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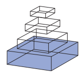

Implications of free Shiga toxin-converting bacteriophages occurring outside bacteria for the evolution and the detection of Shiga toxin-producing *Escherichia coli*

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In this review we highlight recent work that has increased our understanding of the distribution of Shiga toxin-converting phages that can be detected as free phage particles, independently of Shiga toxin-producing bacteria (STEC). Stx phages are a quite diverse group of temperate phages that can be found in their prophage state inserted within the STEC chromosome, but can also be found as phages released from the cell after activation of their lytic cycle. They have been detected in extraintestinal environments such as water polluted with feces from humans or animals, food samples or even in stool samples of healthy individuals. The high persistence of phages to several inactivation conditions makes them suitable candidates for the successful mobilization of *stx* genes, possibly resulting in the genes reaching a new bacterial genomic background by means of transduction, where ultimately they may be expressed, leading to Stx production. Besides the obvious fact that Stx phages circulating between bacteria can be, and probably are, involved in the emergence of new STEC strains, we review here other possible ways in which free Stx phages could interfere with the detection of STEC in a given sample by current laboratory methods and how to avoid such interference.

Keywords: bacteriophages, Shiga toxin, STEC, environment, transduction

Shiga toxin-producing *Escherichia coli* (STEC) are important food-borne pathogens and represent a challenge for the scientific community. Effort has been devoted to developing methods for its isolation, detection and identification, to guarantee the quality of products and the health of consumers. Many studies have aimed at gaining an understanding of the mobility of the important gallery of virulence factors that compose the virulome of the *E. coli* pathogenic strains. The mobility of these factors among strains is a key factor for the genotypic and phenotypic variability of this bacterium, and hinders the process of determining what should be considered a true pathogen and how to detect it effectively (Erickson and Doyle, 2007; Karch et al., 2012; Melton-Celsa et al., 2012).

Thus, the methods for *E. coli* detection have focused on those factors that are clearly related with virulence and, particularly, those that are closely related with high incidence and severity of human infections. Among these factors, the Shiga toxin (Stx) is considered one of the most significant and, if not the only one determining pathogenicity, the one that leads to the most undesirable complications of the infection, such as HUS (Tarr et al., 2005).

Stx is a good example of a mobile virulence factor, since the genes encoding for this toxin are located in the genome of temperate bacteriophages (Newland et al., 1985; Huang et al., 1987; O'Brien et al., 1989). Many phages simply multiply by infecting bacteria, killing the host by lysis and releasing new phages. Temperate phages, however, adopt a benign relationship with their hosts, called lysogeny, which allows an attachment of phage DNA to a bacterial chromosome. For Stx phages, once a phage integrates its genome within the bacterial genome, the bacteria acquires the *stx* gene and with it the capacity to express the toxin; thereby becoming a STEC. Temperate phages can revert from the lysogenic state by entering the lytic cycle, mostly due to environmental conditions or exogenous factors. The lytic pathway starts by generating multiple copies of its genome, causing an increase in the Stx produced by the cell during the process (Neely and Friedman, 1998; Wagner et al., 2001; Tyler et al., 2004). By the expression of the phage genome, new phage capsids are generated, assembled with the phage DNA and, once formed, phage particles are released from the cell by lysis. Once released, phage particles remain free outside the bacterial cell, and here is where our story starts.

The major question we address is: what happens to phages outside the cell and to what extent does their occurrence as free particles play a role in the evolution of new STEC strains?

Phages are rather simple particles and Stx phages are no exception. Their persistence outside the cell is guaranteed by their ability to circumvent natural and artificial inactivation processes (as reviewed in the next section), many of which would inactivate their bacterial host. It is the fact that the life cycle of phages switches to the lytic state that allows phages to be released once the cell has been threatened by factors that activate its SOS response (Muhldorfer et al., 1996; Kimmitt et al., 2000; Köhler et al., 2000; Yamamoto et al., 2003; Aertsen et al., 2005; Toshima et al., 2007; Pacheco and Sperandio, 2009).

Stx PHAGES IN FECALLY POLLUTED WATERS

Several reports have shown the occurrence of free Stx phages in water environments with fecal pollution or directly on homogenates of fecal samples (**Table 1**). Most studies have only focused on the detection of Stx phages in general or Stx2 phages in particular, and only a few have evaluated Stx1 phages (Dumke et al., 2006; Yan et al., 2011; Rooks et al., 2012). Those studies show a clear predominance of Stx2 phages over Stx1 phages and, despite the fact that there is still a lack of information on the abundance of Stx1 phages in the environment, the results correlate with reported data on the predominance of stx2 phages over Stx1 phages in lysates induced from environmental *E. coli* isolates (Muniesa et al., 2004a; Garcia-Aljaro et al., 2009; Yan et al., 2011).

Regardless of the type of toxin variant, the high occurrence of free Stx phages detected suggests that Stx phages could do more than provide intestinal bacteria with a new genetic character. Although a certain level of human fecal contamination is always observed in environments where free Stx phages have been detected (Muniesa and Jofre, 1998; Tanji et al., 2003; Muniesa et al., 2004b; Dumke et al., 2006; Imamovic et al., 2010; Rooks et al., 2010, 2012) (**Table 1**), there is no total correlation between Stx phage occurrence and fecal pollution, as shown by comparison with fecal indicators (Dumke et al., 2006; Imamovic et al., 2010). This lack of correlation suggests that the fecal origin is the main but not necessarily the sole source of Stx phages. Accordingly, the description of Stx2-positive extraintestinal *E. coli* strains (Wester et al., 2013) supports the suspicion that *stx* is not restricted to a fecal origin. Stx phages are not limited to waters containing human contamination and they have also been found to be highly prevalent when analyzing wastewater from animals (Imamovic et al., 2010; Yan et al., 2011). Water with lower levels of fecal pollution, such as river water, also shows the presence of Stx phages (Muniesa et al., 1999; Dumke et al., 2006).

Table 1 | Occurrence and abundance of free Stx bacteriophages in diverse environments.

Sample	Country	Detection of Stx phages			Detection method	References
		Stx1 phages	Stx2 phages	Abundance		
Human wastewater	Spain	–	Positive (I)	10 PFU.ml^{-1}	MPN+PCR	Muniesa and Jofre, 1998
Human wastewater	Germany, Austria, France Ireland South Africa New Zaeland	–	Positive Positive Positive Positive Positive Positive	$\geq$1 PFU.ml^{-1} $>$0.1PFU.ml^{-1} $\geq$10 PFU.ml^{-1} $\geq$10 PFU.ml^{-1} $\geq$10 PFU.ml^{-1} $\geq$10 PFU.ml^{-1}	PCR	Muniesa and Jofre, 2000
Human wastewater	Japan	–	Positive (I)	–	PCR	Tanji et al., 2003
Human wastewater	Spain	–	Positive (I)	4.24 $\log_{10}$ PFU.ml^{-1}	Plaque count+PCR	Muniesa et al., 2004b
Cattle wastewater	Spain	–	Positive (I)	4.45 $\log_{10}$ PFU.ml^{-1}	Plaque count+PCR	Muniesa et al., 2004b
Human wastewater	Germany	Positive (I)	Positive (I)	0.34 PFU.ml^{-1} stx_1 3.4 PFU.ml^{-1} stx_2	Plaque count PCR estimation	Dumke et al., 2006
Human treated waste water	Germany	Negative	Positive (I)	–	Plaque count+PCR	Dumke et al., 2006
River water	Germany	Positive (I)	Positive (I)	–	Plaque count+PCR	Dumke et al., 2006
Human wastewater	UK	Negative	Positive (I)	9.41 $\log_{10}$ PFU.ml^{-1}	Plaque count+PCR	Rooks et al., 2010
Human wastewater	UK	Negative	Positive	2.39 $\log_{10}$ GC.ml^{-1}	qPCR	Rooks et al., 2010
Human wastewater	Spain	–	Positive	1.37 $\log_{10}$ GC.ml^{-1}	qPCR	Imamovic et al., 2010
Cattle wastewater	Spain	–	Positive	2.77 $\log_{10}$ GC.ml^{-1}	qPCR	Imamovic et al., 2010
Pig wastewater	Spain	–	Positive	4.59 $\log_{10}$ GC.ml^{-1}	qPCR	Imamovic et al., 2010
Poultry wastewater	Spain	–	Positive	1.11 $\log_{10}$ GC.ml^{-1}	qPCR	Imamovic et al., 2010
Cattle feces	Spain	–	Positive	2.32 $\log_{10}$ GC.g^{-1}	qPCR	Imamovic et al., 2010
Beef	Spain	–	Positive (I)	4.10 $\log_{10}$ GC.g^{-1}	qPCR	Imamovic and Muniesa, 2011
Salad	Spain	–	Positive (I)	3.36 $\log_{10}$ GC.g^{-1}	qPCR	Imamovic and Muniesa, 2011
Swine feces	China	Positive (I)	Positive (I)	–	Plaque count+PCR	Yan et al., 2011
Wastewater	UK	–	Positive (I)	–	Lysogen isolation	Rooks et al., 2012
Human feces	Spain	–	Positive (I)	4.41 $\log_{10}$ GC.g^{-1}	qPCR	Martinez-Castillo et al., 2013

GC, gene copy; PFU, plaque forming unit; MPN, most probable number. (I) those studies reporting infectious Stx phages.

Stx PHAGES IN FECES

If fecally polluted waters present Stx phages, and assuming that Stx phages could have been generated in the intestinal gut, the question that remains to be answered is whether the phages are released into wastewaters by induction from STEC strains present in these environments, or whether free Stx phages are directly excreted through feces. Recent reports indicate that free infectious (those able to infect and propagate in a host strain), Stx2 phages are present in 62% of human feces (Martinez-Castillo et al., 2013). Sequencing of the PCR amplimers suggested that subtypes stx_{2a}, stx_{2c}, and stx_{2d} were the most frequently detected (Martinez-Castillo et al., 2013). From the positive samples, 90% showed infectious Stx phages. Two facts are to be highlighted from that study: there is not a high incidence of STEC in the area of this study; and the stool samples were taken from healthy individuals without reported gastrointestinal symptoms, and that STEC were not detected in or isolated from these samples.

Stx PHAGES IN FOOD

Assuming that Stx phages are excreted in feces, they must have been ingested, either as free phages or as STEC bacteria that were later induced, once in the intestinal tract. If they are ingested as free phages, then they must be present in food or drinking water, assuming that these are free of significant levels of bacteria. The occurrence of infectious Stx2 phages in commercial samples of beef and salad (Imamovic and Muniesa, 2011), that were acceptable for process hygiene microbiological criteria under EU regulation (Anonymous, 2005), highlights the gap that exists in legislation regarding the presence of phages in food samples.

The fact that phages detected in food were infectious is interesting, considering the likelihood of transduction in food matrices at different pH and temperatures (Imamovic et al., 2009) as well as under dairy processing conditions (Picozzi et al., 2012). This fact is of special relevance considering the observations that many food-processing conditions, such as thermal treatment, high hydrostatic pressures, or UV or other irradiation (Yamamoto et al., 2003; Aertsen et al., 2005; Yue et al., 2012), and the addition of certain compounds during the food production, such as chelating agents (Imamovic and Muniesa, 2011), salt (Harris et al., 2012) or antimicrobials (Kimmitt et al., 2000), not only fail to inactive of the Stx phages, but can enhance Stx phage induction from their STEC hosts.

PERSISTENCE OF Stx PHAGES

Generally speaking, phages could persist in different environments or under disinfection processes or inactivation conditions (Dee and Fogleman, 1992; Durán et al., 2002; Jofre, 2007; Lee and Sobsey, 2011). In habitats in which the host bacteria are alien, as fecal bacteria are in the environment, it is likely that phages persist much better than the bacteria (Ogunseitan et al., 1990; Muniesa et al., 1999; Durán et al., 2002). Because of their structural characteristics, their persistence in the environment is high, and these survival capabilities make bacteriophages especially suited for movement and gene transfer between different biomes.

Certain morphologies can be considered more persistent than others, tailed phages being the ones showing higher persistence (Muniesa et al., 1999; Prigent et al., 2005; Lin et al., 2010). Notably, these are the most abundant in different water environments (Prigent et al., 2005; Lin et al., 2010). Stx phages mostly belong to the *Siphoviridae* and *Podoviridae* morphological types (Rietra et al., 1989; Muniesa et al., 2004a,b; Beutin et al., 2012), showing similar persistence to that of other groups of phages (Muniesa et al., 1999; Dumke et al., 2006; Allué-Guardia et al., 2014) and higher persistence than STEC (Muniesa et al., 1999; Mauro and Koudelka, 2011; Rode et al., 2011; Allué-Guardia et al., 2014). Stx phages have been shown to be very stable under food-processing conditions (Yamamoto et al., 2003; Aertsen et al., 2005; Imamovic et al., 2009; Rode et al., 2011; Harris et al., 2012; Yue et al., 2012; Langsrud et al., 2013; Allué-Guardia et al., 2014). In contrast, they do not seem to persist so well under strict treatments such as that linked to the compost model (Johannessen et al., 2005). Nevertheless, their persistence, as for other phages, enhances their mobility between the different environments (feces-food-water) (Mauro and Koudeljka).

Another question is whether the STEC hosts could survive the time necessary to allow phage release before being killed by the treatment applied, or if phages, once released, could remain infectious. Infectivity of Stx phages has been demonstrated after exposure to certain conditions, and some Stx phages remain able to generate lysogens, hence to transduce *stx* (Muniesa and Jofre, 1998; Imamovic et al., 2009; Rode et al., 2011; Yue et al., 2012). The capacity to remain infectious after being subjected to a given condition will determine the chances of *stx* transduction, the real threat in the generation of new STEC strains.

INTERFERENCE OF Stx PHAGES IN STEC DETECTION

The identification of STEC by culture methods in food or clinical samples is advisable to confirm the presence of the pathogen and to further characterize it. However, sometimes strain recovery is not possible because of the low number of STEC cells in a specific sample, the fact that cells could be stressed or in a non-culturable state, or because of the interference of accompanying microbiota, particularly other *E. coli* strains that could confound the detection of the pathogen even when using a specific culture medium. Culture detection of STEC is, in addition, a time-consuming method that hinders early diagnosis of STEC.

Early diagnosis of STEC infection in humans is nevertheless critical for the treatment of the disease, particularly because of the contraindication for treating STEC using antimicrobial agents, and the intense supportive care needed if renal failure occurs (Wong et al., 2000). The need for fast detection and the associated difficulties of STEC isolation from stools in patients treated with antibiotics makes the use of fast and robust molecular methods advisable for STEC detection.

Current laboratory methods for STEC detection, some standardized and approved by national legislation, include PCR-based techniques: either end-point or real-time PCR (Paton and Paton, 2003; Perelle et al., 2004; Gould et al., 2009; Kagkli et al., 2011; Bibbal et al., 2014). Recently, ISO 13136:2012 (Anonymous, 2012), which uses real-time PCR as the reference technology for

the detection of the virulence and serogroup-associated genes, has been included in the amended EU regulation for microbiological criteria for sprouts and the sampling rules for poultry carcasses and fresh poultry meat (European commission regulation No. 209/2013). With the same aim in mind, molecular techniques, many based on the information obtained by the use of next-generation sequencing (NGS) methods, are being explored and advised (Baquero and Tobes, 2013; Underwood et al., 2013).

As indicated above, the genomic plasticity of STEC represents a challenge for discriminating pathogenic strains from other non-pathogenic *E. coli* strains present in a given sample. Thus, PCR-based molecular methods are mainly focused on genes related with virulence and on genes that identify a specific serotype, such as *rfb* genes encoding different O antigens (Maurer et al., 1999). Identification of the serotype could be useful for epidemiological purposes and, generally, virulent strains belong to one of the serogroups that account for the non-O157 serotype, including O26, O45, O103, O111, O121, and O145, plus O157:H7 (USDA FSIS, 2012). Lessons learnt from the last outbreak in Germany (Karch et al., 2012) forced researchers to include O104:H4 on the list, and showed that identification of serotype, albeit very useful when treating known pathotypes or for epidemiological purposes, is of limited value for dealing with a newly emerging strain. If STEC isolation is not possible, identifying virulence genes, alone or preferably in combination, appears to be the best approach for assessing the presence of STEC in a sample.

However, when dealing with molecular methods applied to a sample, the presence of free Stx phages, and also other phages that could harbor virulence genes, represents a challenge for the use of PCR-based and NGS methodologies. Moreover, when the protocols include a previous step of selective enrichment of the target bacteria, this step could also maintain or even propagate bacteriophages. This is even more significant if we assume that the methods used for bacterial DNA extraction in these complex matrices will also extract phage DNA (Paton and Paton, 2003; Monday et al., 2007; Grys et al., 2009; ISO 13136:2012). Therefore, any virulence genes present in phage DNA, notably *stx* genes, but also other virulence genes reported in prophages (e.g., *cdt*, *cif*, etc.) (Asakura et al., 2007; Loukiadis et al., 2008) would generate amplimers (PCR) or reads (NGS) that will be interpreted as belonging to STEC, while it could be that they originated in phages. Since in STEC these genes belong to prophages, and therefore are flanked by phage sequences, there is no easy way to distinguish whether the target detected is a bacteria or a phage. If it is a phage, the threat of virulence in humans must be discussed, but it obviously has reduced potential for virulence and would not be enough to raise the alarm.

Table 2 | Frequency of STEC isolation by various methods in samples showing positive result for *stx* (either stx_1 or stx_2) by PCR methods.

	No of *stx*-positive samples by PCR	No of *stx*-positive samples by PCR with STEC isolation	Percentage of PCR *stx*-positive samples with STEC isolation (%)	References
Human stools (healthy slaughterhouse workers)	90	8	8.9	Hong et al., 2009
Humans stools (asymptomatic)	196	47	24	Stephan et al., 2000
Human stools (volunteers)	21	1	4.8	Urdahl et al., 2012
Human stools (hospital)	150	1	0.67	Urdahl et al., 2012
Human stools (hospital)	20*	10*	50	Buchan et al., 2013
Children stools (hospital)	21	5	24	Vallières et al., 2013
Children stools (hospital)	19	10	52.6	Pradel et al., 2000
Cattle feces	145	80	55.2	Fremaux et al., 2006
Cattle feces	154	67	43.5	Rogerie et al., 2001
Cattle feces	417	18	4.3	Hofer et al., 2012
Cattle feces	330	162	49.0	Pradel et al., 2000
Bovine hides	301	25	8.3	Monaghan et al., 2012
Bovine carcasses	122	5	4.1	Monaghan et al., 2012
Bovine carcasses	77	16	20.8	Breum and Boel, 2010
Bovine carcasses	91	16	17.6	Rogerie et al., 2001
Cattle environment	179	38	21.2	Fremaux et al., 2006
Beef meat	47	16	34.0	Pradel et al., 2000
Dairy buffalo (feces and milk)	56	20	35.7	Beraldo et al., 2014
Milk (bulk)	32	1	3.1	Trevisani et al., 2014
Milk (filters)	68	7	10.3	Trevisani et al., 2014
Cheese	60	5	8.3	Pradel et al., 2000
Healthy pigs feces	255	62	24.3	Meng et al., 2014
Swine feces	484	196	40.5	Fratamico et al., 2004
Meat products	36	8	22.2	Díaz-Sánchez et al., 2012

Calculated from the % of positive samples.

In addition to those well-characterized phages encoding virulence genes in STEC, many lytic phages could mobilize bacterial genes by generalized transduction. In transduction, after random packaging of bacterial DNA fragments, the genetic material can be mobilized by a phage from a donor bacterium and inserted into a recipient bacterium when it becomes infected by the phage particle. Despite a lack of clear evidence of generalized transduction relating to STEC, any bacterial gene can be mobilized via generalized transduction, including chromosome fragments, though plasmids, transposons and insertion elements, and examples in enterobacteria can also be found (Mann and Slauch, 1997; Schmieger and Schicklmaier, 1999).

Generalized transduction is considered a rare event (Bushman, 2002); however, many approaches for STEC detection based on genomic techniques envisage preliminary steps for the selective or non-selective enrichment of the target microorganism (Hussein and Bollinger, 2008). Enrichment to propagate bacteria can cause the propagation of any sort of phages present in a sample too, a fact that, firstly, would cause a bias in the population of enriched bacteria (Muniesa et al., 2005), and secondly, by increasing the number of phages, would also theoretically cause an increase in the frequency of generalized transduction. As an example to illustrate this point, the protocols for P1 generalized transduction include a propagation step between the donor and the phage to increase the number of generalized transducing particles (Thomason et al., 2007).

Since Shiga toxin is the main virulence threat in STEC, there are many examples of *stx* positive PCR results from samples showing negative detection of culturable STEC. The percentage of samples showing STEC isolates among PCR *stx*-positive samples (either stools of food samples) is in many reports no higher than 50% (**Table 2**). In clinical stools samples this could be attributable to previous treatment with antimicrobial agents or to a disease being diagnosed late in its course. In food samples it could also be attributable to the presence of non-culturable microorganisms or the presence of bacteria in very low numbers. However, considering the presence of phages as described above, their role in these positive PCR detections or their involvement in sequences generated by NGS cannot be excluded.

AVOIDING PHAGE INTERFERENCE

The issue that arises is how to avoid phage interference. In theory, several methods could be applied to eliminate phages from the equation. These are mostly based on dismissing the phage population from the sample without interfering with the bacterial population and avoiding complicated steps in the methodology. Some protocols use a centrifugation step after the enrichment culture, and only the pellet containing bacteria is used for DNA extraction (Feng et al., 2011). This method would reduce the number of phages present in the supernatant of the enrichment; though to what extent, has not been tested. Still, phages present in the original samples would have propagated and would still be present in the bacterial pellet.

Filtration has also been proposed to separate phages from the sample and has been widely used to purify phage stocks or phages from a sample before counting (Brock, 1983; EPA, 2000). Microfiltration is mostly applied to retain viruses using the filter and clarify the sample (Van Reis and Zydney, 2001; Saxena et al., 2008), while here the aim is to eliminate phages by passing them through the filter but retaining the bacteria. The selection of the most efficient membrane, in terms of pore size and chemical structure, would be critical with this purpose in mind. The use of 0.22 or 0.45 μm low protein binding membranes that do not retain phages would be advisable. Among these, polyvinylidene fluoride (PVDF), polyethersulfone (PES) or cellulose ester membranes saturated with beef extract have been proposed (Anonymous, 2000; EPA, 2000; Papageorgiou et al., 2000; Mocé-Llivina et al., 2003), and PVDF membranes have been shown to reduce by 2–3 $\log_{10}$ the phages present in a bacterial enrichment culture (Muniesa et al., 2005). Complete elimination of all phages present in the samples will probably not be accomplished only by adding a single filtration step, but optimization of this approach would reduce the phage population enough to avoid interference in the molecular detection methods without adding complicated steps to the already-established protocols and without reducing the bacterial population. However, it must be borne in mind that filtration would turn out to be more useful when treating aqueous samples or clear homogenates of solid samples and with a variable efficiency depending on the complexity of the matrix, with lower recoveries expected when it is applied to solid or mixed samples that could clog the membranes.

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