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ORIGINAL ARTICLE

Isolation and characterization of bacteriophages for avian pathogenic *E. coli* strains

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Abstract

Aims: To isolate and characterize bacteriophages, and to evaluate its lytic performance against avian pathogenic *Escherichia coli* (APEC) strains with high patterns of antibiotic resistance, in order to select phages for a therapeutic product to treat colibacillosis in chickens.

Methods and Results: Bacteriophages were isolated from poultry sewage and tested against 148 O-serotyped APEC strains. The morphological characterization of the bacteriophages was made by transmission electronic microscopy (TEM) observations and the genetic comparison between bacteriophages DNA was performed by restriction fragment length polymorphism (RFLP) patterns. Results showed that 70·5% of the tested *E. coli* strains were sensitive to a combination of three of the five isolated phages, that seemed to be virulent and taxonomically belong to the *Caudovirales* order. Two of them look like 16–19, T4-like phages (*Myoviridae*) and the third is a T1-like phage and belongs to *Syphoviridae* family. All of them are genetically different.

Conclusions: It was possible to obtain a combination of three different lytic bacteriophages with broad lytic spectra against the most prevalent O-serotypes of APEC.

Significance and Impact of the Study: Data reported in this study, presents an *in vitro* well studied phage product to be used as antimicrobial agent to treat colibacillosis in poultry industry.

Introduction

Escherichia coli is present in the normal microflora of the intestinal tract of chickens. However, some of these *E. coli* strains are able to cause disease under certain conditions, like abnormal predominance over the other gut flora, host depressed immune system or adverse environmental exposure. Extra-intestinal pathogenic *E. coli*, termed avian pathogenic *E. coli* (APEC) possess specific virulence attributes causing invasive infections in poultry (chickens and turkeys), namely colibacillosis (Zhao *et al.* 2005). The pathogenesis of APEC infections include the colonization of the respiratory tract, the crossing of the epithelium and penetration into the mucosa of the respiratory organs, the survival and multiplication in the blood stream and in the internal organs, and the production of adverse effects and lesions in cells and tissues (Dho-Moulin and Fairbrother 1999). These bacteria can be typed according to the somatic cell-wall antigen (O-antigen), or the flagella antigen (H-antigen). In poultry, 10–15% of the serotypes are pathogenic, and are present in the poultry environment causing a variety of disease syndromes including colibacillosis (Barnes and Gross 1997).

Avian colibacillosis is a complex syndrome characterized by multiple organ lesions, typically pericarditis, airsacculitis, perihepatitis and peritonitis, and in its acute form degenerates in septicaemia. The consequent chickens' high mortality rates and carcass rejection at slaughter causes significant economic losses in the poultry industry worldwide (Delicato *et al.* 2003; Ewers *et al.* 2004). The most important source of transmission seems to be faecal contamination through the inhalation of the

microrganism into the respiratory tract (Barnes and Gross 1997).

Escherichia coli isolates from poultry are frequently resistant to multiple drugs (Levy 2001, 2002). An increased concern over the consequences of the mechanisms that bacteria have developed, to prevent the inhibitory effects of the antibiotics in the treatment of animal bacterial infections is widespread (Schwarz and Chaslus-Dancla 2001; Huff *et al.* 2004). The antibiotic capacity to select and allow proliferation of resistant bacteria is an important clinical problem with public health consequences. Antibiotic residues can be found in the environment for long periods of time after treatment (Levy 2002). These active ingredients affect the microbial community as long as they remain intact and at growth inhibitory levels (Levy 2001).

World Health and Life Science institutions are concerned about a range of deleterious effects that antimicrobial resistant bacteria may have on human health, like increased duration of illness, treatment failure, and loss of therapeutic options as a consequence of human exposure to resistant bacteria through ingestion of animal derived food products. There have been three comprehensive reviews and reports on the problem of bacterial antibiotic resistance, each of which comments on the use and abuse of antimicrobials in food animal production, and recommends application of alternative methods of reducing microbial pathogens loads (FDA 2000a; WHO 2001; Isaacson and Torrence 2002). Also in animal production, there is serious consideration being given to restrictions on the use of antibiotics (FDA 2000b).

Phage therapy is presented as an alternative to antimicrobial therapies. Bacteriophages or phages are viruses that exclusively infect bacterial cells. If they are obligate lytic phages, or virulent phages, multiply in the host bacteria and lyse it at the end of the cycle, after immediate replication of new phage particles. As soon as the cell is destroyed, the new phages can find new hosts. Like all viruses, phages are metabolically inert in their extra cellular form. These structures are only able to self-reproduce as long as the host bacteria is present, and thus are not toxic to non specific bacteria, animals or plants. In fact, their replication depends exclusively on the infection of a specific bacterial host and on the utilization of the host intracellular machinery to translate their own genetic code. Phages are part of both gastrointestinal and environmental ecosystems and are among the simplest and most abundant organisms on earth (Carlton 1999; Sulakvelidze *et al.* 2001). Lytic phages are suitable for phage therapy in opposition to temperate phages. The former do not include the integrase genes on their genome, they lack the molecular basis for coexistence with the host and the potentiality to carry harmful genes from one host to another (Karam 1994; Brussow 2005; Skurnik and Strauch 2006).

Recently, well-controlled animal models have demonstrated that phages can rescue animals (chickens, mice, calves, pigs, lambs, fishes, etc.) from a variety of harmful infections, like *E. coli* or *Salmonella* infections (Smith and Huggins 1982, 1983; Smith *et al.* 1987; Berchieri *et al.* 1991; Barrow *et al.* 1998; Park *et al.* 2000; Sklar and Joerger 2001; Huff *et al.* 2002; Bru Ronda *et al.* 2003; Huff *et al.* 2003).

In this study, *in vitro* efficiency of five phages was evaluated based on lytic spectra against 148 avian pathogenic *E. coli* (APEC) strains. The best lytic performance was obtained with a combination of tree phages. In order to characterize these phages, an effective phage sorting scheme based on phage life cycle, lytic efficiency rate, morphology and on phage DNA restriction endonuclease digestion profile (RFLP) was conducted.

Materials and methods

Escherichia coli isolation

Escherichia coli strains were isolated from organs (liver, spleen, lungs) of infected commercial birds, with typical lesions of colibacillosis. Organs were emulsified in sterile saline solution 0·85% NaCl (Sigma, Osterode am Harz, Germany) and 0·1 ml of supernatant was plated in MacConkey agar (Biokar Diagnostics, Pantin Cedex, France), a selective medium for Gram-negative bacilli, which differentiates lactose fermenters (pink-red colonies) from nonfermenters bacteria. As approximately 95% of *E. coli* ferment lactose (Murray *et al.* 1991), pink red colonies were collected from plates and the specie confirmation of the isolates was conducted by using API strips according to manufacturer's instructions (Bio-Merieux, Marcy l'Etoile, France). *Escherichia coli* isolates were stored in Nutrient Broth (Oxoid, Hampshire, UK) with 20% glycerol at −80°C.

Escherichia coli serotyping for the O-antigen

The O-antigen serotyping of *E. coli* strains was performed using a 'kit for serotyping avian septicemic *E. coli* strains', supplied by the 'Laboratorio de Referencia de *E. coli* (LREC)' of the Veterinary Faculty of Lugo, Spain. The kit included 26 antisera: O1, O2, O5, O6, O8, O9, O11, O12, O14, O15, O17, O18, O20, O35, O36, O45, O53, O78, O81, O83, O88, O102, O103, O115, O116 and O132. If the strain was negative for all these antisera, it was considered not typeable (N/T) with this kit. Samples were prepared and procedures were carried out according to the supplied protocol.

Antibiotic susceptibility testing of APEC

The isolated APEC strains were subjected to antibiotic susceptibility testing. The active ingredients with systemic action (relayed throughout the blood circulation) in poultry, generally used for colibacillosis treatment were selected to perform this antimicrobial test. In order to label strains as susceptible, intermediate or resistant, antibiotic discriminating concentrations were used: ampicillin (AMP), 10 μg disc^{-1}, doxycycline (DO), 30 μg disc^{-1}, enrofloxacin (ENR), 5 μg disc^{-1}, sulphamethoxazole/trimethropim (STX), 25 μg disc^{-1}, nalidixic acid (NA), 30 μg disc^{-1}, pipemidic acid (PIP), 20 μg disc^{-1}, tetracycline (TE), 10 μg disc^{-1}, oxolinic acid (OA), 2 μg disc^{-1} and amoxycillin (AML), 30 μg disc^{-1} (Oxoid).

Each strain was plated in Mueller Hinton agar (Biokar Diagnostics), and the discs with the antibiotics were placed over the bacteria layer (Bauer *et al.* 1966). Plates were incubated at 37°C overnight. After this period, the diameter of the clear zone was measured and strains classified according to the sensitivity to each antibiotic. An *E. coli* reference control culture (ATCC 25922) was used for quality control of the test.

Bacteriophage isolation and purification

Bacteriophages were isolated from samples of poultry sewage, collected randomly from Portuguese poultry houses. Under sterile conditions samples were emulsified in Luria Bertani (LB) broth (Sigma), and the decanted supernatant obtained from each emulsion was added to an early-log grown mixture of eight *E. coli* strains selected randomly, from different O-antigene serotypes. Suspensions were incubated overnight at 37°C, with shaking (120 rev min^{-1}) and were then centrifuged at 9000 ***g*** for 10 min (rotor 19776, Sigma 3–16k). The supernatant was then filtered through a 0·22 μm membrane, 33 mm Millex Filter Units, Durapore® (PVDF) (Millipore, Bedford, MA, USA). The spot test method was used as an initial test for the presence of phage. A procedure based on the double layer plaque technique was performed (Carey-Smith *et al.* 2006). Layers of 3 ml of LB 0·6% agar (Sigma), previously inoculated with 100 μl of each *E. coli* strain used above, 6–8 h culture were spotted with 10 μl of the filtered suspension. This procedure was performed over LB 1·5% agar. Plates were incubated at 37°C overnight. A clear zone in the plate, resulting from the lysis of host bacterial cells, indicated the presence of phage.

In order to isolate phages from this clear lysis zone, serial dilutions in phage buffer (100 mmol l^{-1} NaCl (Sigma), 8 mmol l^{-1} $MgSO_4$ (Sigma), 50 mmol l^{-1} Tris (Sigma), pH 7·5) were done from the phage stocks obtained above. A colony of the respective hosts strains were grown 3–4 h (early-log phase culture) in 5 ml of LB broth. A volume of 100 μl of phage-containing sample and 100 μl of host culture were mixed with 3 ml of 0·6% LB agar, overlaid onto 1·5% LB agar plates and incubated overnight at 37°C. Phages were purified by successive single plaque isolation, from the higher dilutions plates where plaques were still distinct. A single plaque was picked from the bacteria lawn, inoculated into an early-log phase host culture, and the lysate plated as described above. After repeating the cycle three more times, lysates from single plaques were treated with chloroform 4 : 1 (v/v), mixed and centrifuged at 5000 ***g*** for 5 min. The phages were recovered from the upper phase suspension and filtered trough 0·22 μm. Phages stocks were stored at 4°C.

Phage lytic spectra of the typed *E. coli* strains

Bacterial susceptibility to bacteriophage was assayed for the 148 isolated *E. coli* strains by adapting a modified procedure of the traditional double-layer technique (Carey-Smith *et al.* 2006). Once the top agar was solidified at room temperature, 10 μl of the phage lysate suspension of about 10^7 PFU ml^{-1} was spotted, incubated at 37°C overnight and examined for the presence of a clear zone of lysis.

Bacteriophage amplification

The amplification of each isolated bacteriophage was performed by inoculating 5 ml of the purified phage suspensions in 10 ml of a 3–4 h culture (in LB broth) of the respective *E. coli* hosts. It was incubated overnight at 37°C, with shaking (120 rev min^{-1}). The suspension was centrifuged at 9000 ***g*** for 10 min and filtered trough a 0·22 μm membrane. This procedure was repeated again, by inoculating the resulting phage lysate volume in 100 ml of 3–4 h culture followed by incubation overnight at 120 rev min^{-1} and 37°C. The resultant phage suspension was filtered through a 0·22 μm membrane and stored at 4°C.

The number of phages present in this suspension was determined according to the Adams (Adams 1959) method with minor modifications. Successive dilutions of the phage suspension were performed in a saline solution (0·85% NaCl) and 100 μl of each dilution together with 100 μl of the respective bacterial host suspension were mixed with 3 ml of LB 0·6% top agar layer and placed over a 1·5% LB agar bottom layer. Plates were incubated overnight at 37°C. Phage titration was performed in triplicate.

Phages life cycle investigation by the induction of infected host strains with mitomycin C

In order to evaluate if phages selected based on the lysis efficiency were able to insert their genome in bacteria

DNA remaining as a prophage, some tests were performed. Lambda (λ) phage (DSM 4499), a *Siphoviridae* temperate phage, and the respective *E. coli* host (DSM 4230) were used as positive controls. Reconstitution, propagation and storage of this phage and *E. coli* host strain were conducted according to the supplier instructions (DSMZ, Braunschweig, Germany).

Each of the host strains were early-log grown in LB broth and 20 μl of the respective phage were spotted on the lawns, as described above. After an overnight incubation at 37°C, bacteria colonies change in the central lytic zone (resistant colonies) were picked (at least five colonies) and purified by successive sub-culturing in MacConkey agar, to remove attached phage particles. Phage resistance of those isolated strains was confirmed by the cross-streaking test and the spot lysis assay, and those phage resistant colonies were stress induced with mitomycin C (Sigma). The strains were grown in 200 ml of LB until an optical density at 600 nm of 0·2 was reached. The induction of phage release was attempted via overnight incubation at 37°C, in the presence of mitomycin C (1 μg ml^{-1}). A negative control, without mitomycin C was prepared. Bacteria lysate was centrifuged at 9000 ***g***. The supernatant was filtered through 0·22 μm and tested against each phage-sensitive host strain [wild-type (WT)] (Keel *et al.* 2002; Harel *et al.* 2003). After an overnight incubation at 37°C, bacterial lawns were checked for clear zones.

Electron microscopy

Phage particles were sedimented at 25 000 ***g*** for 60 min using a Beckman (Palo Alto, CA) J2-21 centrifuge with a JA 18·1 fixed-angle rotor. Phages were washed twice in 0·1 mol l^{-1} ammonium acetate, pH 7·0 (Sigma), deposited on copper grids (Ernest F. Fullam, Clifton Park, NY, USA) provided with carbon-coated Formvar films (Canemco & Marivac, Quebec, Canada), stained with 2% potassium phosphotungstate, pH 7·2 (Sigma) and examined in a Philips (Eindhoven, the Netherlands) EM 300 transmission electron microscope (TEM), operating 60 kV. Magnification was monitored with catalase crystals (performed by Dr H.W. Ackermann, Laval University, Quebec, Canada).

Phage purification by CsCl precipitation

An ultracentrifugation method was performed based on a caesium chloride density gradient. Four different solutions were prepared in phage buffer: 1·70, 1·50 and 1·30 g ml^{-1} CsCl (Sigma). After the volume of each phage suspension was measured, 0·5 g ml^{-1} of CsCl was added. These suspensions were ultracentrifuged (XL-90, Beckman) at 60 000 ***g*** for 2 h at 4°C. A bluish band indicative of phage particles was collected and placed in a microtube (Sambrook and Russell 2001). A Centricon 20 spin filter unit (millipore) was used to reduce the volume of the recovered CsCl purified phage concentrate. The centrifugation was performed at 4000 ***g*** for 10 min at 4°C. The phage concentrate was then washed with the phage buffer 1 : 4 (v/v) and centrifuged with the same settings in the filtration module, three more times, to remove all the CsCl. The resulted suspension was stored at 4°C.

RFLP pattern analysis

Differences between phages were confirmed by comparison between the individual restriction fragment length polymorphism (RFLP) patterns. A volume of 200 μl of the concentrated phage suspension by CsCl precipitation, was preincubated 30 min at 37°C with 1 μl of RNase 20 mg ml^{-1} (Sigma) and submitted to DNA purification according to the protocol provided with a commercial kit, High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Uncut phage DNA was run at 90 V for 45 min, in a 0·8% agarose gel (Qbiogene, Irvine, CA, USA) stained with ethidium bromide (Bio-Rad, Hercules, CA), to verify extraction yield and absence of bacterial genomic DNA. *Xap*I, *Bse*GI and *Sch*I restriction enzymes (Fermentas, St Leon-Rot, Germany) were used in order to obtain phage DNA RFLP patterns. A concentration of 5 U μl^{-1} of each enzyme and the respective enzyme buffers 1 × diluted in RNase and DNase free water (Biological Industries, M.P. Ashrat, Israel) were added to 6 μl of phage DNA, with a final volume of the reaction mixture of 30 μl. Tubes were incubated at 37°C for 3 h, according to supplier instructions. The loading buffer used to improve resolution was 1 × DNA Loading Dye & SDS Solution (Fermentas) and was added to the samples at 1 : 6 (v/v). Tubes were incubated at 65°C for 10 min and chilled on ice. Samples were loaded in a 1 cm thick, 2·0% agarose gel stained with ethidium bromide. Electrophoresis was carried out at 45 V for 5 h in a dark place.

Results

APEC O-serogroup and antibiotics susceptibility

The most common O-serotype of the 148 isolated APEC strains was the O78 with a frequency of 40·5%, followed by O2, O5 and O88 representing from 5·2% to 6·9% of the isolated bacteria. It was not possible with the kit used to type 34·7% of the bacterial strains. *Escherichia coli* serotypes as O1, O8, O99, O15, O20, O53, O86 and O103 were present at a low frequency (from 0·6% to 1·7%), while O6, O9, O12, O14, O17, O18, O35, O36, O45, O81,

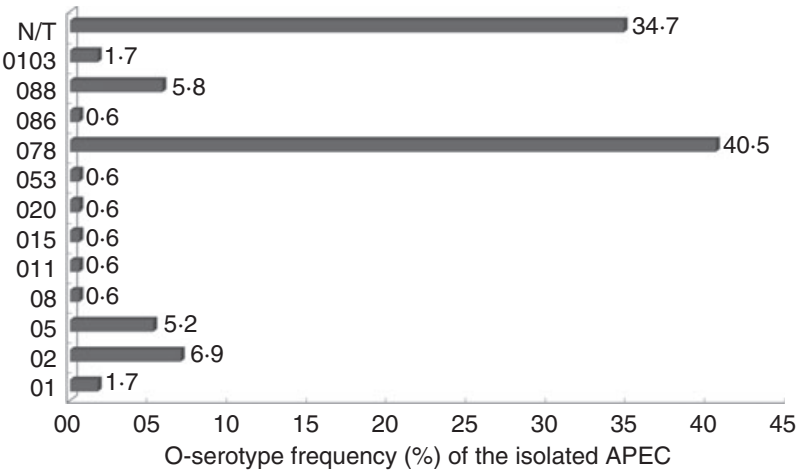

Figure 1 Relative frequency (%) of the APEC O-serotypes.

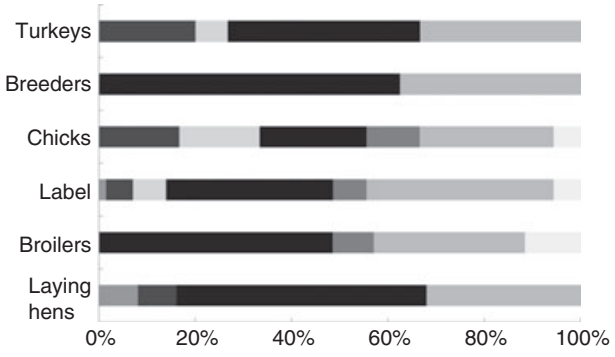

Figure 2 Relative frequency (%) of *E.coli* main isolated serotypes, according to the birds' strain, specie or age. O-serotypes described as 'others' are O8, O11, O15, O20, 053, 086 and O103, and were found in bacteria collected from broilers, 2·9% O8, 2·9% O15, 2·9% O86 and 2·9% O103, label chickens, 1·4% O11, 1·4% O53 and 2·8% O103 and chicks (5·6% O20). (■, O1; ■, O2; □, O5; ■, O78; ■, O 88; ■, NT; □, Others).

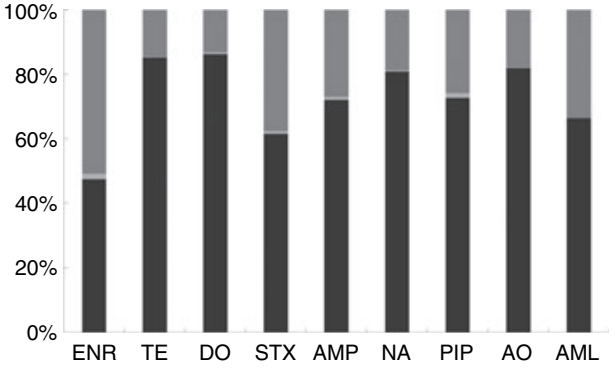

Figure 3 Relative comparison (%) of the isolated strains according to susceptibility, intermediate susceptibility or resistance to a range of antibiotics commonly used for therapy in poultry industry. AMP, ampicillin; DO, doxycycline; ENR, enrofloxacin; STX, sulphamethoxazole/trimethropim; NA, nalidixic acid; PIP, pipemidic acid; TE, tetracycline; OA, oxolinic acid; AML, amoxycillin. (■, susceptible; ■, intermediate; ■, resistant).

O83, O102, O115, O116 and O132 were not detected (Fig. 1).

Escherichia coli strains were also grouped according to the respective O-serotype, by strain of birds, age or species, according to the source of isolation. Figure 2 refers to the relative distribution of O-serotypes per group of birds. It is possible to verify that different O-serotypes infected the same group of birds. For example, serotypes like O1, O2, O78 and N/T were isolated from laying hens, O8, O15, O78, O86, O88, O103 and N/T were isolated from broilers, O1, O2, O5, O11, O53, O78, O88, O103 and N/T from label chickens, O2, O5, O20, O78, O88 and N/T were found in chicks, O78 and N/T in breeders and O2, O5, O78 and N/T were isolated from turkeys. It was observed that the isolated O-typeable strain more frequent in all the groups, was the O78.

The isolated strains were then subjected to an antibiotic sensitivity test and the percentages of susceptible, intermediate or resistant strains to each antibiotic are present in Fig. 3.

It was observed that 80–90% of the strains were resistant to TE, DO, OA and NA, 70–75% were resistant to AMP and PIP, 66·5% to AML, 61·6% to STX and 47·5% to ENR. The active ingredient with higher effectiveness to this group of strains was ENR, active against 50·8% of the APEC.

The bacteriophage lysis efficiency

Five phages were isolated: phiF78E, phiF258E, phiF2589E, phiF61E and phiF5318E. Phage lysis efficiency for the 148 O-serotyped strains is illustrated in Tables 1 and 2. From Table 1, it is apparent that all the phages were effective against O78, O5 and N/T *E. coli* strains and most of them were active for O2 and O88. From the low

Table 1 Bacteriophages lytic score (%) by *Escherichia coli* O-serotype

O-serogroup	phiF78E	phiF258 E	phiF2589E	phiF61E	phiF5318E
O1	0·00	0·00	0·00	0·00	0·00
O2	63·64	9·09	0·00	18·18	9·09
O5	35·29	5·88	5·88	47·06	5·88
O15	100·00	0·00	0·00	0·00	0·00
O20	0·00	0·00	0·00	0·00	0·00
O53	0·00	0·00	0·00	0·00	0·00
O78	8·33	30·73	14·06	23·96	22·92
O88	26·32	0·00	26·32	47·37	0·00
O103	100·00	0·00	0·00	0·00	0·00
N/T	48·39	16·13	6·45	19·35	9·68

Table 2 Bacteriophages sensitive strains (%)

Phage	phi78E	phiF258 E	phiF2589E	phiF61E	phiF5318E
Sensitive strains (%)	35·14	44·59	23·65	47·97	33·11

frequency O-typed strains (Fig. 1), O1, O20 and O53 were not sensitive to any of the tested phages.

Generally, observing the host lysis performance of each phage (Table 2), phiF258E and phiF61E were found to have the broadest host range, 44·6% and 48·0%, respectively.

Figure 4 illustrates the best phage association according to the higher percentage of lysed strains, in groups of two, three, four and five phages. When combining them in groups of two, the strongest lysis association was between phiF78E and phiF61E (60·4%). Groups of three phages, phiF78E, phiF258E and phiF61E, presented a higher lysis percentage, 70·5%. Associations of four and five phages are able to lyse, respectively 71·8% and 72·5% and thus does not bring a relevant advantage for lytic spectra range when compared with an association of three phages. Based on these results, the phages selected for further characterization were phiF78E, phiF258E and phiF61E.

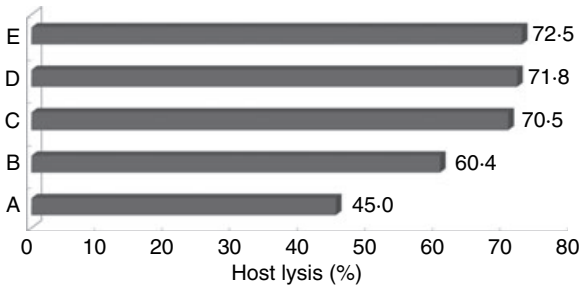

Figure 4 Best phage associations according to the higher percentage of lysis of the tested *Escherichia coli* strains: A – phiF61E (45·0%); B – phiF78E + phiF61E (60·4%); C – phiF78E + phiF258E + phiF61E (70·5%); D – phiF78E + phiF258E + phiF61E + phiF5318E (71·8%); E – phiF78E + phiF258E + phiF2589E + phiF61E + phiF5318E (72·5%).

Characterization of phages phiF78E, phiF258E and phiF61E

Phages phiF258E and phiF61E formed very clear lytic zones on their hosts (H816E and H161E, respectively) exhibiting no resistant bacteria. Conversely, phiF78E induced the formation of resistant colonies on H561E lawns after subculture, which may be an indication of lysogeny. Temperate phages integrate into the DNA hosts and only lyse the cells under certain conditions. Stress induced infected cells with temperate phages usually results in the release of the phage. So, the mitomycin C assay with the phiF78E resistant bacterial cells was performed. Infected *E. coli* DSM 4230 with λ bacteriophage was used as a positive control. In the assay no clear zone was found after stress inducing phiF78E resistant bacteria, which indicated that phiF78E is not temperate.

Electron micrographs demonstrated that all phages do not possess any lipidic envelope. Phages phiF78E and phiF61E had a neck with a tiny collar and a contractile tail. phiF78E has caudal fibres (20×2 nm) (Fig. 5a). phiF78E and phiF61E capsids were 103×42 nm and the tails 100×17 nm (Fig. 5a,c, respectively). phiF258E has a circular head with diameter of 62 nm and is characterized by a flexible tail of 160×8 nm (Fig. 5b).

It was possible to observe discriminatory bands in each phage DNA restriction pattern for a given enzyme (Fig. 6). For example the digestion of the phiF78E DNA with *Bse*GI showed a distinct band between 2300 and 2000 bp, while F258E DNA digested with the same enzyme exhibited a strong band above 23 000 bp and others of lower molecular weight (<2000 bp). The digestion of phiF61E DNA with this enzyme gave rise to four distinct bands under 1000 bp. The same stands for the profiles obtained with *Xap*I: high molecular weight discriminatory bands only above 4000 bp were detected in phiF78E DNA, whereas in the case of phiF258E, there were more distinct bands under this molecular weight (4000 bp). The phiF61E digestion pattern was not very clear, probably due to overlapped bands. Also, divergent RFLP profiles were noticed for *Sch*I digestion: when comparing the two first profiles in Fig. 6 relative to this enzyme, it can be noticed that discriminatory bands of low molecular weight appeared in the gel, only for phiF258E. phiF61E DNA did not present any discriminatory band for this enzyme.

Discussion

APEC possess specific virulence characteristics associated with colibacillosis. This is the primary cause of morbidity, mortality, and rejection of carcasses at slaughter in the poultry industry worldwide, with consequent high economical losses (Dho-Moulin and Fairbrother 1999;

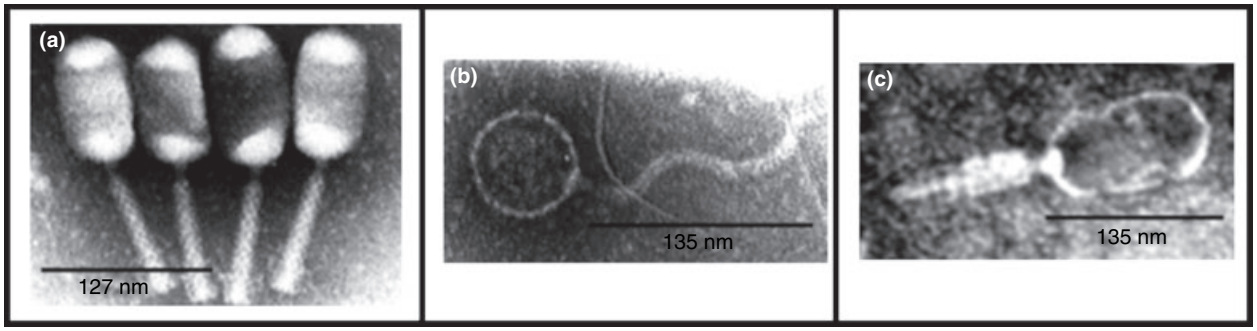

Figure 5 Bacteriophage microphotograph obtained by TEM: (a) phiF78E (magnitude: 297 000×); (b) phiF258E (magnitude 297 000×); (c) phiF61E (magnitude: 148 500×).

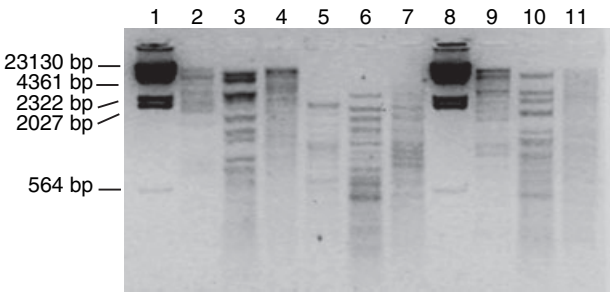

Figure 6 Agarose gel 2% stained with ethidium bromide, 5 h run at 50 V: 1 and 8 – λ DNA/*Hin*dIII; 2 – phiF78E/*Xap*I; 3 – phiF258E/*Xap*I; 4 – phiF61E/*Xap*I; 5 – phiF78E/*Bse*GI; 6 – phiF258E/*Bse*GI; 7 – phiF61E/*Bse*GI; 9 – phiF78E/*Sch*I; 10 – phiF258E/*Sch*I; 11 – phiF61E/*Sch*I.

Delicato *et al.* 2003; Ewers *et al.* 2004). The incidence and severity of colibacillosis has been increasing and might become an even greater problem in the poultry industry (Blanco *et al.* 1997; Altekruse *et al.* 2002).

APEC can cause disease in birds of various ages and strains. The results obtained in the present work, suggests that the O-serotypes affected poultry, independently of specie, strain or age. The most common typeable *E. coli* O-serotype isolated from each defined group of birds was the O78, and generically, the O2, O5 and O88 were also found as common serotypes. This result is supported by several *in vivo E. coli* pathogenicity tests performed in poultry, in which the same O-serotypes were found to be responsible for the most part of the colibacillosis infections (Blanco *et al.* 1997; Altekruse *et al.* 2002; Derakhshanfar and Ghanbarpour 2002; Raji *et al.* 2007; Zhao *et al.* 2005; Hammoudi and Aggad 2008). Data from the antibiograms demonstrated the high capacity of *E. coli* to acquire resistance to the most frequently used antibiotics (Fig. 3). Similar results have been reported by several authors (Ojeniyi 1985; Van den Bogaard and Stobberingh 1999; Van den Bogaard *et al.* 2001).

Bacteriophages have several characteristics that make them potentially attractive therapeutic agents against bacterial infections. One of them is the high specificity and effectiveness in lysing targeted pathogenic bacteria. Due to phages high specificity, they are likely to have a relatively narrow host range, and so, the disease agent has to be isolated and a bacteriophage lysis test must be customized to the specific pathogenic bacteria (Huff *et al.* 2004).

From another perspective, the treatment of a disease with bacteriophage might benefit, if instead of one, a cocktail of phages effective against the most part of the bacteria that are known to cause the disease is used. From this point of view, it would be useful to develop a bacteriophage therapeutic product based on the best phage associations, increasing the antimicrobial range of the product (Slopek *et al.* 1987; Smith *et al.* 1987; Carlton 1999; Sulakvelidze *et al.* 2001; Goodridge and Abedon 2003; Huff *et al.* 2004). This was the underlying reason for testing the efficacy of several isolated *E. coli* phages against a pool of the isolated APEC strains resistant to the most common antibiotics. From the five phages isolated, two revealed broad lytic spectra, being phiF61E the most effective phage, lysing 48·0% of the bacterial strains. The association of phiF78E, phiF258E and phiF61E was effective for 70·5% of the strains. It is important to stress that with only three phages, a large range of APEC strains were covered, which is better than the most effective antibiotic, the ENR with 50·8% of efficacy (Fig. 3). A significant increment in the lysis efficiency combinations of four or five phages was not found beyond the efficacy observed with three phages (71·8% and 72·5% of lysed strains, respectively). In fact, an association of more than three phages would be even disadvantageous, because the economic recourses necessary to characterize and produce different phages would be higher. Based on this assumption, phiF78E, phiF258E and phiF61E were selected for further characterization.

The phages morphological characteristics observed by transmission electronic microscopy (TEM) revealed that phiF78E and phiF61E belong to *Myoviridae* taxonomic family and seem to be 16–19 type phages, roughly like T4. The same phage types have already been isolated from sewage and characterized morphologically by Ackermann *et al.* (1974), and later by Ackermann and Nguyen (1983). Similar to phiF78E and phiF61E, the two phages described by those authors showed contractile tails of 100×7 nm and 94×15 nm and elongated heads with 104×43 or 102×57 nm in diameter. The same authors described that in those phage types, heads resembled superficially those of T-even phages and appeared to be mostly oval. Tails of these phages were complex and consisted generally, of a neck, a base plate and tiny caudal fibres, similar to the phages characterized in this work. Those phages were so far described for *Salmonella*, so, to the authors knowledge, this was the first time that 16–19 phages are described as being effective against *E. coli* strains. phiF258E seemed to be a *Siphoviridae*, T1-like, already described for *E. coli* (Wietzorrek *et al.* 2006).

One of the major concerns in the use of phages for therapy purposes is to guarantee that the phages do not integrate into the DNA hosts. The morphological characteristics of these phages are similar to those described as lytic; nevertheless a mitomycin C stress inducement was performed to confirm that phage phiF78E is not temperate, because resistant colonies were recovered from the interior of the phage clear zone.

The three phages presented different structure and host range, and therefore are distinct. This was also corroborated by their different RFLP patterns.

In short, in this work three phages belonging to the *Myoviridae* and *Siphoviridae* families, isolated from poultry sewage, showed to be effective against 70·5% of the 148 isolated APECs, most of which were resistant to the majority of antibiotics tested. Morphological and genetic characterization of these phages suggests that they belong to different phage-types. Taking together these results suggest these three phages combined in a therapeutic cocktail would be a more efficacious therapy over conventional antibiotic therapy.

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