre losses from 4 to 5·3 orders of magnitude at pH 4; though, among them the isolate MFA60D proved significantly more resistant ($P < 0·05$) at this pH than the other two.

Discussion

In this study, 20 STEC and EHEC bacteriophages were isolated from pork sausage, minced meat, and bovine faecal samples. As meat contamination with *E. coli* mainly derives from its contact with a ruminant's intestinal faeces during slaughter (Elder *et al.* 2000), ground beef and bovine stools were chosen as sources of phages whose natural environment was already the ruminant's gut, as these phages would be expected to survive in the bovine intestine better than those from other environments. This is the first report of the isolation and characterization of STEC and EHEC phages in Argentina.

To initially screen possible candidates for phage therapy in calves, phages able to infect nonpathogenic *E. coli* were discarded for further characterization because such phages could cause an imbalance in the bovine intestinal flora (Tanji *et al.* 2005). Phages of that type, however, might be considered as good candidates for other applications – e.g., biocides for sanitary surfaces and even for food (Kudva *et al.* 1999; O'Flynn *et al.* 2004) or antibiotic agents in the treatment of superficial or other infections (O'Flaherty *et al.* 2009), where a disturbance to other related micro-organisms is irrelevant. This left 16 possible calf phage therapy candidates from two different morphologic families within the order Caudovirales: 10 belonged to the Podoviridae family morphotype C1, all of them isolated from O157:H7 hosts; while the remaining six pertained to the Myoviridae family morphotype A1, with two isolated from a O157:H7 host and the other four from hosts with serotypes O103:H2 and O113:H21.

Molecular characterization of the selected phages was performed by amplification profiles of their genomes by RAPD and rep-PCR and by detection of the *stx1*, *stx2*, and *cI* genes by PCR. The profiles obtained by RAPD with primers RAPD6 and RAPD10 were similar in band number and differentiation power to those obtained by rep-PCR, but a better differentiation was obtained with primer RAPD9, which sequence yielded profiles with a higher number of bands and was the only one that allowed some differentiation among the phages isolated from strain 933, those all being O157 phages with the same microscopic morphology. As previously reported, RAPD proved to be a simple and highly reproducible technique for differentiation among phages specific to a particular bacterial host (Perez *et al.* 1998; Barrangou *et al.* 2002; Shivu *et al.* 2007) and thus constitutes a facile approach to the analysis of phage DNA, especially when working with large numbers of samples. This work represents the first time that rep-PCR has been used for the characterization of phages as a complement to RAPD profiles. The RAPD and rep-PCR results together allowed a marked differentiation among the phages because the relationships among the different isolates were established comparing both the profiles obtained with different primers and those resulting from different amplification methods. RAPD and rep-PCR profiles from non-O157 Myoviridae phages were, as expected, completely different from those from Podoviridae phages because isolates from those two phage families differed not only in their morphology but also in their host ranges. For obtaining consistent results on the PCR profiles – in terms of amplification fragment size and number and the relative intensity of bands – all the experimental conditions were maintained throughout the different experiments and involved DNA template concentrations that were standardized to ensure that the patterns obtained from different phages could be compared to each other.

PCR amplification of the *stx* and *cI* genes revealed that all the phages positive for *stx1* and/or *cI* were non-O157 phages and belonged to the Myoviridae family, and those positive for *stx2* were one Myoviridae and two Podoviridae O157 phages, all three isolated from the same bacterial host. Short-tailed and long rigid-tailed phages have been previously reported as carriers of *stx1* or *stx2* genes (Willshaw *et al.* 1987; Muniesa *et al.* 2000; Allison *et al.* 2003; Muniesa *et al.* 2003a, 2004a,b; Garcia-Aljaro *et al.* 2006).

All phages positive for either *stx* or *cI* are contraindicated for any type of phage therapy, as they represent a hazard of possible horizontal transfer of genes (Brussow 2005; Kropinski 2006; Parisien *et al.* 2008).

In this work, PCR determinations under conditions specific for *stx1*, *stx2* and *cI* were used to screen for undesirable genes. Nevertheless, because PCR results are not an absolute proof of the presence or absence of a given gene, the total sequencing of the genomes of any candidates for use in phage therapy must necessarily be the

last obligatory step before the latter's implementation (Brussow 2005; O'Flaherty *et al.* 2009).

Isolated phages were furthermore characterized by their plaque size and halo formation on their respective isolation host in different culture media. As plaque size in soft agar could be considered an approximation of phage proliferation and spread in natural matrices (such as an animal's intestinal mucosa) where bacterial hosts grow within a structured environment (Abedon and Yin 2008), phages with extensive plaque formation were chosen as the most promising candidates for phage therapy in cattle.

The Myoviridae phages, excepting CA911, formed pinpoint plaques in all media assayed. These kinds of plaques could either result from long latent periods, very small burst sizes, and/or a low rate of adsorption of phage particles onto the host bacterium (Abedon and Yin 2008) or indicate an association with the temperate phage phenotype. This last possibility agrees with the observation that, when spotted on bacterial lawns of almost all of their hosts, those phages formed lysis zones with a high turbidity (Guttman *et al.* 2005), and four of them tested positive for *stx* and/or *cI*. Whatever the reason might be, any or all of those characteristics would render them undesirable as phages for use as therapeutic agents. They were accordingly excluded from further characterization.

The remaining phages formed well-defined plaques, in some instances, surrounded with a halo. The presence of halos has been described as the result of the diffusion of phage depolymerases that degrade capsule exopolysaccharides (Bessler *et al.* 1975; Tomlinson and Taylor 1985; Hughes *et al.* 1998). Depolymerase activity of phages – along with infection and lysis – could be considered an additional mechanism against pathogens as the bacterial capsule itself in *E. coli* is considered to be a virulence factor (Russo 2002) through protecting the bacterium from the animal or human host's immunologic defences and has also been reported to enhance the attachment of this particular bacterial pathogen to a given surface (Hassan and Frank 2004; Sharma *et al.* 2005). Haloes from the same phage on the same host did not have the same size among the three different media, probably as a result of the different growth states of the bacteria. Bigger halos were observed in N agar than in TS agar for almost all the isolated phages assayed, despite the results of Hassan and Frank (2004), who reported no capsule production of O157:H7 in N broth. A possible explanation for this discrepancy is that the capsule formed with bacteria growing in N agar can be thinner than that observed in TS agar, and thus the former appears as a more extensive depolymerase activity in the agar plate, whereas such a thin capsule formed in N broth may have not been detected by light microscopy, as discussed by those same authors. Another characteristic sought in phages to be used for therapy is a broad host range for the target bacteria. Therefore, among the phages with extensive plaque formation, those able to infect as many serotypes as possible should be chosen to obtain a cocktail of phages that infect the widest possible range of serotypes with the lowest number of phages. Phages able to infect genera other than *E. coli* could be useful in that they would eliminate other pathogens as well, but the possibility of infection of other bacteria not contemplated in the host range assays should be taken into consideration. Of all the other Gram-negative pathogens tested, apart from *Shigella*, only *Ps. aeruginosa* was infected and was likewise sensitive to many of the isolated phages from both the Myoviridae and the Podoviridae families. Phages capable of infecting both *Ps. aeruginosa* and *E. coli* have been previously reported (Jensen *et al.* 1998).

The final characteristic analysed was the acid resistance of the phages exhibiting extensive plaque formation since phages selected for oral administration to bovines must resist gastric passage and reach the intestine at a sufficient concentration to target a pathogen (Koo *et al.* 2000; Chibani-Chennoufi *et al.* 2004). To do so, those phages must accordingly withstand acidity levels of *c.* pH 1. With respect to comparative acidity resistance, in agreement with the results obtained in a previous report (Jamalludeen *et al.* 2007), all the phages tested here were acid resistant down to a pH of 5, but then different resistances were observed at pH 4, with titre losses from 10^3 to 10^5 UFP ml^{-1} occurring after 16 h. As no phage particles were detected at pH 3 or lower, for oral administration phages would have to be included in an acid-resistant matrix for protection to serve as a vehicle for phage delivery. In this regard administration with bicarbonate, as other delivery strategy, has been previously proposed (Chibani-Chennoufi *et al.* 2004; Tanji *et al.* 2005). Even under such conditions phages able to tolerate the lowest possible pHs would nevertheless be desirable to use. Alternatively, such acid-resistant phages could be applied as biocides in combination with other acidic treatment agents (Choi *et al.* 2009; Gill 2009; Laury *et al.* 2009) or might be suitable for the decontamination of acidic foods.

In summary, 16 of the original 20 phages isolated failed to infect the nonpathogenic *E. coli* strains assayed; and as determined by screening for the *stx1*, *stx2*, and *cI* genes, only 10 amplified neither *stx* nor *cI*. One of the isolates negative for *stx* and *cI* was a non-O157 myophage, but was discarded as a possible biocontrol agent out of precaution because of its similarity in plaque morphology plus its RAPD and rep-PCR profiles to the other three non-O157 myophages isolated that were in fact positive

for amplification of either of those two genes. Of the nine remaining phages negative for both *stx* and for *cI*, one was from the Myoviridae and eight from the Podoviridae families; and all nine produced well-defined plaques. The widest host ranges were obtained with myophage CA911 and podophage CA933P, although the latter was not specific to STEC and EHEC. CA911, for its part, was one of the phages with the highest acid resistance with a titre loss of 3·15 orders of magnitude at pH 4, MFA933P and CA933N exhibited a similar pH resistance to that of CA911, and one significantly higher than those of the other 933 phages; but MFA933P also had significantly larger plaques in LB agar than did CA933N. Phage MFA45D likewise had a high acidity resistance and moreover was the only phage isolated that showed depolymerase activity in all three media assayed.

Phages chosen for a therapeutic cocktail should have infection mechanisms as different as possible so that, if some of the targeted pathogens become resistant to one of those phages, it will still remain sensitive to the others – a possibility that might be reflected initially by a diversity in their genomic profiles. A high similarity between the RAPD and rep-PCR profiles was observed for the phages from 933, so phage CA933N was discarded as it did not have the highest acidity resistance nor the bigger plaques size among 933 phages. Out of this selection, the isolated phages CA911, CA933P, MFA933P and MF45D would appear to be the best candidates for oral administration as therapeutic agents in cattle. It should be highlighted that, as Podoviridae phages infecting STEC and EHEC are rarely described, they have never been proposed as biocontrol agents of these bacteria. Nevertheless, we found members of this family whose characteristics make them promising candidates as biocontrol agents of STEC and EHEC.

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