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"Aislamiento y caracterización de un bacteriófago que infecta aislados clínicos de *Escherichia coli* enterotoxigénica por medio de la proteína de membrana externa TolC"

Tesis que presenta

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A mi familia,

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INDEX

1.- RESUMEN

Escherichia coli enterotoxigénica (ETEC) es el principal agente causal de la diarrea del viajero, enfermedad que afecta a individuos de países industrializados que visitan países en desarrollo en América latina, África y Asia. Además, se estima que anualmente ETEC es responsable de 400,000 muertes de niños menores de cinco años. ETEC produce las enterotoxinas ST y LT, las cuales se unen al enterocito y producen una diarrea secretoria. La secreción de la toxina ST requiere de la proteína de membrana externa TolC. Se ha mostrado que la deleción de TolC en ETEC impide la secreción de ST. El objetivo de este trabajo fue emplear un sistema para aislar bacteriófagos capaces de infectar aislados clínicos de ETEC utilizando TolC como receptor. 41 cepas obtenidas de niños mexicanos fueron caracterizadas por medio de PCR para toxinas ST, LT y factores de colonización CFA/I, CFA/II y CFA/IV. A partir de éstas se construyeron 15 mutantes de TolC por medio de transducción con el fago P1. Para el aislamiento de fagos se utilizaron muestras de agua de la ciudad de México y College Station, TX. El fago Stilgar, aislado de una muestra de agua obtenida en College Station, fue seleccionado por su habilidad de infectar aislados clínicos de ETEC pero no su mutante en TolC, ya que la ausencia del receptor impide la adsorción del fago. Stilgar es un myovirus con una cápside icosaédrica de 50nm y una cola de 130nm de largo. Stilgar tiene un genoma de 41,047pb con 56 secuencias codificantes y presenta homología con los myovirus EcoM-ep3 y PPpW3, utilizados para el control de cepas patógenas de *E. coli* en aves y *Pseudomonas plecoglossicida* en peces, respectivamente. Stilgar fue capaz de infectar 9 de los 41 aislados clínicos de ETEC. Análisis de PCR confirmó que después de ser infectadas por Stilgar, las bacterias resistentes tienen alteraciones en el locus de t*olC.* El uso de bacteriófagos que utilicen factores de patogenicidad como receptor representa una alternativa para el tratamiento de infecciones bacterianas, ya que va dirigido a blancos exclusivos de cepas patógenas. Esto adquiere gran relevancia en el tratamiento de ETEC, ya que no afecta a las cepas comensales.

2.- ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is the predominant cause of traveler's diarrhea, the most common illness affecting travelers visiting developing countries in Latin America, Africa and Asia. Moreover, ETEC is estimated to be responsible of 400,000 deaths of children under the age of five. ETEC produces ST and LT enterotoxins which must be secreted in order to attack the enterocytes, causing a secretory diarrhea. ST secretion relies on the outer membrane protein TolC. It has been shown that deletions in TolC block the ability of ETEC to secrete ST toxin. The aim of this project was to implement a system to isolate bacteriophages capable of infecting clinical isolates of ETEC using TolC as a receptor. We gathered a panel of 41 strains isolated from Mexican children and performed a PCR characterization for toxin ST, LT, and colonization factors CFA/I, CFA/II and CFA/IV. From these, 15 TolC mutants were constructed by transduction with phage P1. Sewage samples Mexico City and College Station, TX were used for the isolation of bacteriophages. Phage Stilgar, isolated from a water sample obtained in College Station, was selected on its ability to infect ETEC but not its TolC mutant because absence of the receptor impairs the ability of phage to adsorb an initiate infection. Stilgar is a myophage with an icosahedral capsid of 50nm and a 130nm-long tail. Genomic phage DNA characterization reveals that Stilgar has a 41,047bp genome with 56 predicted coding sequences and has presents homology with myophages EcoM-ep3 and PPpW3, phages use for the control of pathogenic strains of *E. coli* in chickens and *Pseudomonas plecoglossicida* in fish. Stilgar was able to infect 9 out of 41 ETEC clinical isolates. PCR analysis confirmed that, after being infected by Stilgar, phage-resistant bacteria had alterations in the *tolC* locus. The use of bacteriophages that use virulence factors as receptors represent an alternative to the treatment of bacterial infections, since it is aimed at targets present only in pathogenic strains. This is relevant in the treatment of ETEC, since it does not affect commensal strains.

3. INTRODUCTION

3.1 Pathogenic Escherichia coli

Escherichia coli is a gram-negative, rod shaped, facultative anaerobe, mostly motile, non-spore forming bacillus that belongs to the relatively homogeneous group of Gammaproteobacteria, family *Enterobactericeae,* genus *Escherichia* (Bettelheim 1994; Madigan et al., 2006). *E. coli* is defined as the type species of its genus, and one of the most extensively studied organisms (Lerner and Lerner, 2003). In humans, *E. coli* colonizes the gastrointestinal track within hours after birth and remains harmlessly confined to the intestinal lumen of the host. This relation derives in a mutual benefit since *E. coli* participates in food digestion and production of vitamin K (Drasar and Hill, 1974; Bentley and Meganathan, 1982).

Commensal strains of *E. coli* constitute normal part of colonic flora and rarely cause an infection, except in a debilitated or immunosuppressed host (Nataro and Kaper, 1998). However, there are several *E. coli* strains that have acquired specific elements that provide them with the ability to colonize or intoxicate the human host, causing a broad spectrum of diseases (Brussow et. al., 2004; Nataro and Kaper 2004). Those elements that separate commensal strains from pathogenic strains are termed virulence factors. These are molecular determinants responsible for the versatility of bacteria as a pathogen. They are present in the *E. coli* genome in the form of virulence-related mobile elements (bacteriophages, transposons and plasmids) or chromosomally-encoded clusters of virulence traits, known as pathogenicity islands (Gyles and Boerlin, 2014). In this way, different combination of genes are acquired, and the most successful ones are maintained and further developed, giving rise to specific *E. coli* pathotypes: strains able to cause illness in human using a common set or virulence factors (Narato and Kaper, 2004).

3.1.1 Diarrheagenic *E. coli*

Infections due to pathogenic *E. coli* strains are grouped in three clinical syndromes: urinary tract infections, sepsis/meningitis and enteric/diarrheal disease (Nataro and Kaper, 1998). Of the latter, there are six well defined categories of diarrheagenic *E. coli* pathotypes (DEPs) that infect humans using different strategies: Enterotoxigenic *E. coli* (ETEC) colonizes the small bowel through colonization factors and then produces enterotoxins to induce watery diarrhea (Echeverria et al., 1986). Enteropathogenic *E. coli* (EPEC) intimately attaches to small bowel enterocytes causing cytoeskeletal arrangements, inflammatory response and diarrhea (McDaniel et. al., 1995). Enteroinvasive *E. coli* (EIEC) penetrates colonic epithelial cells, lyses the endocityc vacuole, multiplies and and moves laterally through the epithelium into adjacent cells (Nataro and Kaper, 1998; Sansonetti, 2002). Enterohaemorrhagic *E. coli* (EHEC) produces Shiga toxin (Stx). Stx travels through the bloodstream to the kidney, where it damages renal endothelial cells through toxicity and cytokine production, resulting in renal inflammation that can lead to diarrhea, bloody diarrhea and hemolytic uremic syndrome (Nakao and Takeda, 2000; Nataro and Kaper 2004). Enteroaggregative *E. coli* (EAEC) colonizes intestinal mucosa, forms a thick biofilm and then produces cytotoxins and enterotoxins (Nataro and Steiner, 1998). Lastly, diffusely adherent *E. coli* (DAEC) binds to the small bowel enterocytes using a fimbrial adhesin and then triggers a signal transduction effect that produces the growth of cellular projections that wrap around the bacteria (Bernet-Carmard *et. al*., 1996; Nataro and Kaper, 1998). The schematized strategies are illustrated in figure 1.

All categories of diarrheagenic *E. coli* carry at least one virulence related property and they follow a common basic strategy for infection: (1) adherence to host cells, (2) multiplication, (3) evasion of host defenses and (4) host damage (Whittam, 2000; Mims, 2001). Most of the virulence factors that participate in this multi-stage process are features of the bacterial surface or

metabolic pathways; e.g. fimbrial antigens for attachment to host cells (Levine *et. al*., 1984), pili or flagella for mobility (Mattick, 2002; Nataro and Kaper, 1998), extracellular carbohydrates and proteins for evasion of host immune response (Finlay and McFadden, 2006), transporters for nutrient uptake (Perna *et. al.*, 2001), toxins and their secretion systems (Sears and Kaper, 1996).

Human infections with DEPs occur through consumption of contaminated food and water (Berger et. al., 2010). They are associated with travelers' diarrhea, a mild and self-limiting infection that affects people who travel to developing countries where these strains are endemic (Clements et al., 2012; DuPont, 2009). However, DEPs are identified as one of the leading causes of bacteria-induced diarrhea in the developing countries (Nataro and Kaper, 1998). This represents a serious health problem, since diarrheal diseases are the second cause of mortality among children under the age of five worldwide (Levine et. al., 2012). Among DEPs, ETEC stands as the main cause of *E. coli*-mediated diarrhea (Gaastra and Svennerholm, 1996), and it is reported as one of the most commonly isolated enteropathogens responsible for childhood diarrhea (Kotloff et. al., 2013) and travellers' diarrhea (DuPont, 2009).

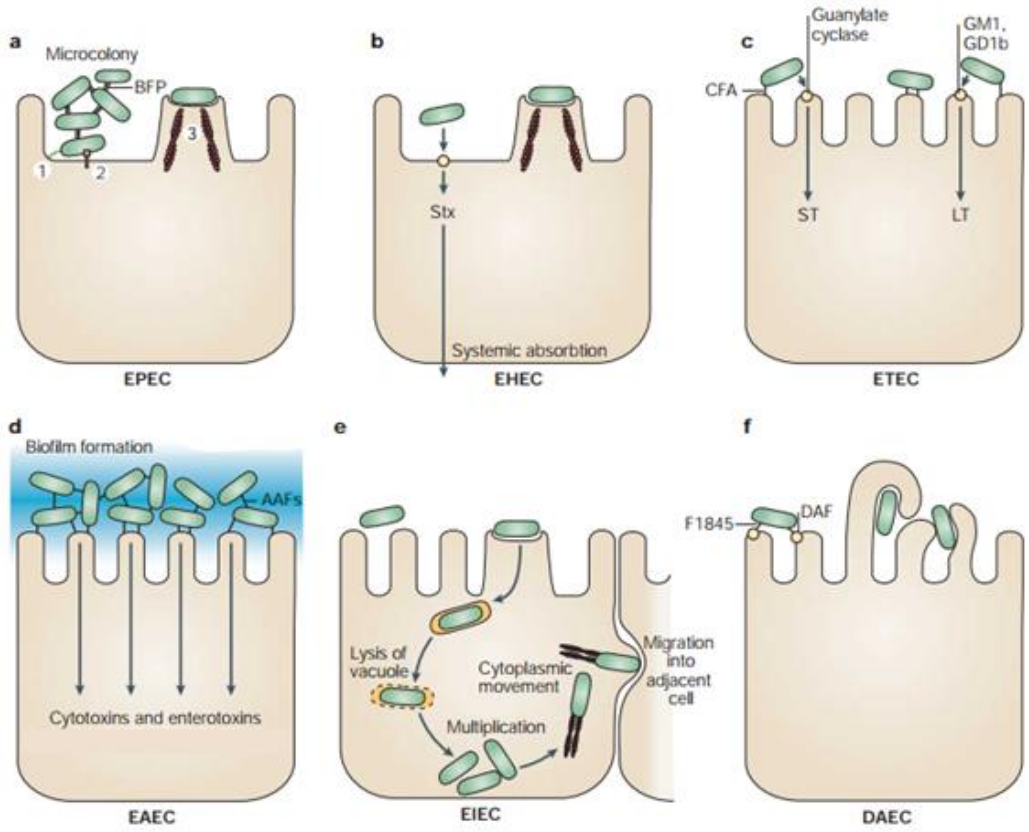


Figure 1. - Pathogenic categories of diarrheagenic *E. coli*. Interaction of each category with target cell is schematically represented. a) EPEC b) EHEC c) ETEC d) EAEC e) EIEC f) DAEC. Abbreviations: AAF: aggregative adherence fimbriae; BFP, bundle forming pilus; CFA, colonization factor antigen; DAF, decay accelerating factor; LT, heat-labile enterotoxin; ST, heat-labile enterotoxin; Stx, Shiga toxin. (Nataro, 2004)

3.2.1 Enterotoxigenic *E. coli*

Pathotype ETEC is defined as a group of *E. coli* strains that produces at least one of two types of plasmid-encoded enterotoxins: Heat-labile (LT) and Heat-stable (ST) (Levine, 1987). The identification of ETEC as a cause of diarrhea and its mechanism of action was first recognized in pigs, calves and rabbits (Smith and Halls, 1967; Gyles and Barnum, 1969), and later on it was demonstrated that ETEC strains where also able to cause diarrhea in humans (DuPont et. al., 1971). ETEC's major virulence factors are colonization factors (CFs) and enterotoxin production (Yamamoto and Yokota, 1983; Echeverria et al., 1986). To initiate infection, they use CFs to attach and colonize the surface of the

small bowel mucosa (Levine *et. al*., 1984). After that, ETEC secretes ST and/or LT, or both enterotoxins to induce secretory diarrhea (Nataro and Kaper, 1998). To have a better understanding of their role and importance in the process of ETEC's pathogenesis, we will discuss this virulence factors in detail.

3.2.2 Colonization Factors

CFs is a term that encompasses a large group of adhesin molecules used, as its name suggests, to colonize host tissue. In ETEC, CFs are acquired through virulence-related plasmids, which can also encode for ST and/or LT enterotoxins (deGraaf and Gaastra, 1994). They are proteinaceous appendages located in the surface of the bacteria that allows them to attach to small bowel enterocytes (Cassels and Wolf, 1995). To date, more than 20 CFs have been identified and characterized among human ETEC strains. The morphology of CFs is mainly fimbrial, fibrillar, helical, or nonfimbrial (Gaastra and Svennerholm, 1996). CFs play a major role in host specificity and infection. Human ETEC strains have sets of CFs that differ from other organisms, however, both in human and animal models, loss of this trait results in bacteria unable to colonize and cause diarrhea (Evans et. al., 1978; Svennerholm et. al., 1990; deGraaf & Gaastra, 1994).

Nomenclature for CFs can be cumbersome since they were first designated as colonization factor antigen (CFA), or putative colonization factor (PCF) when their role in colonization had not been determined (Cassels and Wolf, 1995). Currently, CFs are designated as coli surface antigen, represented by the initials CS and followed by a number according to their chronological order of identification, with CFA/I being the exception (Gaastra and Svennerholm, 1996). CFA/I was the first CF described from a human ETEC. It is a fimbrial rod of 7nm of diameter and a molecular weight of 15 kDa (Evans et. al, 1975; Evans et. al., 1979). CFA/II was the second CF described, and it was later found out to be a group composed of CS3 (flexible fibrillae with a diameter of 2-3nm and a MW of 15.1 kDa) alone or combined with CS1

(fimbrial rod with a diameter of 7nm and a MW of 16.5 kDa) or CS2 (fimbrial rod with of 7nm of diameter with a MW of 15.3 kDa) (Cassels et. al., 1993; Perez-Casal et. al., 1990; Gaastra and Svennerholm, 1996). The group CFA/IV is composed of CS6 (nonfimbrial adhesin with a MW of 14.5/16 kDa) alone or in combination with CS4 (fimbriae with a diameter of 6-7nm and a MW of 17 kDa) or CS5 (flexible fimbriae with a diameter of 5-6nm and a MW of 21 kDa) (Knutton et. al, 1989; Clark et. al., 1992). Overall, CFA/I, CFA/II and CFA/IV are the most prevalent and intensively studied CFs worldwide, present in almost 75% of the ETEC clinical isolates (Gaastra and Svennerholm, 1996; Wolf, 1997). However, it is important to note that not all ETEC strains have detectable CFs, either because it has not been identified yet, lost on a subculture, or the lack of proper detection tools (Quadri et. al., 2005; Turner et. al., 2006; Levine et al., 1984).

Studies on the genetics of CFs have served to establish two major models for the synthesis, assembly and expression of these adhesins: The chaperone-usher-dependent pathway and Type IV pilus assembly. The first depends on a four-gene cluster consisting of an outer membrane usher protein, a periplasmic chaperone, and major and minor fimbrial subunits (Ananta et. al., 2004). The minor subunit stands at the tip and the pilus grows with the addition of several molecules of the major subunit (Voegele et. at., 1997). This system has been described in CFA/I, CS1, CS3, CS5, and CS6 (Kusters and Gaastra, 1994; Nataro & Kaper, 1998). Type IV pilus assembly is a fourteen-gene operon consisting of a major and minor pilin units, prepilin peptidase, and accessory genes that play a major part in secretion and polymerization of the pilus (Pugsley, 1993). This system is common in CFA/III and Longus (Peabody et. al., 2003).

Once expressed and located on the cell surface, CFs are ready to interact with their receptors and attach to the host cells. Due to the high diversity of CFs, a lot of receptors remain uncharacterized, but they are known

to be glycoconjugates of the cell membrane (Cassels and Wolf, 1995; Turner et. al., 2006). Having stablished contact, ETEC elaborates and secretes enterotoxins for the induction of watery diarrhea (Nataro and Kaper, 1998).

3.2.3 Heath-labile enterotoxin

LT is an oligomeric toxin of 84 kDa of molecular mass. It is closely related to cholera enterotoxin (CT) of *Vibrio cholerae*, sharing over 80% of protein identity, primary receptor and enzymatic activity (Spangler, 1992; Sixma et. al., 1993; Nataro and Kaper, 1998). LT can be divided in two groups that do not cross react immunologically and have some differences in their sequence: LT-I and LT-II (Spangler, 1992). LT-II shares less similarity with CT and is found mainly on animal isolated ETEC strains, although it is not associated with disease. LT-I is predominantly present in human isolates and it is pathogenic for both humans and animals (Fukuta et. al., 1988; Nataro and Kaper, 1998).

LT-I (or LT) consist of 6 subunits: an A subunit of 28 kDa, and a pentameric ring of five identical B subunits of 11.6 kDa (Streatfield et. al., 1992). The A subunit is responsible for the enzymatic activity and possess two structural domains: A1 with ADP ribosylase activity and NAD glycohydrolase activity, and A2 domain that acts as a link with B subunits in order to form an AB_5 complex (Spangler 1992; Kaper et. al., 2004). Both A and B subunits are produced with N-terminal signal peptide targeted for translocation across the inner membrane via Sec-dependent translocon (Hirst and Holmgren 1987; Pugsley, 1993). Once in the periplasm, they fold into the mature holotoxin in a process dependent of the enzyme DsbA (Yu et al., 1992) and then the toxin is secreted across the outer membrane by a Type-II secretion apparatus recently identified on ETEC strains (Tauscheck et al., 2002).

After secretion, B subunits mediates the binding of the toxin to the cell surface gangliosides GM1 and GD1B, and the subsequently entry of the A subunit to the cell (Teneberg et at., 1994). The A subunit must be proteolytically cleaved in order to separate it from B domain (Lencer et al.,

1997). Once inside the cell, the ADP ribosylase activity of the A1 subunit transfers an ADP-ribosyl moiety to the α-subunit of the stimulatory G protein, leading to a permanent activation of adenylate cyclase complex, which in turns leads to increased levels of cAMP, activation of cAMP-dependent kinases and phosphorylation of the main chloride channels of epithelial cells, the cystic fibrosis transmembrane conductance regulator (CFTR). The net result of this signaling cascade is the secretion of Cl^- and reduced adsorption of Na^+ (Gill and Richardson, 1980; Sears and Kaper, 1996; Nataro and Kaper, 1998).

3.2.4 Heat-stable enterotoxin

ST is a small, plasmid-encoded single-peptide toxin of 2 kDa, which contains 18 or 19 amino acid residues on its mature form (Nataro and Kaper, 1998; So and McCarthy, 1980). ST can be subdivided in two classes that differ in structure and mechanism of action: methanol-insoluble, protease sensitive STb or methanol-soluble and protease resistant STa (Peterson and Whipp, 1995), being the latter associated with human disease (Nataro and Kaper, 1998). Human isolated ETEC strains present two variants of STa: STp (porcine) and STh (human), according to the organism where the strains where originally isolated (Nair and Takeda, 1998). Both types of toxin are synthesized as 72 amino acid precursor consisting of a pre region (residues 1-19), pro region (residues 20 to 54) and a mature region (residues 55 to 72) (Rasheed et al., 1990). The pre region functions as a signal peptide for translocation of the ST precursor to the inner membrane via Sec proteins. The pre region is cleaved during the process and the resulting peptide is released into the periplasm (Okamoto and Takahara, 1990). Subsequently, DsbA protein catalyses the formation of three intramolecular disulfide bridges in the mature region of the peptide, a process that confers heat stability and it is also crucial to toxin activity (Yamanaka et al., 1994). In an undefined proteolytic event, the pro region is removed before the mature toxin is exported through the outer membrane using the TolC protein transporter (Yamanaka et al., 1998).

Guanylate cyclase C (GC-C), a protein found in the apical membrane of intestinal epithelial cells, is the main receptor of STa (de Sauvage et al., 1992). Binding of STa promotes a conformational change in GC-C that activates its guanylate cyclase domain, leading to increased concentrations of intracellular cGMP, activation of GMP-dependent protein kinases, and ultimately, stimulation of the CFTR chloride channel (Hirayama et al., 1989; Field et al., 1978; Nair and Takeda, 1998). The net result of this signaling cascade is the inhibition of Na^+ absorption and stimulation of Cl^- secretion (Sears and Kaper, 1996).

3.2.5 Epidemiology

The primary vehicle of ETEC is food or water contaminated with human or animal feces. Thus, ETEC is a severe pathogen in places where hygiene conditions are poor and sanitation systems are deficient (Black et al., 1982). Studies have shown that ETEC contamination is endemic and extremely prevalent in developing countries, with children under the age of two being the more susceptible group (Flores-Abuxapqui et al., 1994; Albert et al., 1995; Quadri et al., 2000). The age at which the first ETEC infection occurs, and the characteristics of the strains vary among different countries (Cravioto et al., 1988; Steinsland et al., 2002; Rao et al., 2003; Estrada-Garcia et al., 2009). On the other hand, adults and children over the age of 5 rarely present an ETEC infection (Nataro and Kaper, 1998).

A few factors are important to understand this epidemiological pattern of ETEC: (i) the infection requires an infectious dose of at least 10^8 CFU, (ii) exposed individuals develop immunity to ETEC infections, and (iii) there are some immune asymptomatic individuals despite having high numbers of ETEC in their stool (DuPont et al., 1971). In this scenario, infants in developing countries will be exposed to ETEC upon weaning (Nataro and Kaper, 1998), and repeated infections in early life may help develop immunity, since the incidence of infection decreases after 5 years of age and mature individuals

are less likely to present a symptomatic ETEC infection. In fact, there is lack of epidemiologic information regarding adults living in the same areas (Stoll et al., 1982; Nataro and Kaper 1998; Quadri et al., 2005). Nonetheless, ETEC does represent a threat to the adult population, being the main etiological agent responsible for traveler's diarrhea, an infection contracted by immunologically naive adults that travel to developing countries in Latin America, Africa and Asia, where ETEC contamination is endemic (DuPont, 2009).

3.2.6 Clinical considerations and treatment.

The Incubation period of ETEC-caused diarrhea goes from 14 to 50 hours (DuPont et al., 1971). Loss of water and electrolytes from the stool causes dehydration which can range from mild to severe (Black et al., 1981). This can lead to dry mouth, decreased blood pressure, cramps, and in severe cases, shock (Quadri et al., 2005). Fever and vomiting may not be present in all of the cases (Levine, 1987). Diarrhea from ETEC can be a mild and self-limiting disorder that last from 3 to 4 days, usually without sequelae (Nataro and kaper, 1998).

Even though ETEC is reported to cause an estimate of 400'000 deaths per year in children under the age of five (WHO, 1999), and the main etiological agent out of 10 million cases of traveler's diarrhea per year, there is still a lack of proper treatment. The most effective way to treat ETEC diarrhea is either oral or intravenous rehydration. In fact, maintaining hydration can keep mortality rates under 1% (Nataro and kaper, 1998; Quadri et al., 2005). However, this has no effect on the pathogen itself.

Efforts to develop an effective ETEC vaccine have not yield satisfactory results (Beodeker, 2005). The use of antibiotics is problematical because of the diminishing number and efficacy of available drugs and the wide spread emergence of multi-drug resistant strains (Al-Abri et al., 2005). ETEC's resistance to tetracycline, ampicillin, gentamicin, trimethoprim-sulfamethoxazole, chloramphenicol, ciprofloxacin, and multi-drug resistance

has been documented (Chakraborty et al., 2001; Matsushita et. al., 2001; Estrada-Garcia et. al., 2005). Moreover, since the infection resolves within a few days, antibiotic treatment is largely useless. Antibiotics such as fluoroquinolones and rifaximin are still effective, but both are important for the treatment of severe enteric disease, and the adverse effects weight strongly against their prophylactic use. Thus, there is a need for useful prophylaxis that does not involve antibiotics for the treatment of life-threatening enteric disease. In this light, bacteriophage prophylaxis can be an important strategy.

4.1 Bacteriophages

Bacteriophages (phages) are the viruses that infect bacteria. Their discovery is controversial, as the first descriptions where done by F. W. Tworth in 1915 and Felix d'Herelle in 1917 (Douglas, 1975; Duckworth, 1976). However, the latter is widely recognized as the father of phage biology and promoted the idea of using phages for the treatment of bacterial infections, or phage therapy (Calendar, 2006).

Phages are constituted by proteins and nucleic acids, although a few types have lipids (Ackerman, 1999). Phage proteins form an icosahedral structure, or head, where genetic material is stored. This is made up of many copies of a head protein and a major capsid protein. Virions can have binary symmetry, cubic, helicoidal or pleomorphic. Phage genome can be dsDNA (double stranded), ssDNA (single stranded), ssRNA or dsRNA. The vast majority has a tail structure used for fixation to bacterial host (Ackerman, 2006).

Scientist estimate phage population to be in the order of 10^{31} individuals in the biosphere, which makes them the most abundant form of life (Hendrix, 2003). They are ubiquitous and can found in Archea as well in bacteria, suggesting that phages originated before the separation of the kingdoms, meaning that their population is not only big, but really dynamic (Ackerman, 2006).

4.1.2 Life cycle.

Phages follow a life cycle than can be described as a four step process that includes: adsorption, infection, release and decay. To initiate an infection cycle, phages adsorb to a specific surface feature of bacteria and then eject its genetic material into the permissive host. Only bacteria with that surface determinant will be sensitive to phage infection, making them host-specific (Abedon, 2006). Infection period is divided into eclipse and maturation. Phages hijack the host's cellular machinery in order to duplicate its genome and produce the virion components in a process than can last from 15-60

minutes depending on the host's physiology (Hadas et. al., 1997). Eclipse is used to describe the period before the appearance of the first mature virions (Adams, 1959) and is followed by maturation.

For the release of mature virions, bacterial cells burst inside out, liberating 50-200 phages. This process is accomplished with a set of lysis proteins that form a "hole" in the membrane at specific time, attacking the structural bonds of peptidoglycan and disrupting the outer membrane (Young and Wang, 2009). In some cases virions are extruded across the cell envelope without causing host cell death (Russel and Model, 2006) or released by budding through the membrane (Martin et al., 1984). A new infection cycle can begin if the newly released phages are able to find a susceptible bacterial host. However, if the density of the bacterial host is low, phage populations are not able to survive indefinitely and will decay, losing their ability to infect bacteria and/or produce progeny (Abedon, 2006).

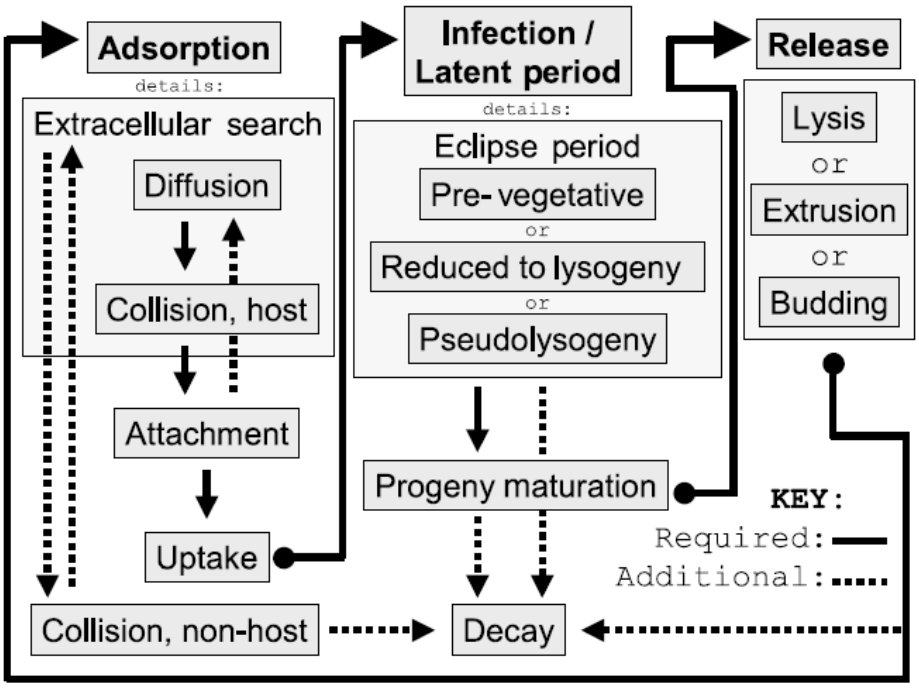


Figure 2. Phage life cycle. Continuous lines indicate necessary paths to complete phage cycle. Dashed lines represent optional pathways. (Calendar, 2006)

Two main types of phage emerge from differences in their lifestyle: virulent (or lytic) and temperate. Both start with adsorption to bacteria and ejection of DNA into the cytoplasm. In virulent phages, eclipse period is immediately followed by maturation, release and new infection cycles. On the other hand, temperate phages can either continue the lytic pathway or integrate its DNA into a specific site in the host chromosome. In this scenario, phage genome is now called prophage and the harboring bacteria a lysogen. Prophage replicates along with the chromosome of lysogenic bacteria which continues to grow normally, depending on the phage (Campbell, 2003). Physical or chemical stimuli can disrupt the temperate life-style and cause the prophage to be cleaved from the genome and enter the lytic pathway (Watson, 2003). This plays a major role in bacterial evolution since bacterial genomes are known to carry several prophage sequences (Brussow et al., 2004). A classic example for the description of the temperate life-cycle is phage lambda (schematized in figure 3).

Lastly, another alternative is a pseudolysogenic state in which the phage has ejected its DNA into the bacterial host but this has not integrated nor initiated the lytic cycle, but remains episomal inside the host (Ripp and Miller, 1997).

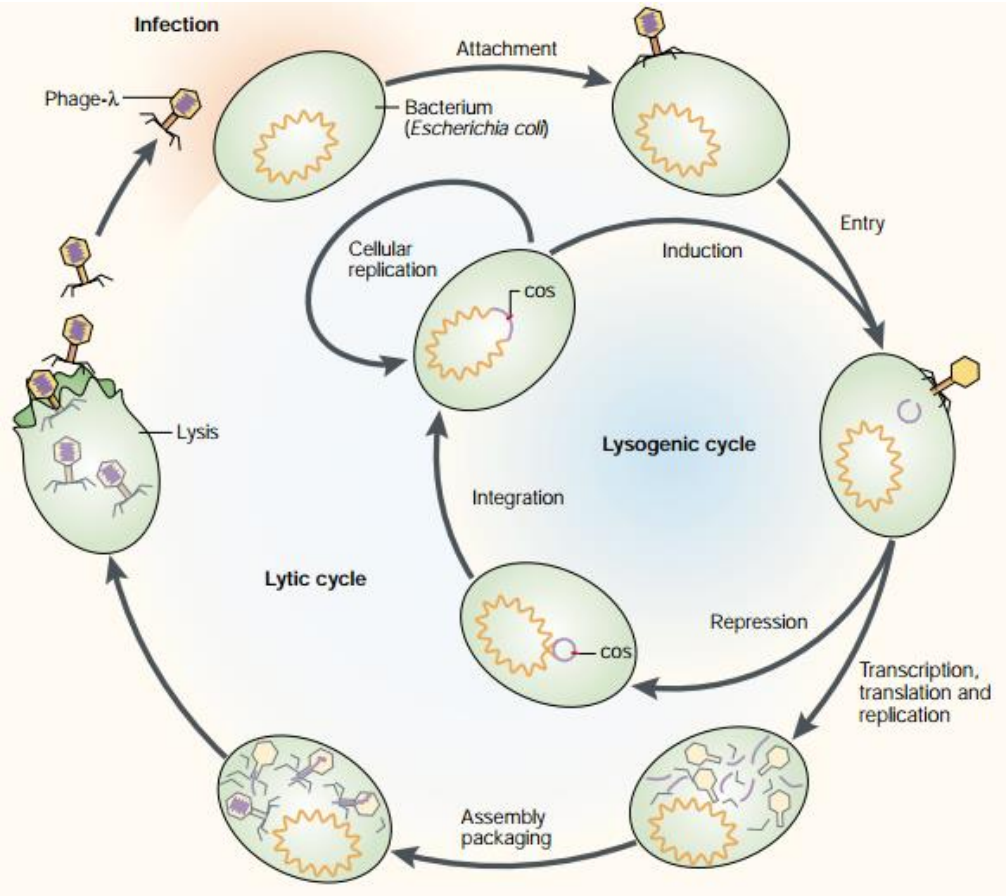


Figure 3. Lysogenic and lytic cycle of temperate phage Lambda. DNA ends join through cohesive ends (cos) to form a circle and then is transcribed, translated and replicated. The lysogenic cell can replicate indefinitely until an induction event and activation of the lytic pathway. (Campbell, 2003)

4.1.3 Classification

Virus classification can be somewhat troublesome, and phages are not the exception. The International Committee on Taxonomy of Viruses (ICTV) recognized 1 order, 13 families and 31 genera of phages (van Regenmortel et al., 2000). Filamentous, cubic and pleomorphic phages have less than 190 and are classified in 10 small families. Tailed phages are classified in the order *Caudovirales* and comprehend more than 5000 viruses (+90% of phages), which makes them the interest group (Ackerman, 2006).

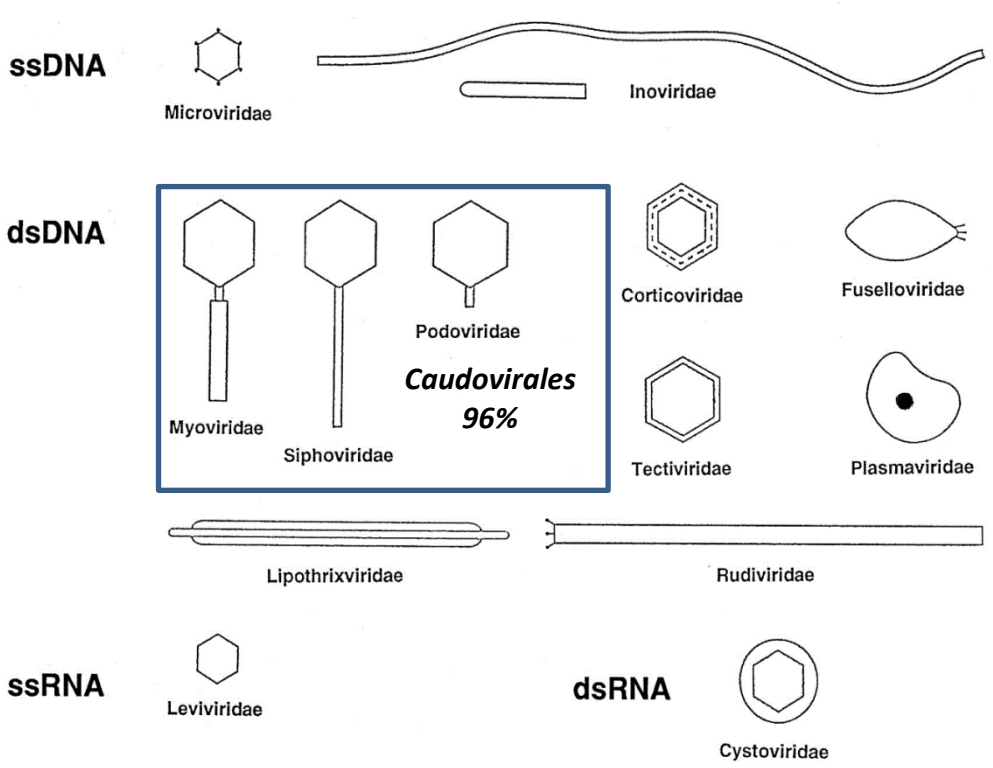


Figure 4. Representation of major phage groups. Modified from Calendar, R. 2006.

Symmetry	Nucleic acid	Order and families	Members	Particulars
Binary (tailed)	DNA, ds, L	*Caudovirales*	4950	
		Myoviridae	1243	Tail contractile
		Siphoviridae	3011	Tail long, noncontractile
		Podoviridae	696	Tail short
Cubic	DNA, ss, C	*Microviridae*	40	
	ds, C, T	*Corticoviridae*	3?	Complex capsid, lipids
	ds, L	*Tectiviridae*	18	Internal lipoprotein vesicle
	RNA, ss, L	*Leviviridae*	39	
	ds, L, S	*Cystoviridae*	1	Envelope, lipids
Helical	DNA, ss, C	*Inoviridae*	57	Filaments or rods
	ds, L	*Lipothrixviridae*	6?	Envelope, lipids
	ds, L	*Rudiviridae*	2	Resembles TMV
Pleomorphic	DNA, ds, C, T	*Plasmaviridae*	6	Envelope, lipids, no capsid
	ds, C, T	*Fuselloviridae*	8?	Spindle-shaped, no capsid

Figure 5. Basic properties of bacteriophages. Modified from Calendar, R. 2006.
C, circular; L, linear; S, segmented; T, superhelical; ss, single-stranded; ds, double-stranded

It is important to point out that families are defined by the nature of their nucleic acid and morphology of the virion (figures 4 and 5). However, there's no universal criteria that delimits genera or species. In the ICTV scheme species are defined by a set of characteristics that may or may not be present in all of the members (Van Regenmortel, 1990), making this classification cumbersome.

4.1.4 Caudovirales (Tailed phages)

Caudovirales are defined as phages with binary symmetry, meaning that they have an icosahedral head attach to a tail. This head-tail structure is responsible for host specificity and the efficient ejection of DNA into the cytoplasm. Virions consist on dsDNA and proteins. Tails have baseplates, spikes and fibers. They are divided into 3 families: (i) Myoviridae: phages with a contractile tail formed by a sheath and central tube that is separated from the head, (ii) Siphoviridae: phages with non-contractile tails, and (iii) Podoviridae: phages with short tails (Ackerman, 2006). Represented in fig. 4 blue box

As we mentioned before, this classification is arbitrary. The division of *Caudovirales* in 3 families is misleading because suggests non-existent evolutionary relationships. Besides the tail, the only shared characteristic among all *Caudovirales* is a DNA packaging mechanism consisting of a portal protein and a terminase used to pump DNA into the virion capsid (Catalano, 2005). Phages between different families can share life-style, genome size and arrangement, and regulatory strategies despite having different tail structures. Therefore the latter, although easy to identify, is not the best relatedness indicator (Casjens, 2005).

Some research groups use the concept of "phage type" for the classification of *Caudovirales*, eliminating families and classifying phages according to their genome arrangement, transcriptional regulation and genome size. This concept introduced by S. Casjens (Casjens, 2008), organized *Caudovirales* into 13 types, most of them typified by a paradigm phages such

as T1, T7, T4, T5, N4, Felix-01, SETP3, 9NA, Lambda, P2, Mu, P1 and E15. For example, the term “Lambda-like” indicates that the phage genomic characteristics fit into the lambda type, a criteria that indicates more relatedness than tail morphology.

4.1.5 Importance of characterization and classification.

Phages have been found in more than 179 bacterial genera and are easily found on industrially and clinically relevant bacteria such as Firmicutes and γ-Proteobacteria (a group that includes pseudomonads and enterobacteria) (Ackerman and Prangishvili, 2012). However, phage research is limited to about 15 countries where its focus is only in a few viruses, which account for the fact that as today, many important bacterial phyla remain without described phages (Ackerman, 2000; Ackerman, 2006). With the enormous diversity of phages, we can doubtlessly say that the many important habitats for phage are yet to be explored.

Precise phage characterization is necessary to perform phylogenetic studies and comparison between other organisms in order to place them correctly within their biological group. Basic characterization includes electron microscopy for morphology, DNA restriction patterns to determine individuality of phages, sequencing and genome annotation, and host range. This facilitates the analysis of phages with industrial and medical relevance. For example, temperate phage P27 is carried EPEC and encodes Shiga-toxin (Stx), responsible for hemorrhagic disease. Moreover, with the revival of phage therapy is absolutely necessary to characterize phages with therapeutic potential in order to avoid phages that carry virulence factor or potentially harmful genes.

4.1.4 Phage therapy

The use of phages as a therapeutic agent was proposed by d'Herelle almost 100 years ago when he demonstrated the ability of virulent phages to infect and kill bacteria, and prompted the idea of their for the treatment of human infections (d'Herelle, 1917).

Because of their nature, phages represent a good candidate for phage therapy: (i) host range is specific and sometimes reduced, which results in the ability to kill pathogenic bacteria without harming the gut microbiota, (ii) low toxicity to plants or animals, and (iii) they increment their numbers as they infect and kill their bacterial target. After lysis of their bacterial host phages can continue their cycle and migrate to other sites of infection within the body (Bruttin and Brussow, 2005; Summers, 2001).

The transition from their discovery to animal trials and then human studies was rather fast. Although crude and without proper controls, the first human trials gave reasons to believe that phage therapy could be effective, but it was not long after phage therapy hit a wall. One of the first observations was the emergence of phage-resistant bacteria, which has become the most frequently cited limitation of phage therapy (Stent, 1963). Coupled with inconsistent results due to the lack of knowledge in microbiology and phage biology, absence of proper standards and controls, imperfections in bacteriological diagnosis at the time and the success and industrial production of antibiotics from 1940, helped to end initial excitement for phage therapy (Summers, 2001). After this period there is no scientific literature on phage therapy on the western world. Phage research shifted away from therapy to become one of the main experimental systems used during the early development of molecular biology (Summers, 2006).

Although dead in the western world, phage therapy continued to be a useful resource in the Eliava Institute in Tbilisi, founded by George Eliava, and other institutes such as the Hirszfeld institute of immunology and

experimental therapy in Poland (Deresinski, 2009). Phage therapy is of common use in Poland and Belgium, where it is consider an experimental treatment working under the Helsinki declaration, and a routine treatment in Russia and Georgia (Pirnay et al., 2011).

However, the world faces new therapeutic challenges as there is an increase in multi-drug resistant bacteria, coupled with the lack of new antibiotics (Giamarellou, 2010; Freire-Moran et al., 2011) . The aforementioned has contributed to a renewed academic, commercial and therapeutic interest on phage therapy.

5. BACKGROUND

The standard methodology for phage isolation begins with a collection of water, sewage or soil samples. Filtered samples are mixed with the bacterial host of interest to enrich for phages able to infect the bacteria and facilitate their detection, further purification and propagation. The basic characterization includes electronic microscopy for morphology, DNA restriction patterns to determine the individuality of phages, sequencing and genome annotation to determine if the phage of interest does not contain genes that encode for toxins or virulence factors, and host range to determine the ability of the phage to infect other strains *in vitro*, before moving to *in vivo* studies.

In the last 30 years, studies in animal models where phages are administered intravenously or intraperitoneal have shown the capacity of phages to infect multi-drug resistant bacteria *in vivo*. Several reviews that sum up those works have been published (Sulakvelidze and Kutter 2005;Gorski et al., 2009; O'Flaherty et al., 2009). However, most studies where carried out with relatively uncharacterized phages or do not meet current scientific standards, and recent phage work requires a more rigorous characterization processes.

Smith and cols conducted experiments for the treatment of *E. coli* diarrhea in calves, mice, piglets and lambs (Smith and Huggins, 1982; Smith and Huggins, 1983), demonstrating that single-dose phage treatment was more effective to several antibiotics. A study to compare the ability of phage to control bacterial in vivo and in vitro was carried out by Soothill (Soothill, 1992). Septicemia and meningitis was successfully treated in chickens and calves using phage (Barrow et al., 1998). Phages have also been used for the treatment of plant and fish pathogens (Flaherty et al., 2000; Nakai and Park, 2002).

Several studies have addressed gastrointestinal infections where oral administration is the most used model of phage activity against gut bacteria (Chibani-Chennoufi et al., 2004; Denou et al., 2009; Maura and Debarbieux, 2011; Maura et al., 2012; Tanji et al., 2005). Models for the treatment of E. coli infection have been described in calves, pig and mouse (Bruttin and Brussow, 2005; Chibani-Chennoufi et al., 2004).However, one of the limitations of this kind of studies is the lack of a proper diarrhea model in mouse. After being colonized by diarrheagenic *E. coli* pathotypes, it resembles an asymptomatic infection in humans (Denou et al., 2009).

A recurrent phenomena observed is the replication of phage in their bacterial host in vivo, however, bacterial levels remain constant (Maura et al., 2012), or in some cases, bacteria that was susceptible in vitro remained without alteration when exposed to phage in vivo (Chibani-Chennoufi et al., 2004). Which indicates that we still have a lot to learn when it comes to nature and characteristics of phages to use for therapy, as well as the nature of interaction phage-bacteria in vivo.

Phase I clinical trials of phage therapy in humans have been done (Bruttin and Brussow, 2005; Kutter et al., 2010). Some research groups focus in obtaining a phage cocktail for the treatment of diarrheal disease that attacks the main *E. coli* diarrheagenic pathotypes (Alam et al., 2011; Bruttin and

Brussow, 2005; Denou et al., 2009; Maura et al., 2012), and there is also and undergoing phase II clinical trial for the treatment of childhood ETEC and EPEC caused diarrhea by a T4 phage cocktail (ClinicalTrials.gov identifier: NCT00937274).

This kind of work and their future results will give information about the true potential and limitations of phage therapy in humans. However, ETEC strains are endemic to developing countries, and composition of the cocktail must specific for each region. This represents a problem because it will require the design of treatments for specific areas, and moreover, due to the emergence of phage-resistant bacteria it might require some adjustments over time. Those are some of the problems that we wanted to address with our experimental approach.

5.1 SPEAR

Bacterial target can become resistant to phage infection by mutation, most likely eliminating or changing the surface receptor used for phage adsorption. Different to the common methodology in which several phages are used to infect a set of pathogenic strains, our approach to counter the problem of resistance is to exploit the enormous diversity of phages to construct receptor-specific phage cocktails, composed of phages that recognize virulence factors as receptors.

SPEAR (Selection of phage for the emergence of avirulent resistance) strategy, developed in the Young Laboratory at the Texas A&M University, focuses on the construction of a phage cocktail that infects pathogenic bacteria using virulence factors as receptor. In this way, if resistance does arise by mutation of the receptor, there is a strong probability that the resistant bacteria have attenuated or ablated virulence.

The target for this project is the chromosomically-encoded outer membrane protein TolC, a conserved protein ubiquitous among Gram negative bacteria involved in many cellular activities. TolC plays a central role in

translocation of toxins, antibiotics and other deleterious agents through the outer membrane, as is required for the functioning of the AcrAB efflux pump in *E. coli*, responsible for the multiple antibiotic resistant phenotype (Okosu et al., 1996; Fralick, 1996). This feature makes TolC an interesting therapeutic target for multi-drug resistant bacteria (Koronakis et al., 2004). In ETEC strains, TolC is responsible for the secretion of ST toxin (Yamanaka et al., 1998), necessary for the process of pathogenesis but not for bacterial survival as deletions of the TolC gene have already been achieved. *E. coli* K-12 mutants lacking TolC present are hypersensitive to antibiotics and detergents (German and Misra, 2001), whereas in ETEC TolC deletion ablated the ability to translocate ST toxin across the outer membrane, becoming unable to cause host damage (Yamanaka et al., 1998).

The use of a phage cocktail that recognizes TolC as a receptor in ETEC strains will lower the frequency of resistance. In this way, if resistance does arise by mutation of TolC, is highly likely to have attenuated virulence.

6. JUSTIFICATION

Enterotoxigenic *Escherichia coli* (ETEC) is the predominant cause of traveler's diarrhea, the most common illness affecting travelers visiting developing countries. ETEC is estimated to be responsible of 400,000 deaths of children under the age of five, and overall represents a serious health threat. The most effective way to treat ETEC diarrhea is either oral or intravenous rehydration, however, this has no effect on the pathogen itself. The use of antibiotics after the symptoms develop is useless and not recommended because the disease is self-limiting and will resolve within a few days. Moreover, this can cause the emergence of drug-resistant strains. Therefore, there's need for prophylaxis that does not involve any of the few useful antibiotics for the treatment of life-threatening enteric disease, such as phage therapy.

7. HYPOTHESIS

Mutations in TolC ablate the ability of enterotoxigenic *Escherichia coli* to secrete ST toxin. Therefore, the use of bacteriophages that recognize TolC as a receptor in ETEC strains is likely to produce avirulent resistance.

8. OBJECTIVES

8.1 General objective:

Isolation of a bacteriophage that infects clinical strains of enterotoxigenic *Escherichia coli* (ETEC) through the outer membrane protein TolC

8.2 Specific objectives:

1.- Creation and characterization of a panel of clinical ETEC strains isolated from patients in Mexico.

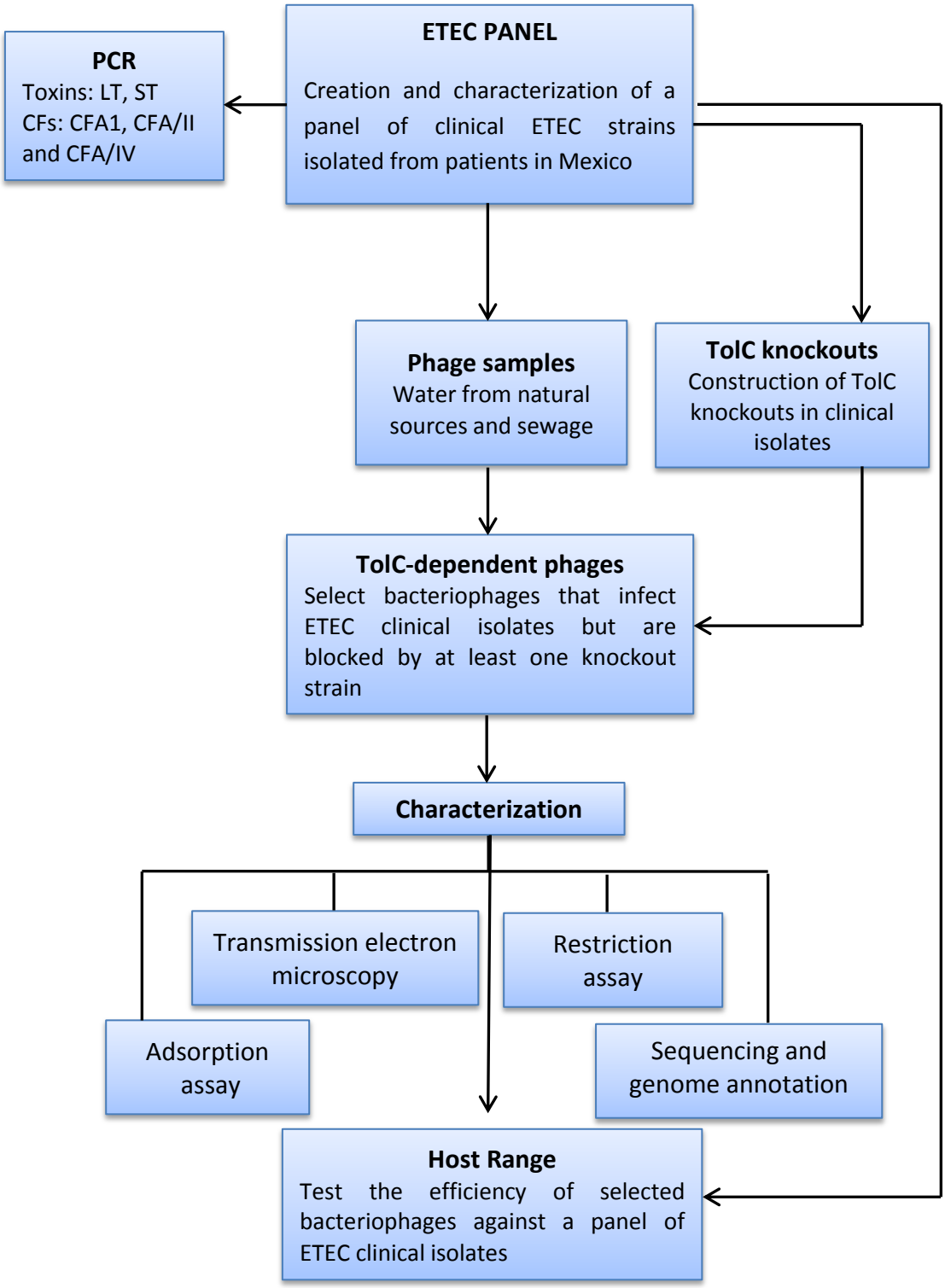
2.- Construction of knockouts for TolC gene in ETEC clinical isolates.

3.- Selection of bacteriophages that infect ETEC clinical isolates but are blocked at least by one of TolC knockout strains.

4.- Characterization of TolC-dependent phages by an adsorption assay, transmission electron microscopy, restriction assay, sequencing and genome annotation

5.- Determine the efficiency of selected bacteriophages against a panel of ETEC clinical isolates

9. EXPERIMENTAL STRATEGY



10. MATERIALS AND METHODS

10.1 Characterization of an ETEC panel

41 Lactose fermenting, biochemically identified enterotoxgenic *E. coli* strains from Dra. Maria Teresa Estrada's collection (Department of Molecular Biomedicine at CINVESTAV) where analyzed. *E. coli* H10407, the prototypical strain of ETEC was used as a control. Lactose-fermenting colonies with *E. coli* morphology were selected from MacConkey agar plates. Preparation of bacterial lysates was performed by resuspending single colonies in 1ml of deionized water (Milli-Q system, Millipore, Bedford, MA), boiling them for 1 min and freezing until needed. A multiplex PCR that detects pathogenic genes for heat-stable and heat-labile enterotoxins was performed. PCR reaction contained 23µl of reaction mix, a mixture of ST and LT primers and 2µl of bacterial lysate. Cycling conditions were as follows: 50°C (2 min, 1 cycle); 95°C (5 min, 1 cycle); 95°C, 50°C, and 72°C (45 sec each temperature, 40 cycles) and a final extension of 72°C (10 min) in a thermal cycler. PCR products (5 µl) were visualized after electrophoresis in a 2% agarose gel in Tris-borate-EDTA buffer and ethidium staining. ST and/or LT positive colonies were inoculated in peptone agar and stored at room temperature. Later, PCR characterization for CFA/I, CFA/II and CFA/IV was performed. PCR reaction contained 23µl of reaction mix, a mixture of CFAs primers and 2µl of bacterial lysate. Cycling conditions: hot start at 95°C (3 min, 1 cycle), 95°C (1 min), 54°C (45 sec), 72°C (1.5 min) (30 cycles), and a final extension of 72°C (5 min) in a thermal cycler. PCR products (5 µl) were visualized after electrophoresis in a 2% agarose gel in Tris-borate-EDTA buffer and ethidium staining.

Table 1. List of primers used for characterization of ETEC strains

Target	Primer	Sequence 5' to 3'	Amplicon (bp)
LT	**LT fw**	GGCGACAGATTATACCGTGC	**450**
	LT rv	CGGTCTCTATATTCCCTGTT	
ST	**ST fw**	ATTTTTCTTTCTGTATTGTCTT	**190**
	ST rv	CACCCGGTACAAGCAGGATT	
CFAI	**CFAI fw**	GCTCTGACCACAATGTTGA	**364**
	CFAI rv	TTACACCGGATGCAGAATA	
CFAII	**CFAII fw**	GGTGGGTGTTTTGACTCTT	**264**
	CFAII rv	TGTTCGTTACCTTCAGTGG	
CFAIV	**CFAIV fw**	ATCCAGCCTTCTTTTGGTA	**321**
	CFAIV rv	ACCAACCATAACCTGATCG	

10.2 Construction TolC knockouts in ETEC clinical isolates

E. coli BW25113 tolc::kan strain was used as the source of TolC deletion for the construction of ETEC mutants. BW25113 tolc::kan belongs to the Keio collection, a set of single-gene knockout mutants where coding regions are replaced with a kanamycin cassette. The strain was kindly provided by Dr. Dimitris Georgellis from the Institute of Cellular Physiology at UNAM. TolC mutants were constructed by transduction with P1 *vir* a temperate phage that packs bacterial DNA into its virion. The resulting particle is used to infect another strain and the captured DNA fragmets can be integrated into the new host genome by recombination.

10.3 P1 lysate and transduction

To obtain P1 *vir* particles containing the TolC deletion, donor strain BW25113 tolc::kan was grown on a petri dish with LB media by cross streak method and incubated overnight at 37°C. A single colony was inoculated in 5ml of LB media and incubated overnight at 37°C and 200rpm. 50µl of the overnight culture were transferred to 5ml of LB media with 0.2% glucose and 5mM of $CaCl_2$ and grown until OD_{600} of .1-.2. 100µl of a P1 *vir* stock of 10^9 – 10^{10} PFU/ml were added and then incubated until the culture was lysed (approximately 3 hours).

3 drops of chloroform were added and vortex thoroughly. Lysate was centrifuged at 10'000rpm, 4°C for 10 minutes.

Several ETEC clinical isolates were used as recipient for the TolC deletion. 5 ml of LB media were inoculated with the recipient strain (ETEC clinical isolates) and incubated overnight at 37°C, 200rpm. Next day, 2.5ml of culture was centrifuged at 14'000rpm for 2 minutes. Supernatant was discarded and pellet resuspended in 1.25ml of LB media with 10mM $MgSO_4$ and 5mM $CaCl_2$. 100µl of recipient strain were added to 2 sterile Eppendorf tubes. 10µl of P1 lysate was added to tube 1, and 100µl to tube 2. Two controls were used; a tube with 100µl and another with 100µl of P1 lysate. Tubes were incubated for 30 minutes at room temperature. 1ml LB media with 200µl of 1M sodium citrate was added to each sample and then incubated for 1 hour at 37°C. Samples were centrifuged at 14'000rpm for 2mins. Supernatant was discarded and samples re-suspended in 100µl of LB media with 20mM of sodium citrate. Samples were plated on a petri dish with LB + kanamycin (50µg/ml) and incubated 24-48 hours at 37°C.

10.4 Colony characterization

Colonies where selected on their ability to grow on LB media with kanamycin (50µg/ml), but not on LB with 0.01% of SDS, as the TolC mutation makes bacteria hypersensitive to detergents. Selected colonies were characterized to check the correct chromosomal structure by PCR using a combination of locus and kanamycin specific primers. PCR reaction tube contained 23µl of reaction mix, primers and 2µl of bacterial lysate. Kanamycin-specific primers are named K1 and K2, whereas TolC locus-specific primers are A1 and C2 (figure). Cycling conditions were as follows: Hot start at 94°C (5mins, 1 cycle), 94°C (45 seconds), annealing (45 seconds, specified in table) and 72°C (2 min) for 30 cycles, followed by a final extension at 72°C (10 min). PCR products (5 µl) were visualized after electrophoresis in a 2% agarose gel in Tris-borate-EDTA buffer and ethidium staining.

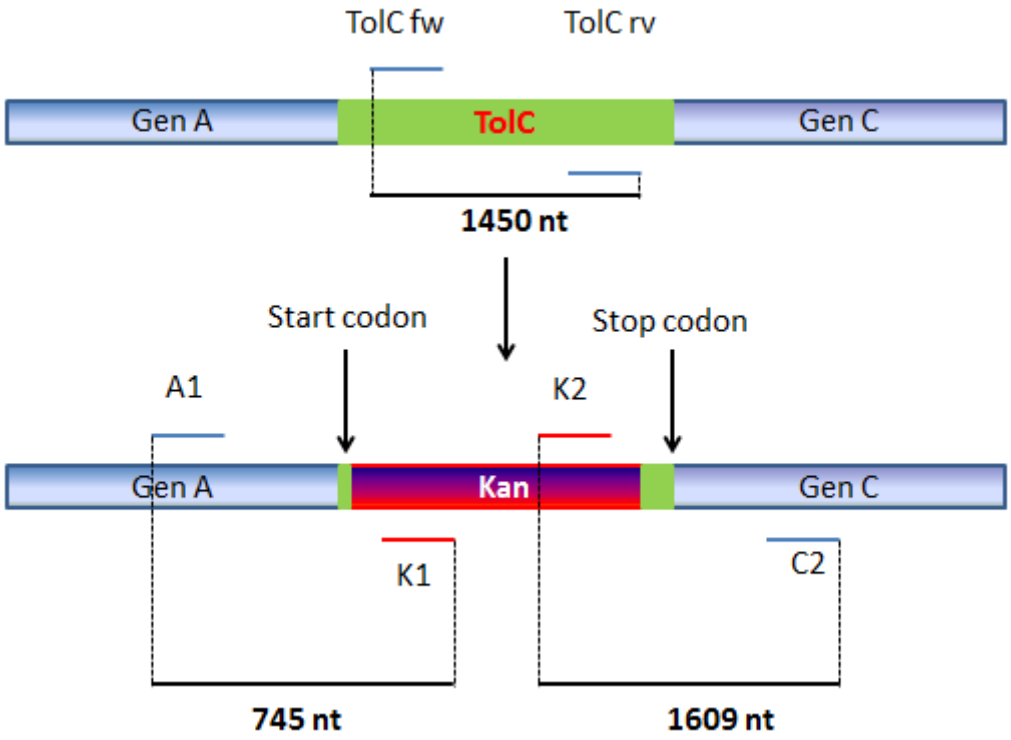


Figure 6. Chromosomic structure of TolC locus. Structure of donor strain *E. coli* BW25113 TolC locus before (up) and after replacement with Kanamycin cassette (below). K1 and K2 are kanamycin-specific primers. A1 and C2 primers target upstream and downstream regions of TolC, respectively. TolC fw and TolC rv are primers for the inner sequence of TolC.

Table 2.- List of primers used for the characterization of TolC::kan strains

Target	Primer	Sequence 5' - 3'	Amplicon (bp)	Annealing T°
TolC	TolC fw	TGCTCCCCATTCTTATCGGC	1450	58°C
	TolC rv	CGTTACTGGTGGTAGTGCGT		
TolC upstream	A1	TTGCCAAATGTAACGGGCAG	745	56°C
and	K1	CAGTCATAGCCGAATAGCCT		
Kanamycin				
Kanamycin	K2	CGGTGCCCTGAATGAACTGC	1609	57°C
and TolC				
downstream	C2	GTGGGCTTTCTCACGCCAGT		

10.5 Isolation and characterization of bacteriophages that use TolC as a receptor

Bacterial strains, media and growth conditions.

Bacterial strains in table 5 (TolC knockouts) and their wild type counterpart were used for phage isolation. All bacterial strains were grown in liquid LB broth at 37°C with shaking. Solid medium was prepared by adding 1.5% agar to LB. Top agar was prepared using 0.75% agar

Bacterial lawns were prepared with the soft agar overlay. 100µl of a bacterial overnight culture were mixed with 4ml to molten agar medium (55-60°C). Mixture vas vortexed and then poured on a petri dish with solid LB medium. Mixture was allowed to solidify and then incubated on inverted position at 37°C.

Plaque assays for phage infection were performed in petri dishes containing solid LB medium overlaid with 4ml of LB top agar (0.75% agar). Lawns of bacteria were prepared from overnight cultures in late logarithmic phase and resuspended in SM buffer.

Sampling site and sample preparation

Sewage samples (-500ml) from different sources in Mexico City and College Station, TX were collected. Each liquid sample was centrifuged at 8'000 rpm for 10 min at 4°C. The supernatant was then filter sterilized using 0.22µm filters, transferred to sterile bottles and stored at 4°C. 20ml of the filtered sample and 20ml of an ETEC tolc::kan overnight culture were mixed without vortexing and incubated for 10 min on ice. The idea of this step was to allow phage adsorption and deplete the sample from phages that don't use TolC as receptor. The sample is then centrifuged at 10'000rpm for 10 min at 4°C. The supernatant was recovered and filter sterilized using 0.22µm filters and stored at 4°C until used.

Direct plating

The standard protocol for phage detection was the agar overlay technique. 100µl of an overnight culture and 100µl of processed sewage sample or phage lysate were mixed without vortex and incubated for 10 min at room temperature. The mix was transferred to 4ml of melted soft agar (55-60°C), mixed gently and poured onto a LB agar plate and kept for solidification. The plates were incubated at 37°C and plaques counted after 12 h.

Enrichment

The goal of this method is to enrich the water sample for phages infecting ETEC. 20ml of the processed and pre-adsorbed with ETEC tolc::kan sewage sample were placed in a 50ml sterile Falcon tube containing 5ml of 5X LB broth. 100µl of an ETEC overnight culture were added and then incubated at 37°C with shaking for 24-48h. After incubation, 500µl of chloroform were added to kill the remaining bacteria. The sample was centrifuged for at 10'000 rpm for 10 min at 4°C. The supernatant (phage lysate) was transferred to a sterile tube.

Phage titering:

Serial 10-fold dilutions of 100µl of phage lysate were transferred to eppendorf tubes containing 900µl of LB medium, until reaching dilution 10^{-8}. 10µl of each dilution were spotted in a lawn of both ETEC and ETEC tolc::kan. The plates were kept 15 min in the flame until drops were adsorbed. Plates were incubated at 37°C in inverted position. Individual plaques in the spots were counted by the formula ((# Plaques / Vol. (ml)) x Dilution) and expressed as Plaque forming units (PFU)/ml.

Phage selection

If any differences in efficiency of plating, we performed a full-plate titration of the phage in both ETEC and its ETEC tolc::kan counterpart. 100µl of phage

dilution containing 1x10³ PFU were placed in 2 sterile eppendorf tubes. The first was mixed with 100µl of the ETEC overnight culture and the other with 100µl of ETEC tolc::kan overnight culture. Each mix was transferred to 4ml of melted soft agar (55-60°C), mixed gently and poured onto an LB agar plate and kept for solidification. The plates were incubated at 37°C and plaques counted after 12 h. Each plaque obtained on an ETEC strain was toothpicked into replica grid plates with lawns of ETEC tolc::kan bacteria. Phage plaques are selected for their ability to infect the ETEC strains but not the ETEC tolc::kan.

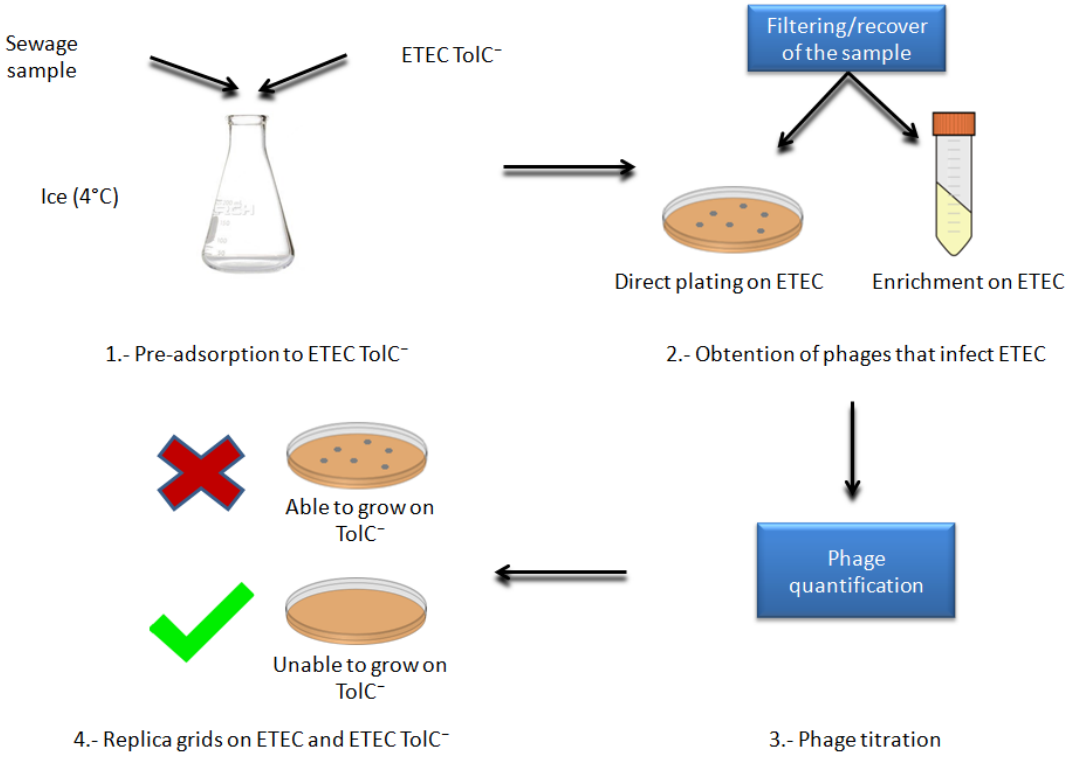


Figure 7. Isolation of TolC specific phages strategy

Phage purification and lysate

Selected phage plaques were toothpicked in the center and then streaked across the surface of a bacterial lawn of the host strain. The plates are incubated at 37°C for 12-24 h. This procedure was repeated 3 times to purify a clonal population. An isolated plaque was picked by slight suction with micropipette tips. The picked plaque was resuspended in 1ml SM gelatin-free

buffer in an eppendorf tube. The tube was kept on a rocker for 1 hour to release the phages from the agar. 50µl of chloroform were added, the sample was centrifuged and supernatant recovered. This sample contains the purified phage.

Large scale amplification of phage was performed by plate lysates. 5 eppendorf tubes with 100µl of an overnight culture of the host bacteria were mixed with $1x10^5$ PFU of the isolated phage. Each mix was transferred to 4ml of LB top agar and poured onto LB agar plates. Plates were incubated overnight at 37°C. For harvesting, each plate was flooded with 5ml of SM buffer. The lawn surface was disrupted with a pipette and then placed in a sterile 50ml Falcon tube. The sample was centrifuged at 10'000 rpm for 10 min. The supernatant is filter sterilized with a 0.22 filter and stored at 4°C.

Phage concentration and CsCl gradients

To concentrate phage lysates, we used high-speed centrifugation. Lysate was centrifuged at 10’000rpm at 4°C for 24hrs using a Sorvall GSA rotor. Supernatant was discarded and 5-10ml of gelatin-free SM-buffer was added to loosen up the pellet. Sample was stored overnight at 4°C. Next day phage was resuspended thoroughly and transferred to a 50ml centrifuge tube. Phage suspension was centrifuged at 12’000rpm at 4°C for 10mins to pellet cell any cell debris and supernatant was transferred to a new tube to continue with CsCl gradient.

Solid CsCl was added to a concentration of 0.75g/ml of phage suspension. Phage-CsCl solution was transferred to ultracentrifuge tubes and ran at 45’000rpm, at 5°C for 24hrs using a Beckman 70.1 Ti rotor. Phage formed a gray band in the tube and it was collected using a 10cc syringe. Phage was transferred to a dialysis cassette and dialyzed against 1 liter of gelatin-free SM buffer containing 1M NaCl at 4°C overnight. Phage prep was filter-sterilized and stored at 4°C

10.6 Phage characterization

Phage adsorption assay

We used a similar protocol that Hendrix and Duda, 1992. 10ml of LB media containing 5mM of $MgCl_2$, $1x10^4$ PFU of bacteriophage mixed with $1x10^7$ cells/ml of exponentially grown ETEC 22 or ETEC 22 tolC::kan bacteria, incubated at 37°C with shaking. Samples were taken every 3 min, centrifuged at 10'000rpm x 4 mins and plated on ETEC 22 WT for plaque counting.

DNA extraction

Genomic DNA extraction was performed using the modified Promega wizard method (Promega A7280). Large scale Phage preparation was treated with DNAse and RNAse at a final concentration of 10µg/ml and incubated for 2 h at room temperature. Precipitation solution is added to a final concentration of 10% PEG-8000 and 1M NaCl and incubated overnight at 4°C. The precipitated phage lysate was centrifuged at 10'000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet resuspended in 500µl of resuspension buffer (10mM $MgSO_4$), then transferred to a 1.5ml tube and centrifuged for 10 s to pellet insoluble particles. Phage suspension is transferred to a new tube and 1ml of purification resine (Promega wizard kit) was added and mixed gently. The resine/phage was passed through a minicolumn. 2ml of isopropanol 80% were used to wash the column and centrifuged at 10'000rpm for 2 min. The minicolumn was transferred to a new tube. DNA was eluted with 50µl of prewarmed (80°C) water and centrifuged at 10'000rpm for 20 s. Minicolumn was discarded and the DNA stored at 4°C. Quality of the DNA was examined by running 2µl of sample on a 0.8% agarose gel stained with etidium bromide

Transmission electron microscopy

Phage samples for microscopy were prepared by negative staining and mounted on a carbon-formvar grid. 10µl of phage lysate were mixed with 40µl of 5mM $MgSO_4$ solution to obtain a 5x dilution of the phage sample and placed

on parafilm. Carbon grids were placed on top of the sample and rested for 1 min. After that, carbon grids were placed on top of 40µl of 2% uranyl acetate (UA) for 20 seconds. Excess of UA was removed and sample was allowed to dry. Morphology was determined using transmission electron microscopy performed at the Texas A&M University Microscopy and Imaging Center.

Restriction assay

Phage DNA was digested with restriction endonucleases EcoRV, XbaI, kpnI and SmaI. A reaction mix consisted of 2µl of DNA, 1µl of enzyme, 2µl of universal green buffer and sterile water for a 20µl reaction volume. Samples were rested overnight at 4°C. Digestion products were examined by running the sample on a 0.8% agarose gel, 0.5X TBE stained with ethidium bromide at 120v for 45mnins.

Genome sequencing and annotation

Phage DNA was sequenced in an Illumina MiSeq 250-bp paired-end run with 550-bp insert library at the Genome Sequencing and Analysis Facility at the University of Texas (Austin, TX). Genes were predicted using GeneMark v4.3 (Borodovsky and Lomsadze, 2011). Ribosome-binding sites were detected with RBS_finder (Suzek et al., 2001). Rho independent terminators were predicted with TransTermHP v2.07 (Kingsford et al., 2007) and tRNA sequences with tRNA scan-SE v1.21 (Lowe & Eddy, 1997). Amino sequences of predicted open reading frames were compared with protein sequences of known function deposited in the NCBI non-redundant protein database using BLASTp v2.2.30 (Altschul et al., 1997). Protein conserved domains were predicted using InterProScan v5.0 (Jones et al., 2014), Signal IP v4.0 (Petersen et al., 2011) and Lipo P v1.0 (Juncker et al., 2003)

10.7 Host Range

To determine the ability of each phage to infect 41 clinical ETEC strains, phage suspensions at 10^8 are mixed with 100µl of an overnight culture. 4ml of melted

soft agar are added and then poured onto an LB agar plate and kept for solidification. Plates are incubated overnight at 37°C. Lysis patterns were recorded for susceptible strains.

11. RESULTS

11.1 Characterization of an ETEC panel

The prototypical ETEC strain *E. coli* H10407 (Serotype O78:H11) was used as control for the ETEC panel. H10407 expresses toxins ST, LT and CFA/I. A multiplex PCR analysis confirmed the presence of ST (190bp) and LT (450bp) fragments (Figure 8). Whereas an independent PCR was performed to demonstrate the presence of CFA/I (364bp) fragment (figure 9), which according to the literature, *E. coli* H10407 encodes in the same plasmid as ST toxins.

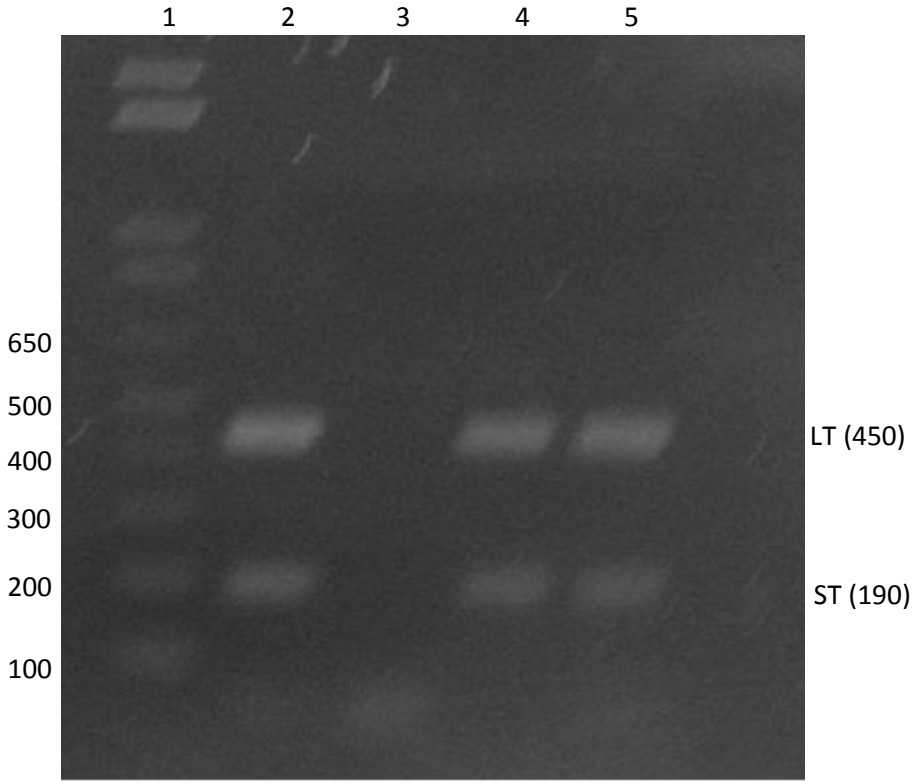


Figure 8. 2% agarose gel of PCR products for ST and LT locus of ETEC H10407. Lane 1: 1kb molecular weight marker in base pairs. Lane 2: Positive control. Lane 3: Negative control. Lane 4: *E. coli* H10407. Lane 5: Positive control. ST and LT fragments are indicated.

Forty one ETEC strains isolated from patients in different parts of Mexico, biochemically characterized and with established ST and LT toxin type were used for the creation of the panel. To confirm the presence of

enterotoxin genes, bacterial lysate was used to perform the multiplex PCR for ST and LT toxins. PCR results confirmed that all of the ETEC clinical isolates harbored genes encoding ST, LT or both enterotoxins, necessary criteria for being ETEC strains (Figures 10-12). Colonization factors are the other major virulence factor present in ETEC. Studies estimate that CFA/I, CFA/II or CFA/IV are present in up to 75% of clinical isolates (Gaastra & Svennerholm, 1996), so we sought to determine the presence of this colonization factors in our previously identified ETEC panel. We use the same bacterial lysate as a template for 3 separated PCR assays performed in the 41 clinical isolates.

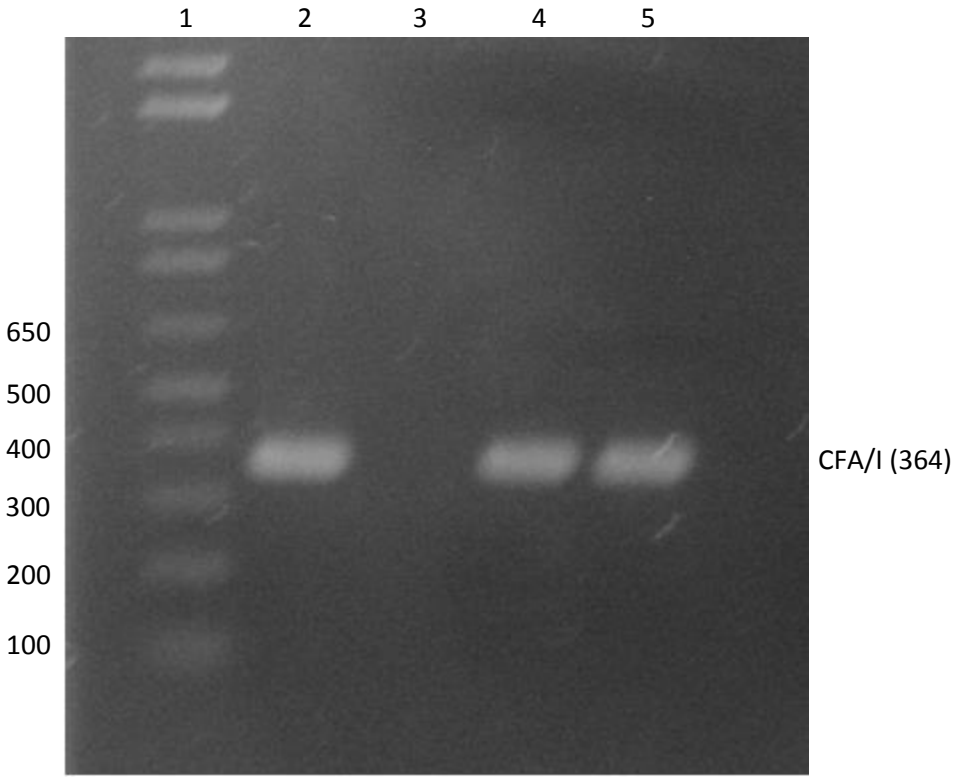


Figure 9. 2% agarose gel of PCR product for CFA/I locus of ETEC H10407. Lane 1: 1kb molecular weight marker in base pairs. Lane 2: Positive control. Lane 3: Negative control. Lane 4: *E. coli* H10407. Lane 5: Positive control. CFA/I fragment is indicated.

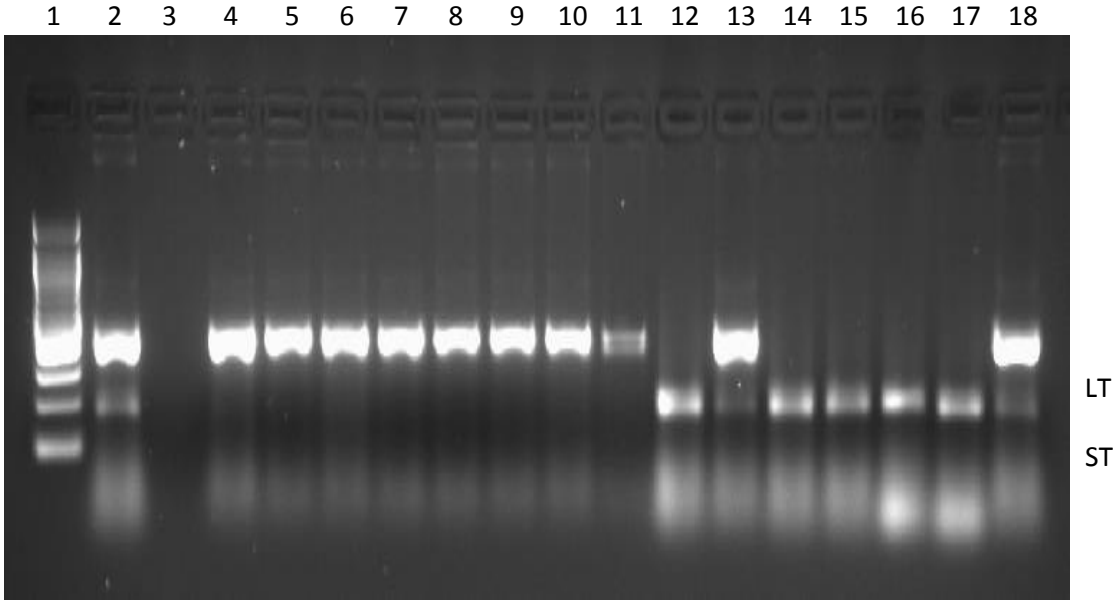


Figure 10. 2% agarose gel of PCR products for ST and LT locus of ETEC clinical isolates. Lane 1: 1kb molecular weight marker. Lane 2: ETEC H10407. Lane 3: Negative control. Lane 4 - 18: ETEC isolates 16, 17, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, 31, 30.

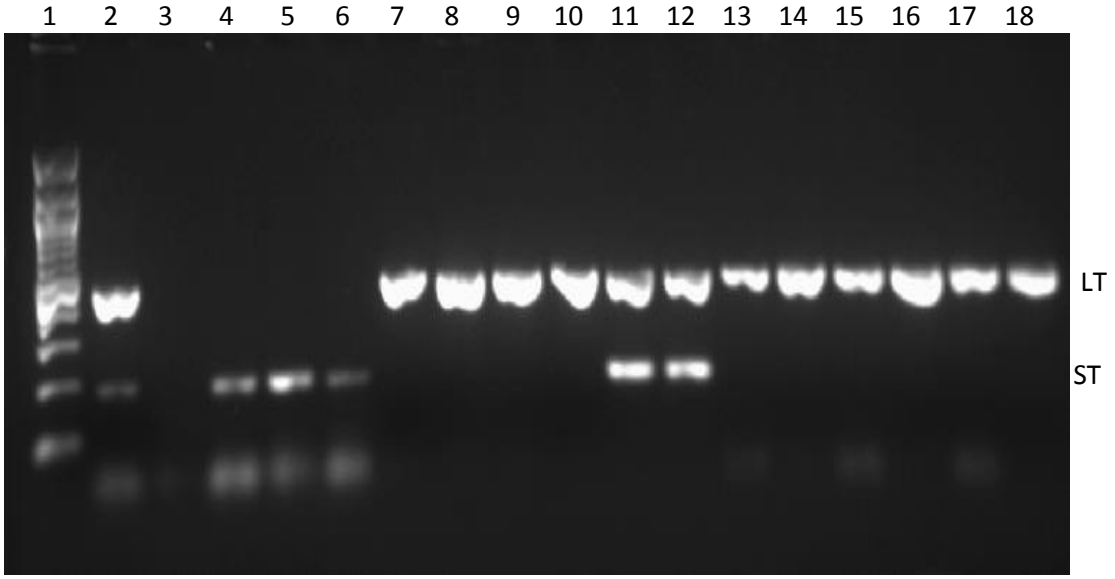


Figure 11. 2% agarose gel of PCR products for ST and LT locus of ETEC clinical isolates. Lane 1: 1kb molecular weight marker. Lane 2: ETEC H10407. Lane 3: Negative control. Lane 4 - 18: ETEC isolates 1, 2, 3, 4, 5, 6, 7, 8, 12, 9, 10, 11, 13, 14, 15.

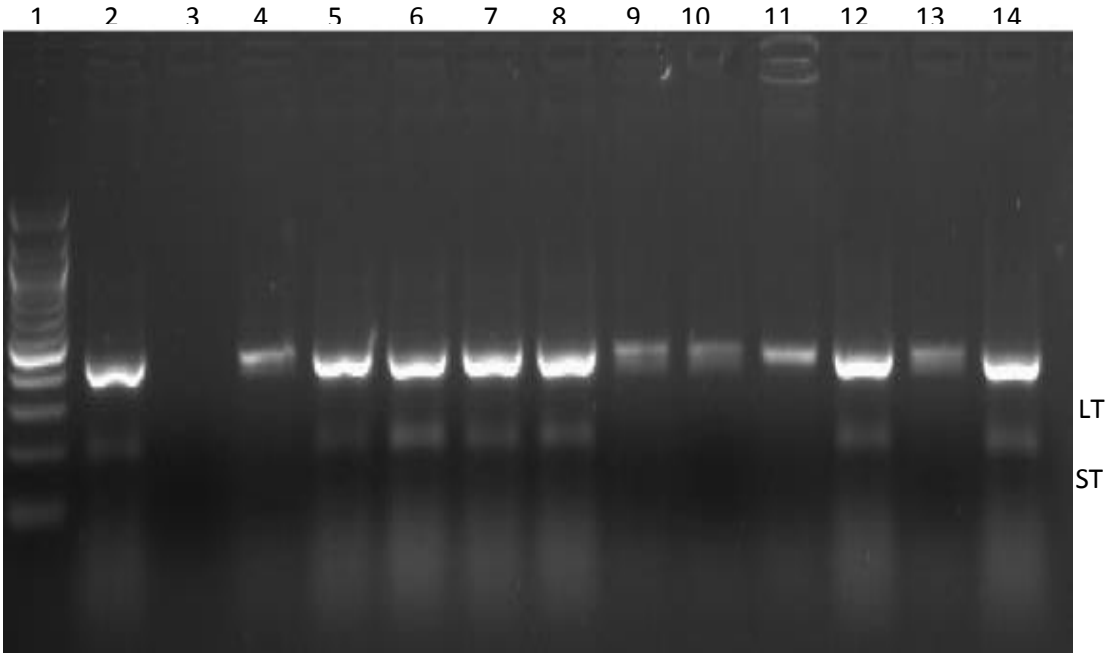


Figure 12. 2% agarose gel of PCR products for ST and LT locus of ETEC clinical isolates. Lane 1: 1kb molecular weight marker in base pairs. Lane 2: Positive control ETEC H10407. Lane 3: Negative control. Lane 4 - 14: ETEC isolates 24, 33, 35, 36, 39, 32, 34, 37, 40, 38, 41.

Four out of 41 strains are positive to CFA/I (figure 13). 7 strains possess that gene that encoded for CFA/II (figure 14) and, lastly, CFA/IV was the most prevalent, present in a total of 14 strains (figure 15). The 3 CFs were present in 25 out of 41 clinical isolates, representing 60% of the total.

In summary, we constructed a panel of a total of 41 ETEC clinical isolates, of which 8 are positive for ST toxin, 23 for LT toxin, and 10 for ST and LT toxins. These results are summarized on tables 3 and 4.

PCR characterization of ETEC clinical isolates

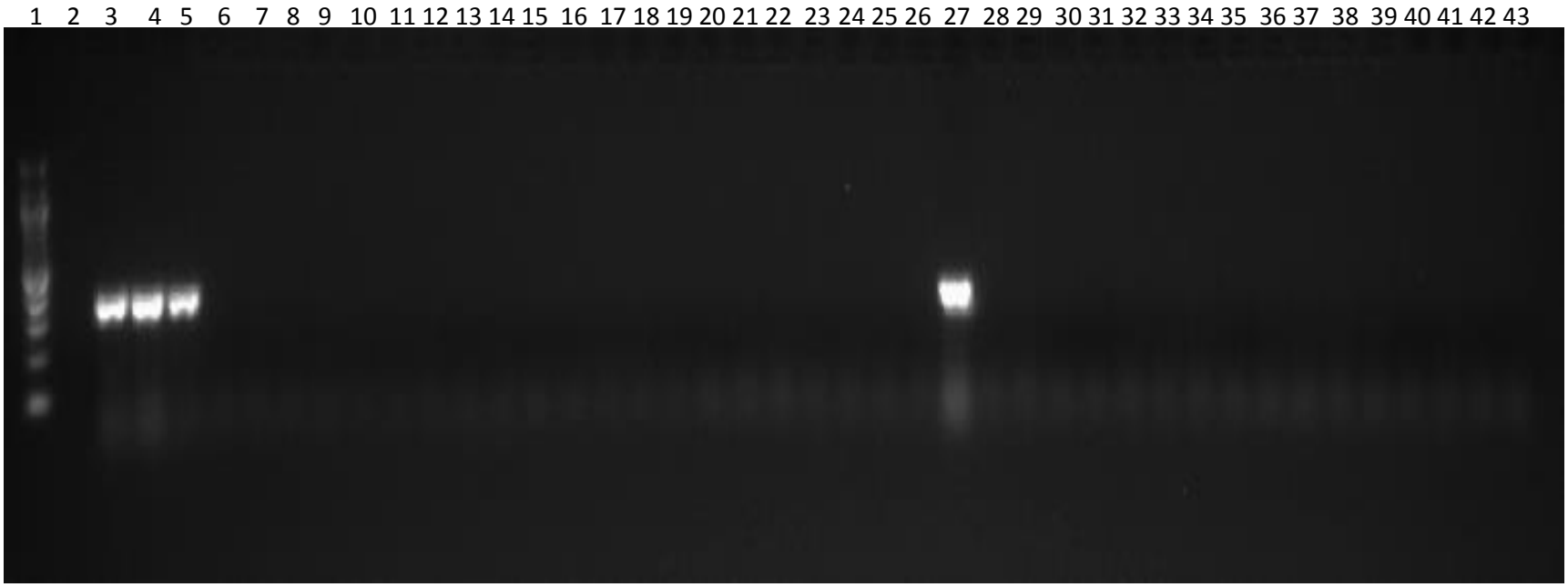


Figure 13. 2% agarose gel of PCR products for CFA/I locus of ETEC clinical isolates. Lane 1: 100bp DNA ladder. Lane 2: Negative control (no DNA). Lanes 3 - 43: ETEC clinical isolates in order from 1 to 41. 4 clinical isolates amplified for CFA/I.

PCR characterization of ETEC clinical isolates

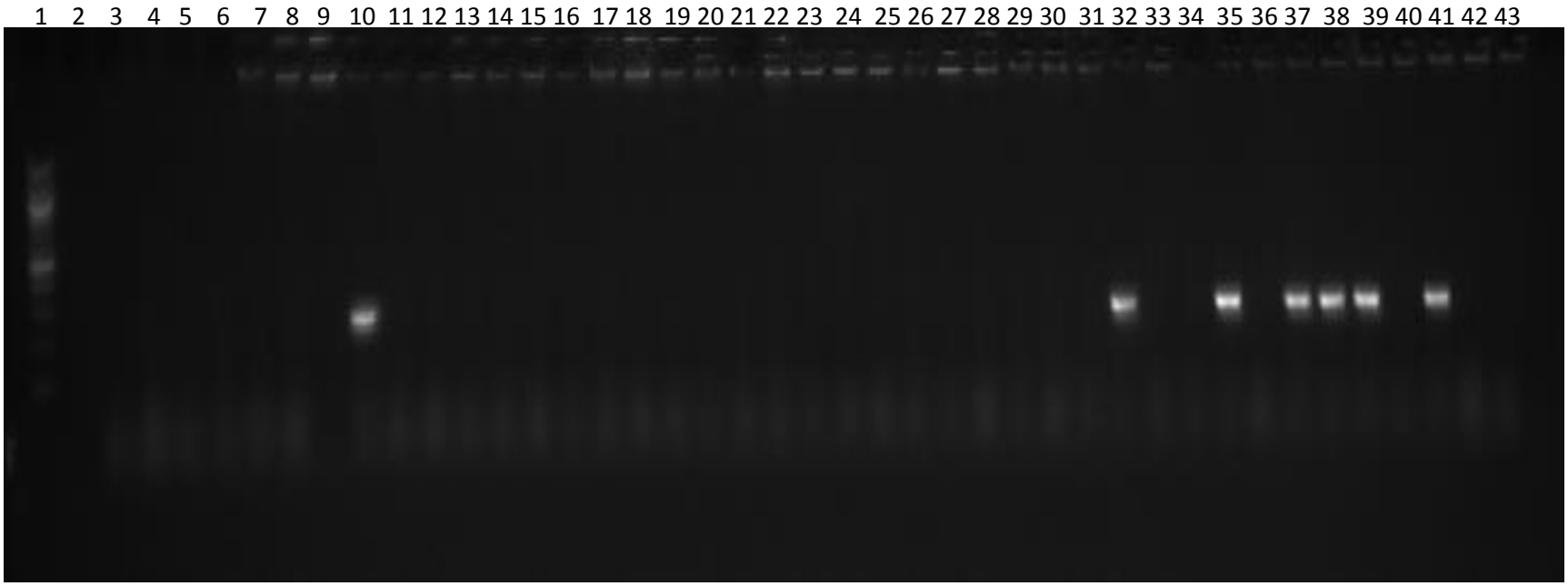


Figure 14. 2% agarose gel of PCR products for CFA/II locus of ETEC clinical isolates. Lane 1: 100bp DNA ladder. Lane 2: Negative control (no DNA). Lanes 3 - 43: ETEC clinical isolates in order from 1 to 41. 7 clinical isolates amplified for CFA/II.

PCR characterization of ETEC clinical isolates

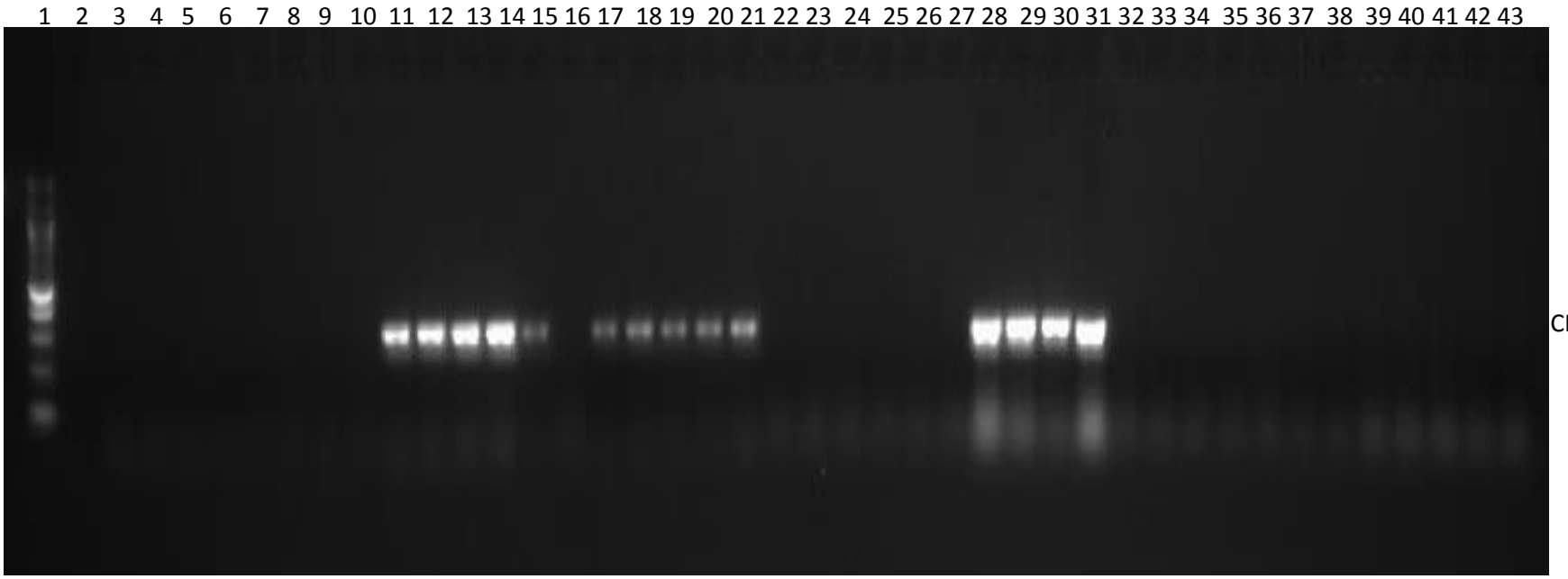


Figure 15. 2% agarose gel of PCR products for CFA/IV locus of ETEC clinical isolates. Lane 1: 100bp DNA ladder. Lane 2: Negative control (no DNA). Lanes 3 - 43: ETEC clinical isolates in order from 1 to 41. 14 clinical isolates amplified for CFA/II.

Table 3. PCR assay of common virulence factors of ETEC clinical isolates.

	Gene				
Strain	**ST**	**LT**	**CFAI**	**CFAII**	**CFAIV**
ETEC 1	+		+		
ETEC 2	+		+		
ETEC 3	+		+		
ETEC 4		+			
ETEC 5		+			
ETEC 6		+			
ETEC 7		+			
ETEC 8	+	+		+	
ETEC 9		+			+
ETEC 10		+			+
ETEC 11		+			+
ETEC 12	+	+			+
ETEC 13		+			+
ETEC 14		+			
ETEC 15		+			+
ETEC 16		+			+
ETEC 17		+			+
ETEC 18		+			+
ETEC 19		+			+
ETEC 20		+			
ETEC 21		+			
ETEC 22		+			
ETEC 23		+			
ETEC 24		+			
ETEC 25	+		+		
ETEC 26	+	+			+
ETEC 27	+				+
ETEC 28	+				+
ETEC 29	+				+
ETEC 30	+	+		+	
ETEC 31	+				
ETEC 32		+		+	
ETEC 33	+	+			
ETEC 34		+		+	
ETEC 35	+	+			
ETEC 36	+	+			
ETEC 37		+		+	
ETEC 38		+		+	
ETEC 39	+	+			
ETEC 40	+	+			
ETEC 41	+	+		+	

*First column identifies the ETEC strains by number. The following columns marks bacteria positive for ST and LT enterotoxins and colonization factors CFA/I, CFA/II and CFA/IV from a PCR analysis.

Table 4. ETEC clinical isolates genotypes and number of positive strains by gene.

Number of strains	Strain genotypes	Positive strains
41	ST	8
	LT	23
	ST-LT	10
	CFA/I	4
	CFA/II	7
	CFA/IV	14

*ETEC strains are defined as ST, LT or ST-LT positives. 25 out of 41 strains were positive for the 3 most common colonization factors CFA/I, CFA/II and CFA/IV.

11.2 P1 transduction on ETEC H10407

For the construction of TolC deletions we choose to use P1 transduction. Briefly, the procedure uses Bacteriophage P1, a phage that occasionally packages DNA of the host bacteria into the protein capsid instead of its own. When P1 is used to infect another host, it will transfer a DNA fragment from the previous host into the new strain, in which it can be incorporated through recombination (Thomason, 2007). However, there's a lack of literature on P1 transduction in pathogenic *E. coli* strains. Therefore, we set out to determine if P1 phage is able to perform gene transduction in ETEC strains using prototypical strain H10407 and non-pathogenic *E. coli* strains BW25113 and P90c as controls. Strain *E. coli* B25113 tolc::kan was the parental strains for the TolC deletion.

After transduction, mutant colonies were directly selected as Kanamycin resistant and SDS sensitive. We performed a PCR analysis by flanking the inner region of TolC (primers TolC fw and TolC rv) that would yield a fragment of 1450bp. fourteen candidate colonies were obtained in strain BW225113 (figure 16). Lane 2 shows the fragment amplified from BW25113 and lanes 4 to 17 show that none of the 14 colonies obtained had the correspondent fragment, meaning that transduction was successful. Seven candidate colonies were obtained in strain P90c (Figure 17, lanes 4 to 10). None of the 7 obtained colonies had TolC. Only 3 candidate colonies were obtained in strain ETEC H10407 (Figure 17, lanes 14 to 16). Lane 12 is the

amplified fragment of TolC in H10407 and lanes 14 to 16 show that neither candidate has TolC. It is worth mention that the transduction efficiency was five times lower compared to BW25113, and less than half with P90c, the non-pathogenic control used in this experiment. Also, P1 was not able to form plaques or lyse the culture in H10407.

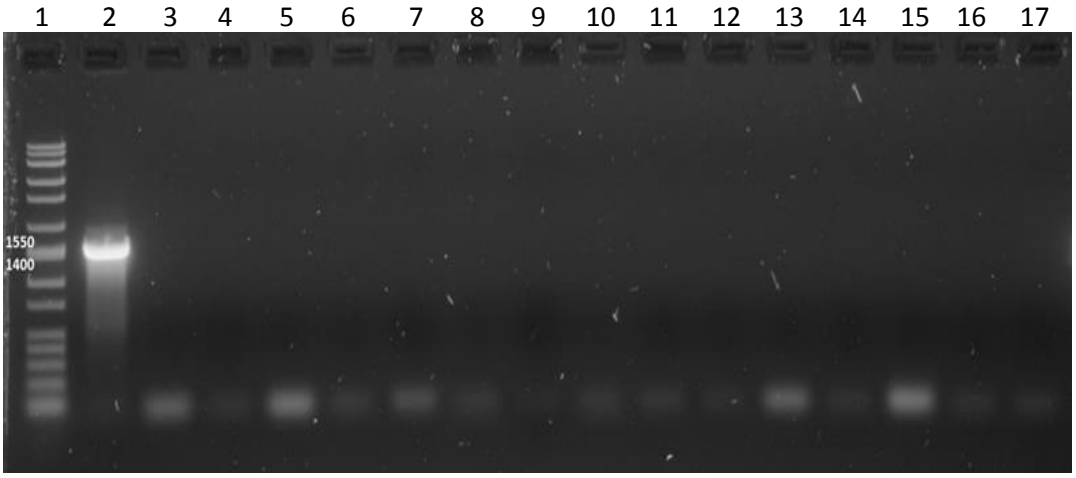


Figure 16. 1% Agarose gel of PCR products for TolC locus in BW25113 transductants. Lane 1: Molecular weight marker in base pairs. Lane 2: E. coli BW25113. Lane 3: Negative control. Lanes 4-17 BW25113 transductans.

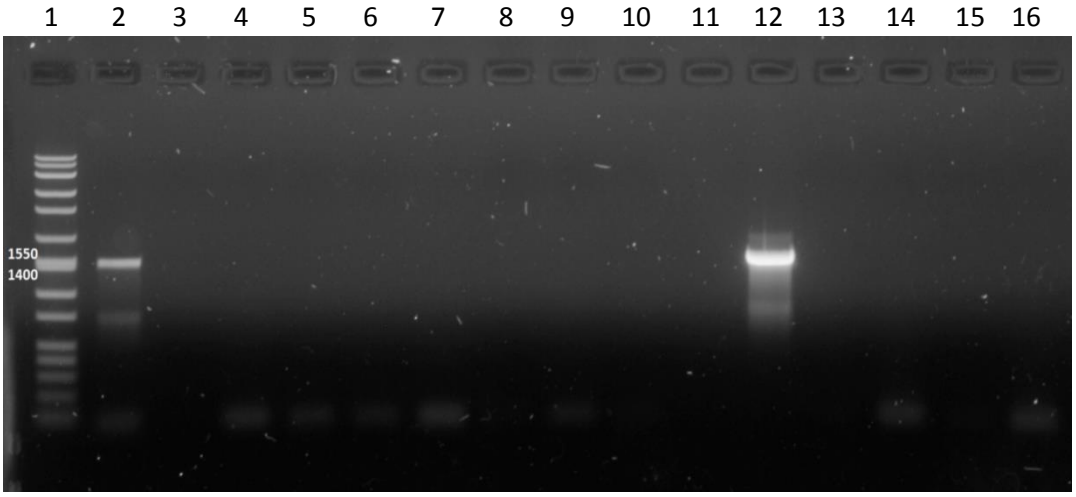


Figure 17. 1% Agarose gel of PCR products for TolC locus in E. coli P90c and ETEC H10407 transductants. Lane 1: Molecular weight marker in base pairs. Lane 2: E. coli P90c. Lane 3: Negative control. Lanes 4-10: P90c transductants. Lane 12: ETEC H10407. Lane 13: Negative control. Lanes 14-16: ETEC H10407 transductants.

Then, we checked if ETEC H10407 transductant had the correct genomic structure. We performed 2 PCR reactions using primers A1 and C2 coupled with kanamycin specific primers K1 and K2 (Baba et. al., 2006) as in figure 6. Presence of a 745bp product on the upstream region (figure 18) and a 1600bp (figure 19) downstream indicate that Kanamycin cassette had correctly replaced TolC locus in H10407. Donor strain BW25113 tolc::kan was used as positive control

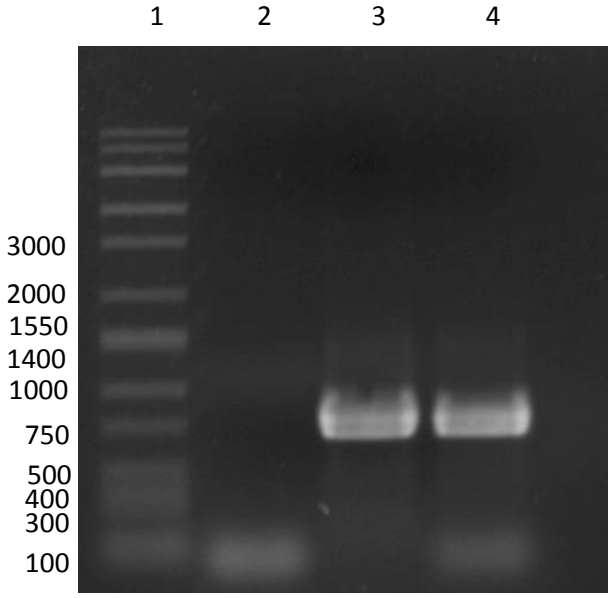


Figure 18. 1% agarose gel of PCR product of tolc::kan upstream region in ETEC H10407. Lane Wide range DNA marker in base pairs. Lane 2. Negative control (no DNA). Lane 3. E. coli BW25113 tolc::kan. Lane 4. ETEC H10407 tolc::kan.

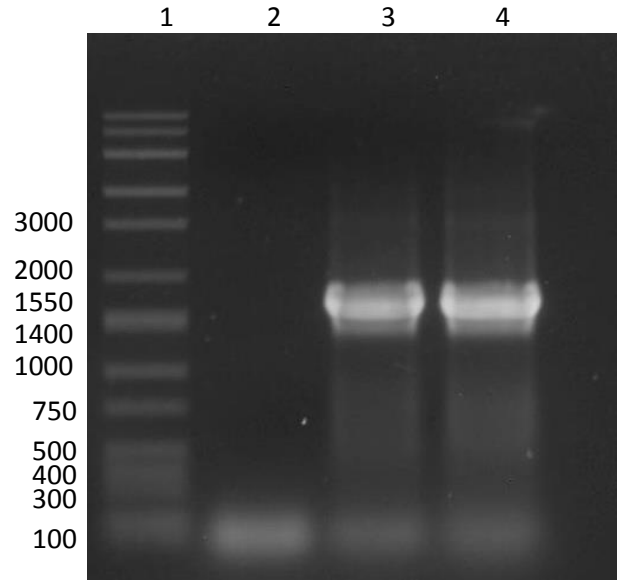


Figure 19. 1% agarose gel of PCR product of tolc::kan downstream region in ETEC H10407. Lane Wide range DNA marker in base pairs. Lane 2. Negative control (no DNA). Lane 3. E. coli BW25113 tolc::kan. Lane 4. ETEC H10407 tolc::kan.

11.3 Construction of knockouts for TolC gene in ETEC clinical isolates

Once demonstrated that it is possible to perform P1 transduction in the prototypical ETEC strain H10407 we proceeded to construct a panel of TolC knockouts using the ETEC clinical isolates. Due to the fact that 40% of clinical isolates were not positive for the CFs tested, and the lack of data about the antigens present in the surface of clinical isolates, we decided to use as many strains possible for the construction of the ETEC *tolc::kan* panel, in this way we would be able to overcome unexpected diversity in the cell surface of the bacteria that could interfere with TolC exposure and bacteriophage adsorption.

Transduction experiments yielded different rates between the clinical strains, with an estimate of 10-100 KmR colonies. Nevertheless, we found a high proportion of antibiotic-resistant transformants without a tolC gene replacement. Each Petri dish was streaked in replica grids of LB alone and LB with SDS at a 0.01% concentration. SDS sensitive colonies were verified by a PCR assay for the presence of the locus-Kanamycin specific primers of predicted sizes.

Fifteen mutants (Figure 20, lanes 4-18) amplify for the correspondent fragment of 745nt corresponding to the upstream region of *tolc* and Kanamycin specific primer and also for the fragment of 1609nt that corresponds to the downstream region of TolC (figure 21, lanes 4-18).

Listed on table 5 are the different characteristics of the transduced clinical isolates. We were able to transduce in strains with CFA/I (3), CFA/II (2), CFA/IV (6) and 4 strains not positive for the CFAs tested.

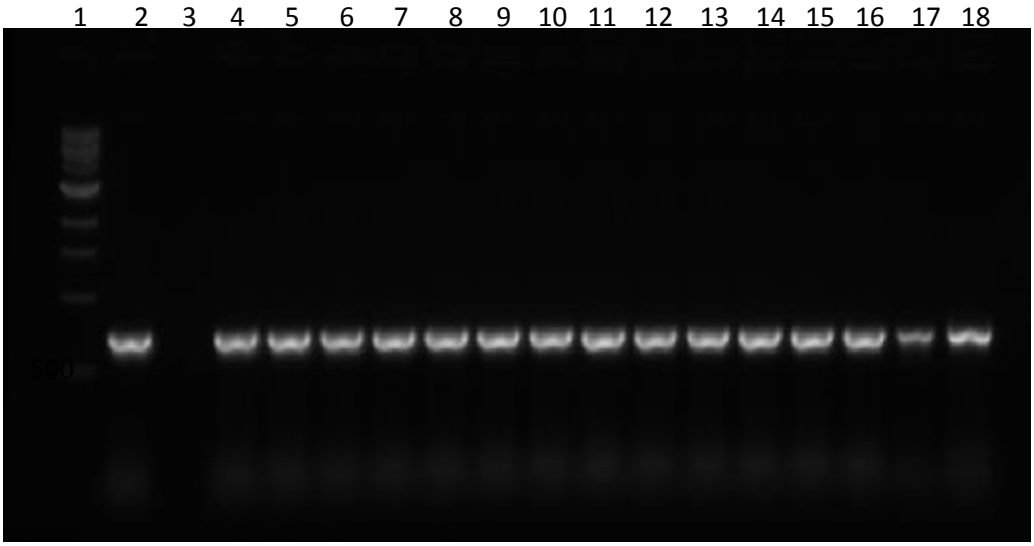


Figure 20. 1% agarose gel of PCR product of tolc::kan upstream region in ETEC clinical isolates mutants. Lane 1 Molecular weight marker. Lane 2. ETEC H10407 tolc::kan. Lane 3 Negative control. Lane 4 - 18. ETEC tolc::kan clinical isolates.

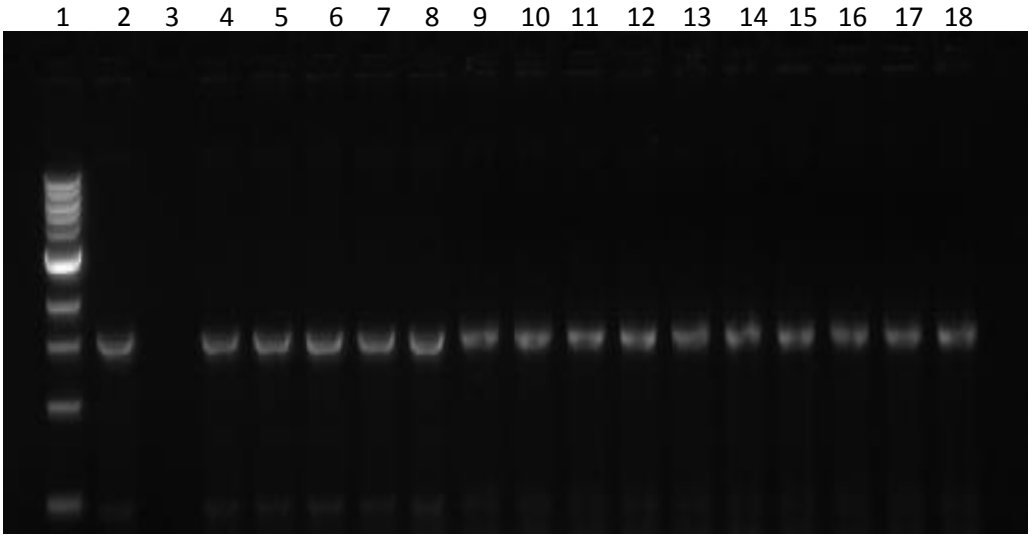


Figure 21. 1% agarose gel of PCR product of tolc::kan downstream region in ETEC clinical isolates mutants. Lane 1 Molecular weight marker. Lane 2. ETEC H10407 tolc::kan. Lane 3 Negative control. Lane 4 - 18. ETEC tolc::kan clinical isolates.

Table 5. Characteristics of ETEC clinical isolates transduced with P1.

	Gene				
Strain	**ST**	**LT**	**CFAI**	**CFAII**	**CFAIV**
ETEC 1 tolc::kan	+		+		
ETEC 3 tolc::kan	+		+		
ETEC 8 tolc::kan	+	+		+	
ETEC 11 tolc::kan		+			+
ETEC 12 tolc::kan	+	+			+
ETEC 13 tolc::kan		+			+
ETEC 15 tolc::kan		+			+
ETEC 20 tolc::kan		+			
ETEC 22 tolc::kan		+			
ETEC 24 tolc::kan		+			
ETEC 25 tolc::kan	+		+		
ETEC 26 tolc::kan	+	+			+
ETEC 27 tolc::kan	+				+
ETEC 30 tolc::kan	+	+		+	
ETEC 36 tolc::kan	+	+			

11.4 Bacteriophage isolation

Enrichments from 10 water samples obtained from different locations in College Station, TX, were analyzed for differential activity against the 15 wild-type ETEC strains and their isogenic tolC::kan mutants.

One of the enrichments had a titer of $9\pm4x10^{8}$ PFU in strain ETEC 22 (Figure 22, lanes 1 and 2), but had no activity against the isogenic mutant ETEC 22 tolC::kan (Figure 23, lanes 1 and 2). In order to have a pure clonal population of the phage purified phage, one of the plaques was streaked and further purified. This stock had a titer of $29\pm5x10^{8}$ PFU in strain ETEC 22 (Figure 22, lanes 3 and 4) and no activity against the isogenic mutant (Figure 23, lanes 3 and 4). This suggests that the enrichment population was mostly constituted by a single phage type.

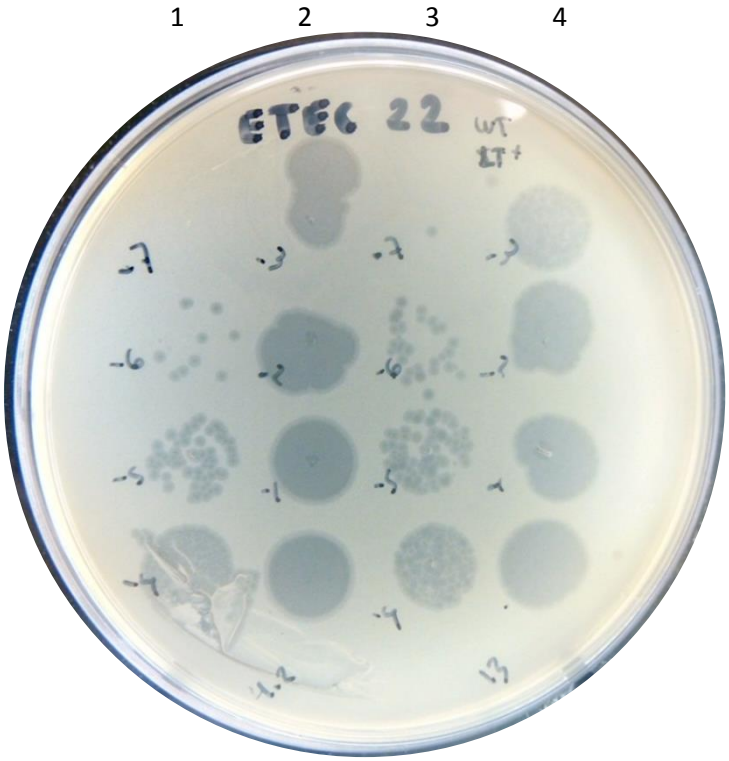


Figure 22. Double agar layer plate of ETEC 22. Lanes 1 and 2 show the plating of phage enrichment for ETEC 22, starting with 10µl undiluted sample (Lane 2, bottom) to 10^{-7} dilution (Lane 1, top). Lanes 3 and 4 show the plating of a purified plaque, starting with 10µl of undiluted sample (Lane 4, bottom) to 10^{-7} dilution (Lane 3, top).

1 2 3 4

Figure 23. Double agar layer plate of ETEC 22 tolC::kan. Lanes 1 and 2 show the plating of phage enrichment for ETEC 22, starting with 10µl undiluted sample (Lane 2, bottom) to 10^{-7} dilution (Lane 1, top). Lanes 3 and 4 show the plating of a purified plaque, starting with 10µl of undiluted sample (Lane 4, bottom) to 10^{-7} dilution (Lane 3, top).

Phage morphology shows a clear 1-2mm diameter plaque with a translucent halo, (Figure 24) and was named Stilgar, according to the rules of the Center for Phage technology (Dr. Young's lab). The isolated phage stock was stored in SM buffer and kept at 4°C, this was used for further characterization.

Figure 24. Double layer agar plate of ETEC 22. Purified bacteriophage plaque.

Phage adsorption assay

To initiate the infection process, the phage uses a specific surface determinant on the host cell as a receptor to which to absorb. Lack of the receptor will lead to impaired adsorption, and therefore failure to infect the bacterial host. To confirm that the phage uses TolC as a receptor, we performed an adsorption assay to compare between ETEC 22 WT and ETEC 22 tolC::kan.

Concentration of free phage declined up to 40% at 18mins when incubated with ETEC 22 (Figure 25), meaning that nearly 60% of the phage was already adsorbed to the bacteria at the time. In contrast, with ETEC 22 tolC::kan a decline in the concentration of free phage was barely noticeable compared to control group. The absence of the receptor does impair the ability of the phage to adsorb to the bacteria, and therefore unable to initiate an infection process.

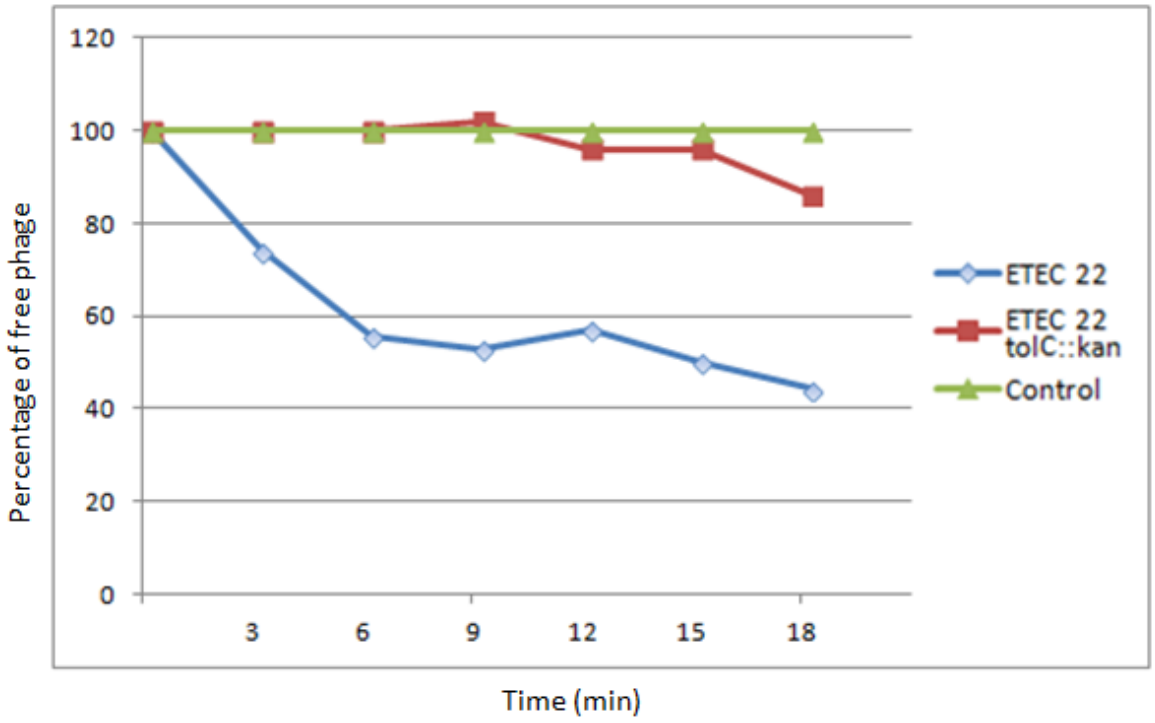


Figure 25.- Adsorption assay of phage Stilgar with ETEC 22 and ETEC 22 tolC::kan. Percentage of unabsorbed phage plotted against time. Blue line with rhombs indicate the assay for ETEC 22. Red line with squares indicates the assay for ETEC 22 tolC::kan. Green line with triangles is the control (no bacteria).

11.5 Phage Characterization

Restriction Assay

To further characterize the phage, purified genomic DNA was treated with EcoRV, HindIII, KpnI, Xbal, EcoRI, SmaI to generate restriction fragment patterns. Phage Stilgar was resistant to EcoRI, HindIII (data not shown) and KpnI. Phage was cleaved by EcoRV, XbaI and SmaI. DNA fragments were resolved by 0.7% agarose gel electrophoresis (Figure 26). Enzymes were able to cleave Stilgar in 1 site, producing 2 DNA fragments.

Electron Microscopy

Transmission Electron Microscopy was performed to determine Stilgar's morphology. It has an icosahedral capsid with a diameter of 50nm, a long

contractile tail of approximated 130nm, meaning that is a Myophage (figure 27).

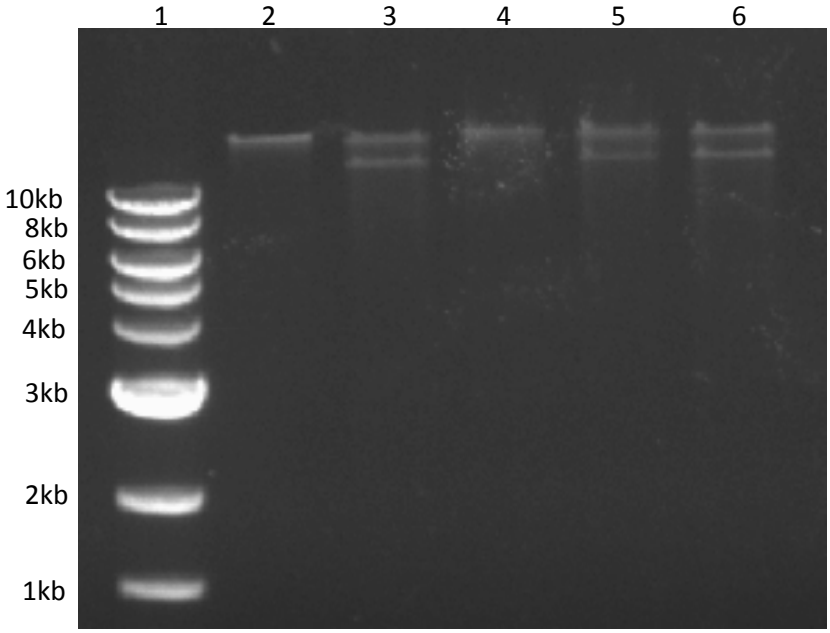


Figure 26. 0.7% agarose gel of phage Stilgar genomic DNA restriction digest. Lane 1: Molecular marker in base pairs. Lane 2: Untreated DNA. Lane 3: DNA + EcoRV. Lane 4: DNA + KpnI. Lane 5: DNA + XbaI. Lane 6: DNA + SmaI.

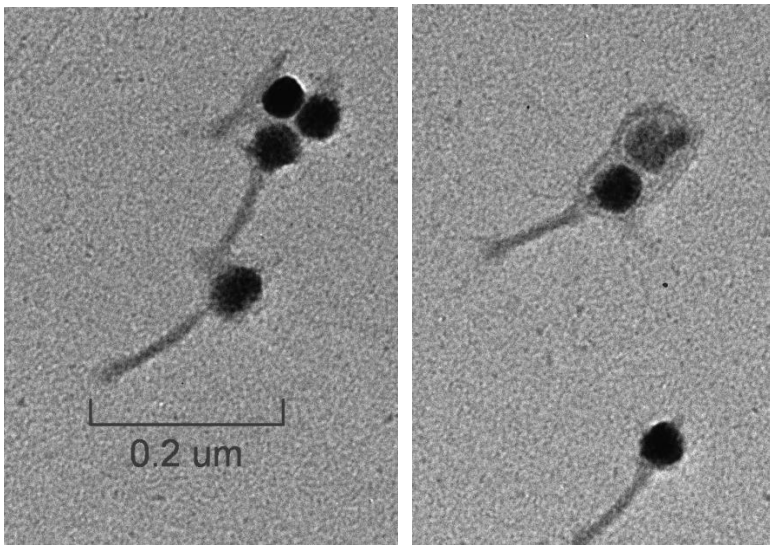


Figure 27.- Transmission electron microscopy images of phage Stilgar. Both images show a magnification of 50K. Scale bar correspond to 200nm.

Genome annotation

We continued with phage characterization by sequencing and genome annotation. Besides classification, this help us to identify if Stilgar has potentially harmful genes or carries virulence factors, unwanted features of a phage with therapeutic purposes. Stilgar is a 41,047-bp phage with a G+C content of 52.57%, 90.9% coding density, and 56 predicted coding sequences (cds), out of which 30 are of known function. It has 11 predicted rho-independent terminators and 1 coding sequence with a Rho termination factor domain. No tRNA genes where found in Stilgar's genome.

Figure 28 shows a map of the 56 predicted genes in Stilgar. Genome map starts with the DNA packaging portion, which includes small (cds 1) and large subunits of the terminase. Genes involved in morphogenesis include the portal protein and major capsid protein, followed by a protein with a Rho termination factor domain. Genes 9 to 24 include tail fibers, tail tube, tail sheath, tail-collar fiber, tail tape measure protein, and baseplate assembly protein. After the tail genes, cds 25 and 27 represent two transcriptional regulators. From cds 30 to 46 we have a few DNA replication/recombination proteins identified: a single-strand binding protein, putative replicative DNA helicase, an exonuclease involved in the recE recombination pathway, helicase, primase, and an endonuclease. From cds 50 to 56 we have the lysis cassette consisting of a predicted holin with three transmembrane domains (class I), a λ-like R endolysin and a spanin complex consisting of an i-spanin and o-spanin present in embedded genes, homologous to Rz and Rz1 (Summer et al., 2007). Stilgar also encodes a GpW/Gp25/anti-adapter protein IraD (cds 13), a structural component of the outer wedge of the baseplate with acidic lysozyme activity. It also contains a tail spike-like protein with a pectin lyase domain fold (cds 16), a microbial virulence factor (Mayans et al., 1997).

A phylogenetic analysis reveals that Stilgar terminase large subunit's closest relatives among well characterized phages are phage Lambda and N15,

known for producing 5' extended cohesive ends with no circular permutation or terminal redundancy (Casjens et al., 2005). Stilgar shares a 51.6% nucleotide sequence identity to Escherichia phage EcoM-ep3 (NC_025430.1). Blastx reveals homology in the terminase subunits, capsid and tail proteins. The presence of 2 predicted transcriptional regulators after tail genes is a feature in both phages. Replicative DNA helicase, helicase and primase are some recombination/replication genes that share homology. In the lysis cassette, EcoM-ep3 has a predicted holin and a hypothetical protein that shares homology with the predicted spanin complex in Stilgar (Figure 29).

Pseudomonas phage PPpW3 (NC_023006.1) also shares homology with Stilgar. They share homology in the terminase subunits, both small and large, portal and major capside proteins, baseplate assembly and tail proteins. Tail spike-like protein with a pectin lyase domain fold present on Stilgar does not share homology with genes in PPpW3. Different to both Stilgar and ep3, PPpW3 only has one transcriptional regulator after the tail genes. Genes involved in recombination/replication include a ssDNA binding protein, replicative DNA helicase, helicase and primase. Finally, PPpW3 and Stilgar share homology in the lysis cassette, with a predicted holing and spanin genes, but not on the endolysin. PPpW3 has a larger genome than Stilgar and presents several predicted coding sequences between the lyses cassette and genes for recombination/replication (Figure 29).

Matches with well characterized phages include Enterobacteria phage P2 (NC_001895.1) and Lambda. However, in both cases homology is present in few genes, and is weak in most of the cases (Figure 30).

This analysis reveals that Stilgar resembles two myophages, not only in the genomic organization but in the ability to infect pathogenic bacteria. *Escherichia* phage EcoM-ep3 is able to lyse multi-drug-resistant clinical strains of pathogenic *E. coli* from chickens (Wang et al., 2015), while Pseudomonas phage PPpW3 is used for its therapeutic effect against *Pseudomonas*

plecoglossicida infection in ayu fish (Park and Nakai, 2003). No virulence factors or potentially harmful genes were identified in Stilgar's genome, so we conclude that this phage is safe for therapeutic use.

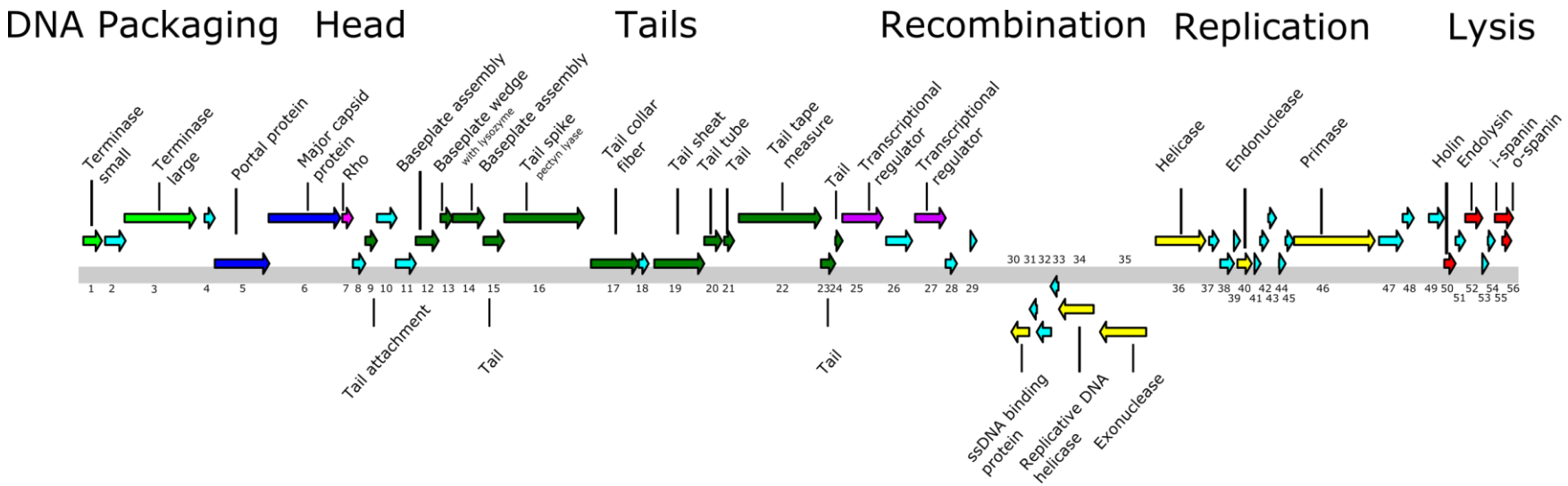


Figure 28. Genome map of phage Stilgar. Predicted genes are represented by arrows. Positive strand genes are above the gray line that represents dsDNA; negative strand genes are below the gray line. Position of the arrows represent different reading frames. Predicted function is given for some genes, represented in different colors according to the functional unit they belong to, highlighted above. Conserved hypothetical proteins or predicted genes of unknown function are represented in light blue. Sequence start is represented by the terminase small subunit.

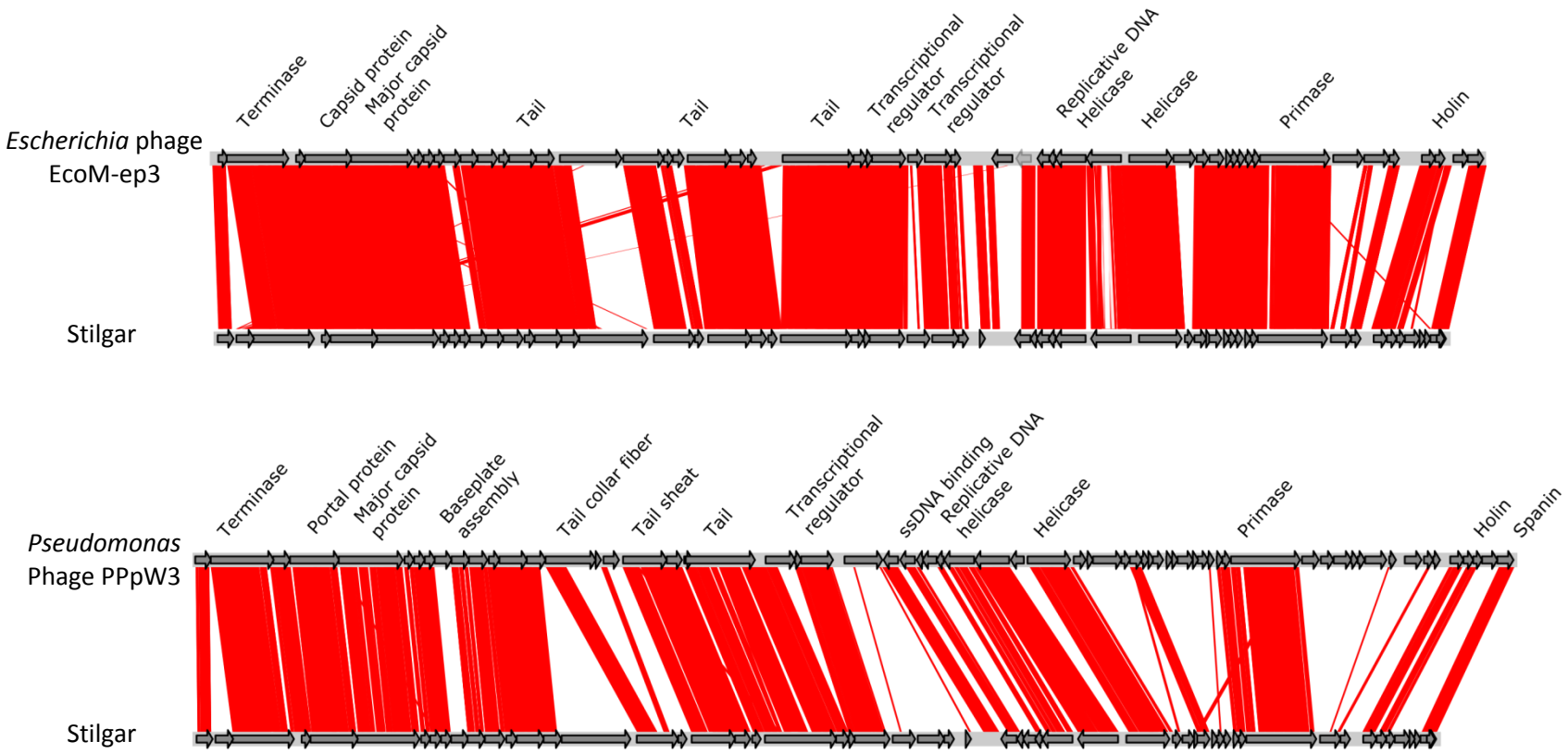


Figure 29. Genome comparison of phage Stilgar with *Escherichia* phage EcoM-ep3 (top) and *Pseudomonas* phage PPpW3 (bottom). Predicted genes are represented by arrows. Positive strand genes are represented by rightward arrows; negative strand genes are represented by leftward arrows. Red blocks vertically connect genes with similar function, stablished by protein sequence homology. Thin red lines indicate weak similarity. Predicted function for genes with homology between phages is given on top of the genome of EcoM-ep3 and PPpW3.

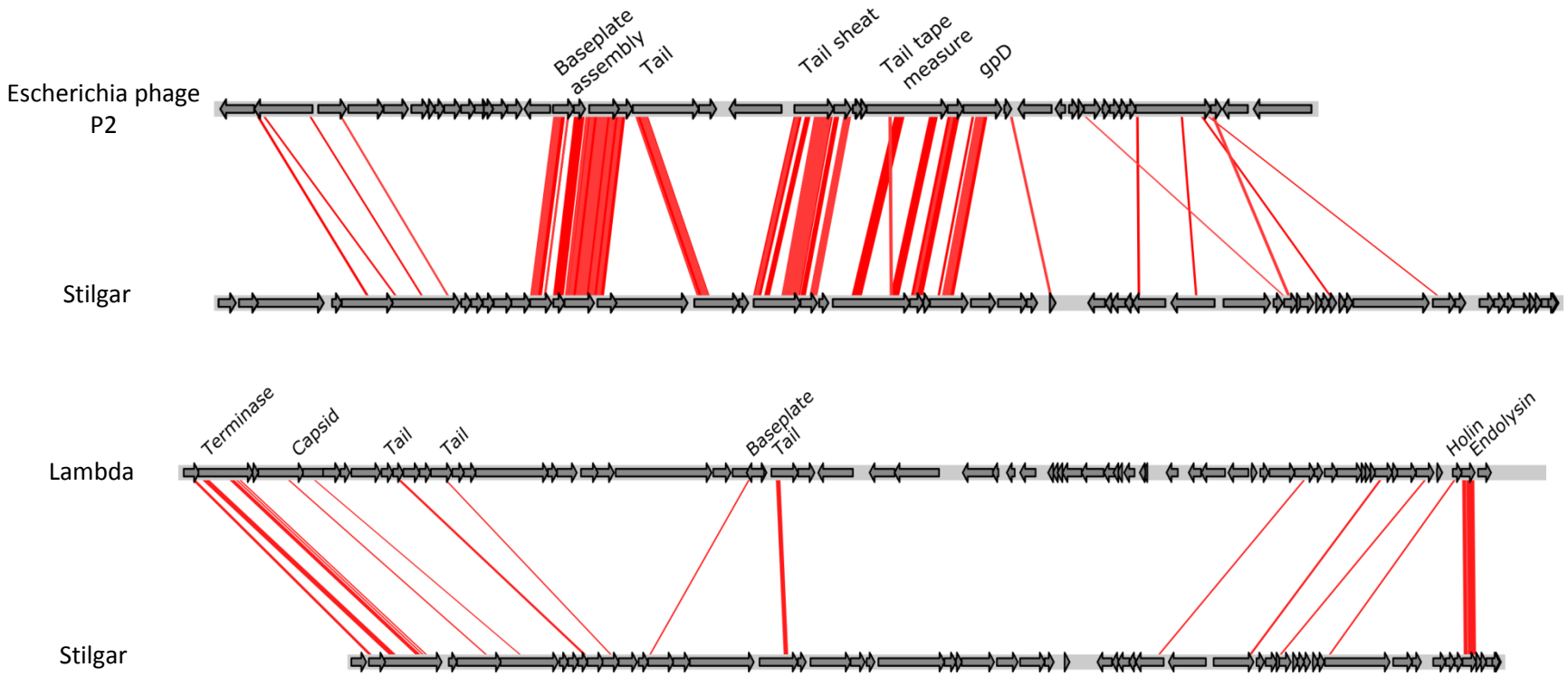


Figure 30. Genome comparison of phage Stilgar with P2 (top) and Lambda (bottom). Predicted genes are represented by arrows. Positive strand genes are represented by rightward arrows; negative strand genes are represented by leftward arrows. Red blocks vertically connect genes with similar function, stablished by protein sequence homology. Thin red lines indicate weak similarity. Predicted function for genes with homology between phages is given on top of the genome of P2 and Lambda

11.6 Host Range

The ability of phage Stilgar to infect 41 ETEC isolates was investigated. Phage suspensions at 10^8 are mixed with 100µl of an overnight culture. 4ml of melted soft agar are added and then poured onto an LB agar plate and kept for solidification. *E. coli* H10407 and BW25113 (K strain) were used as controls in this experiment.

Phage Stilgar was able to infect ETEC 8, 9, 11, 12, 13, 20, 22 (Host), 24 and 30, a total of 9 out of 41 clinical isolates. It was not able to infect strain H10407 or BW25113, showing it has a narrow host range and is specific for ETEC clinical isolates (Table 6).

Among the susceptible bacteria, they had different toxins and colonization factor profiles. It is important to assess the fact that this phage has a very narrow host range, this could be determined by different factors such as prophages present in the bacterial genome, restriction enzymes, surface determinants that obscure the receptor or mutations in the gene encoding the receptor.

Out of the strains susceptible to phage infection (other than ETEC 22), ETEC 8, 11, 12, 13, 20, 24, and 30 had an isogenic tolC::kan mutant. We next sought to determine if phage Stilgar would be able to infect other TolC null bacteria using the same procedure. None of the 7 mutant bacteria were susceptible to phage infection, confirming the idea that the phage uses TolC as a receptor to initiate an infection process.

Strain	Stilgar
ETEC 1	-
ETEC 2	-
ETEC 3	-
ETEC 4	-
ETEC 5	-
ETEC 6	-
ETEC 7	-
ETEC 8	+++
ETEC 9	+++
ETEC 10	-
ETEC 11	+++
ETEC 12	+++
ETEC 13	+++
ETEC 14	-
ETEC 15	-
ETEC 16	-
ETEC 17	-
ETEC 18	-
ETEC 19	-
ETEC 20	+++
ETEC 21	-
ETEC 22	+++
ETEC 23	-
ETEC 24	+++
ETEC 25	-
ETEC 26	-
ETEC 27	-
ETEC 28	-
ETEC 29	-
ETEC 30	+++
ETEC 31	-
ETEC 32	-
ETEC 33	-
ETEC 34	-
ETEC 35	-
ETEC 36	-
ETEC 37	-
ETEC 38	-
ETEC 39	-
ETEC 40	-
ETEC 41	-

Table 6. *In vitro* susceptibility of ETEC clinical isolates to phage Stilgar. First column identifies the ETEC bacteria. The following column marks bacteria sensitive to phage infection. +++ = confluent lysis ++ = partial lysis + = single plaques - = no lysis

Strain	Stilgar
ETEC 8 tolC::kan	-
ETEC 11 tolC::kan	-
ETEC 12 tolC::kan	-
ETEC 13 tolC::kan	-
ETEC 20 tolC::kan	-
ETEC 24 tolC::kan	-
ETEC 30 tolC::kan	-

Table 7. In vitro suceptiblity of ETEC tolC::kan strains to phage Stilgar. Left column identifies the strain. right column marks the bacteria sensitive or insentive to phage infection. +++ = confluent lysis ++ = partial lysis + = single plaques - = no lysis

Phage resistant mutants

The most common mutations that lead to phage resistance are deletions, insertions or truncations of the receptor protein. We sought to determine the frecuency by which phage-resistance bacteria is obtained and if this bacteria contained alterations within the *tolC* locus. $1x10^8$ bacteria of ETEC 22 were mixed with $1x10^7$ PFU of phage Stilgar, incubated for 10 min and then plated either on LB. It is known that *E. coli* mutants lacking TolC have a hightened sensitivity to SDS, so we plated the same mixture on LB + SDS 0.01% as selection method. The rationale behind this is that frecuency of spontaneous mutations will be lower on media with SDS.

Spontaneous mutants were obtained at a frecuency of 10^{-6} (Figure 31). The use of SDS as a selection method reduced tenfold the frecuency of phage resistant mutants, to 10^{-7} (figure 32). To determine if the phage resistant bateria had alterations within the TolC locus, we performed a PCR analisys using primers A1 and C2, that corresponds to a 2.4kb fragment that comprehends upstream and downstream regions of *tolC*. 8 resistant colonies were selected and analyzed. ETEC 22 amplifies the correspondent fragment of 2.4kb (Figure 33, lane 2), but phage resistant colonies do not show the presence of the same fragment (lanes 4-11), some colonies showed the presence of a 500bp fragment (lanes 4,5,6,9,11) while other did not amplify anything at all (lanes 7,8,10). This results show that bacteria resistant to phage

bacterial have alterations on the *tolC* locus, which confirms its role as a receptor.

Figure 31. Double agar layer plate of ETEC 22 mixed with phage Stilgar. 10^8 bacteria were mixed with 10^7 PFU of phage Stilgar and plated on LB media.

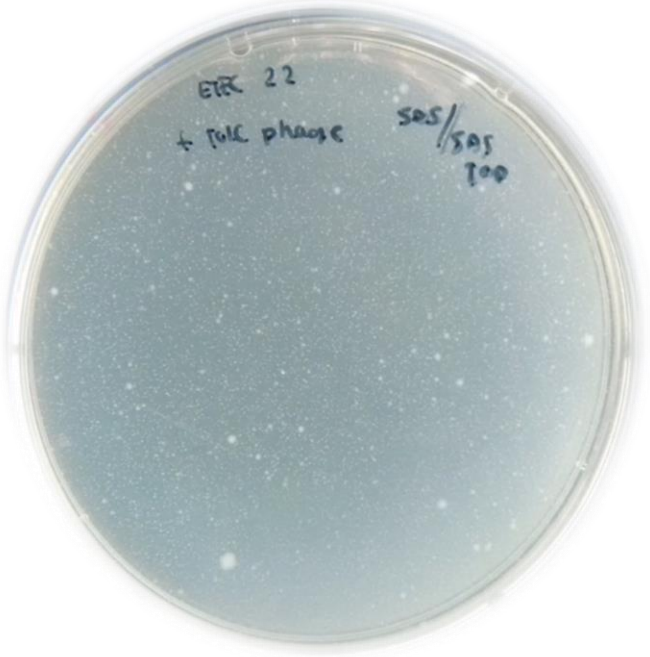


Figure 32. Double agar layer plate of ETEC 22 mixed with phage Stilgar. 10^8 bacteria were mixed with 10^7 PFU of phage Stilgar and plated on LB media containing SDS at a 0.01%

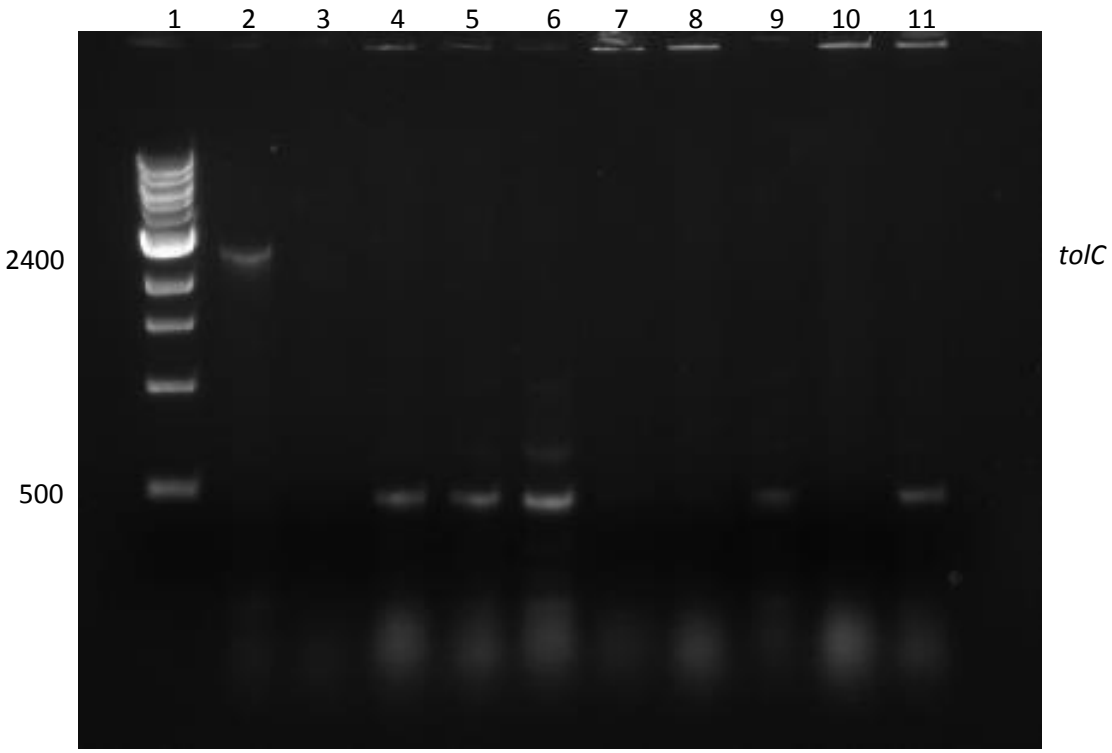


Figure 33. 1% agarose gel of PCR products for TolC locus of ETEC 22 phage resistant bacteria. Lane 1: Molecular weight marker in base pairs. Lane 2: ETEC 22 WT. Lane 3: Negative control. Lanes 4-11: ETEC 22 phage resistant bacteria.

12. DISCUSSION

Nowadays, ETEC stands as the main cause of *E. coli* mediated diarrhea and one of the most commonly isolated enteropathogens responsible for childhood diarrhea and traveller's diarrhea in developing countries (Gaastra and Svennerholm, 1996; DuPont, 2009; Kotloff et. al., 2013). ETEC production of enterotoxins ST and LT that results in loss of water and electrolytes from the stool, leading to dehydration that ranges from mild to severe (Black et al., 1981). Only if untreated could be fatal, however, it is still responsible for 400'000 deaths per year in children under the age of five (WHO, 1999).

Whereas different mechanisms have been developed for the diagnostic and identification of diarrheagenic *E. coli*, the easiest and most common technique is detection of genes through polymerase chain reaction (Estrada et al., 2003). Although there are several different factors that contribute to ETEC's virulence, we limited our characterization to toxins ST, LT and CFs, features that remain as its major determinants (Turner et al., 2006). All of the clinical isolates possess genes for at least one of the toxins, necessary criteria to be ETEC (Levine, 1987). CFs are present in the surface of bacteria, so in order to further characterize the ETEC panel and to gain insight on the possible challenges that phage would have to overcome in order to adsorb, we decided to search for the presence of CFA/I, CFA/II and CFA/IV. Although more than 20 CFs exist, these three groups are reported to be present on most clinical isolates worldwide (Gaastra & Svennerholm, 1996). In our case, only 60% of the ETEC panel had one of this CFs, being CFA/IV the most predominant. Further screening is required to determine the profile of the remaining 16 strains. This is important because CFs can also be a target for phage adsorption, as a phage specific for CS7-expressing strains of ETEC has been isolated (Begum et al., 2010).

For the isolation of a TolC-specific bacteriophage for, we needed to construct a panel of TolC knockouts in ETEC. Not only important in phage

selection, a panel of isogenic mutants would allow us to compare phage-sensitivity among ETEC strains. Recombineering with P1 is the method of regular use in the laboratory; however, there is a lack of literature for P1 transduction in ETEC. Some groups report virtually no success on ETEC, and others indicate that many pathogenic strains are incompatible with P1 transduction due to extensive surface antigens that block access to phage receptors (Lee, 2009). Therefore, we set out to determine if P1 was able to transduce in ETEC prototypical strain H10407. We obtained 3 H10407 transductants that had the correct genomic architecture. Interestingly, P1 was not able to form plaques when grown on H10407 in solid medium nor lyse a liquid culture. To discard the possibility of contamination, we performed a PCR for ST, LT and CFA/I on the H10407 transductants. They still had these virulence determinants, concluding P1 transduction is possible in ETEC. After that we set out to construct our TolC knockouts. We decided to use as many strains possible for the construction of the ETEC *tolc::kan* panel, in this way we would be able to overcome unexpected diversity in the cell surface of the bacteria that could interfere with TolC exposure and bacteriophage adsorption.

Out of 41 strains we were able to construct 15 TolC mutants. Transduction experiments yielded different rates between clinical strains, with an stimate of 10-100 kanamycin resistant colonies (kmR). Nevertheless, a high proportion of transformants without *tolc* gene replacement were found. This could be due to the fact that many pathogenic strains present increased antibiotic resistance and selection with kanamycin as the only selection method resulted cumbersome. Therefore, candidates obtained in LB + Kan were re-streaked LB + SDS 0.01%, a cut off value to select TolC deletions stablished by DeVito, 2008. The 15 mutants obtained had different toxin and CFs profiles.

Regarding phage isolation, the idea of mixing sewage samples with *tolc* null bacteria as the first step of the methodology was to deplete the sample of those bacteriophages that use any receptor other than TolC. After isolation of phage Stilgar we performed an adsorption assay comparing ETEC WT vs *tolC::kan* to determine if TolC is necessary for adsorption and infection. Interestingly, after 18 minutes 40% of phage remained free in WT, whereas other experiments indicate that most of the adsorption occurs during the first 10 minutes (Hendrix and Duda, 1992). After 1 hour phage lysed the WT but the TolC culture gained turbidity, meaning that bacteria was growing. This confirms the role of TolC as a receptor because its absence impaired phage ability to adsorb and therefore to cause cell lysis.

DNA characterization included sequencing and genome annotation. It is important to highlight the fact that Stilgar resemble EcoM-ep3 and PPpW3, phages that infect pathogenic strains and are being studied as candidates for phage therapy ((Wang et al., 2015; Park and Nakai, 2003). It is interesting to highlight that *Pseudomonas* phage PPpW3 is more related to Stilgar than well-characterized *Escherichia* phages. As we discussed in the chapter of phage classification, this points out the fact that there's a lot to do to have a proper and more inclusive organization of phages. Tail-like spike with pectin lyase domain and a component of the outer wedge of baseplate with acidic lysozyme domain might confer the ability to surpass the surface features present in ETEC in order to find and attach to its receptor.

Stilgar had a narrow host range, able only to infect 9/41 clinical isolates. Six of those strains had a *tolC* mutant, which Stilgar was not able to infect, to add more to the fact that Stilgar is TolC-dependent. Bacterial resistance mechanism such as surface determinants to block phage adsorption, mechanism to prevent DNA entry or restriction modification systems, could explain Stilgar's reduced host range against ETEC isolates (Labrie et al., 2010). Also, *E. coli* K-12 was resistant to Stilgar, which agrees to

the idea that pathogenic-specific phages would not affect commensal strains. To further prove this idea it would be necessary to test Stilgar against a set of commensal gut strains.

The main idea behind the project was to solve one of the main cited problems of phage therapy, which is the emergence of phage resistant bacteria. To determine the frequency and nature of those mutants, we performed a simple experiment mixing bacteria and phage (1:100) and plated them on LB or LB + SDS 0.01%. As TolC mutants present a hypersensitive phenotype to detergents (DeVito, 2008), the number of resistant bacteria present on LB + SDS reduced on a 10-fold (compared to LB), suggesting that they had alterations in the *tolc* locus in order to impair phage adsorption. This was confirmed by a PCR analysis. Since it has been previously demonstrated that *tolC* mutations impair the ability of bacteria to secrete ST toxins (Yamanaka et al., 1998), we conclude that the SPEAR methodology is a promising improvement in the selection of bacteriophages with therapeutic purposes.

This doesn't come as a surprise as another phage TLS has been described as TolC-specific (German and Misra, 2001). Mutant bacteria obtained after phage exposure had deletions in *tolC*, although some mutants impaired phage adsorption but kept the ability to pump out deleterious substances out of the cell. In our case, further analysis and sequencing of *tolC* locus will be require in resistant bacteria to determine the nature of this mutations and its effect on cell physiology.

It would be important to investigate other ETEC'S virulence factors such as Type 2 secretion system, responsible for the secretion of LT (Tauscheck et al., 2002), and colonization factors as candidates for the SPEAR methodology.

Lastly, although most of the strains isolated from patients in Mexico had LT toxin rather than ST, the selection of TolC is based on its importance in cell physiology. TolC has been discussed as a therapeutic target for multi-drug

resistant bacteria because plays an important role in pumping out deleterious substances through the outer membrane and is responsible for the multi antibiotic resistant phenotype in *E. coli* (Okosu et a., 1996; Fralick, 1996). It would be important to evaluate the antibiotic sensitivity of resistant bacteria to common use antibiotics.

13. CONCLUSIONS

- Phage Stilgar was selected on its ability to infect clinical isolates using the outer membrane protein TolC as a receptor.
- Adsorption assay showed that to certain extent, phage is unable to adsorb to bacteria when TolC is absent.
- Morphological characteristics determine that Stilgar is a myophage.
- Stilgar's DNA was digested with EcoRV, XbaI and SmaI, which cleave in one site, producing 2 DNA fragments.
- Stilgar genomic organization resembles myophages EcoM-ep3 and PPpW2, used for the control of pathogenic strains in chicken and fish.
- No virulence factors or potentially harmful genes where identified in Stilgar, therefore is a viable candidate for phage therapy.
- Stilgar was able to infect 9/41 ETEC clinical isolates, showing a reduced host range
- PCR analysis shows that phage resistant mutant bacteria have alterations on the *tolC* locus and present a hypersensitive phenotype to detergents, according to what is reported on literature.
- SPEAR methodology for the isolation of virulence factor-specific phage is feasible

14. PERSPECTIVES

- Sequencing of *tolC* in ETEC clinical isolates sensitive to Stilgar
- Determine sensitivity of phage resistant bacteria with *tolC* deletions to antibiotics
- Determine Stilgar host range against a set of commensal gut microbiota
- Further characterization for different colonization factors in the ETEC panel
- Apply the SPEAR methodology to different virulence factors such as colonization factors and Type two secretion systems (LT toxin secretion)

15. REFERENCES

- Abedon, S. T. (2006). Phage ecology. p: 37-46. In Calendar, R. The bacteriophages, 2nd edn. New York: Oxford University Press.
- Ackerman, H.W. (1999). Bacteriophages. p: 398-400. In J.P. Lederberg, Encyclopedia of Micriobiology. 2nd edn, vol. 1. Academic Press, New York.
- Ackerman, H.W. (2006). Classification of bacteriophages. pp: 8-16. . In Calendar, R. The bacteriophages, 2nd edn. New York: Oxford University Press.
- Ackermann, H. W. (2001). Frequency of morphological phage descriptions in the year 2000. *Archives of virology*, *146*(5), 843-857.
- Ackermann, H. W., & Prangishvili, D. (2012). Prokaryote viruses studied by electron microscopy. *Archives of virology*, *157*(10), 1843-1849.
- Adams, M. H. (1959). Bacteriophages. *Interscience Publishers, Inc., New York*, *NY*, 450-454
- Al-Abri, S.S, Beeching, N.J., Nye, F.J. (2005). Traveller's diarrhea. *The lancet infectious Diseases 5*:349-360
- Alam, M., Farzana, T., Ahsan, C.R., Yasmin, M., Nessa, J. (2011). Distribution of coliphages against four E. coli virotypes in hospital origin ated sewage sample and a sewage treatment plant in Bangladesh. *Indian journal of microbiology*, *51*(2):188-93.
- Albert, M.J., Faruque, S.M., Faruque, A.S., Neogi, P.K., Ansaruzzaman, M., Bhuiyan, N.A., Alam, K., Akbar, M.S. (1995). Controlled study of Escherichia coli diarrheal infections in Bangladeshi children. *Journal of clinical Microbiology, 33*(4), 973-977.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research*, *25*(17), 3389-3402.
- Ananta, R.P., McVeigh, A.L., Lee, L.H., Agnew, M.K., Cassels, F.J., Scott, D.A., Whittam, T.S., Savarino, S.J. (2004). Evolutionary and functional relationships of colonization factor antigen I and other class 5 adhesive fimbriae of enterotoxigenic Escherichia coli. *Infection and Immunity, 72*(12), 7190-7201.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology*, *2*(1).
- Barrow, P., Lovell, M., Berchieri, A. (1998). Use of lytic bacteriophage for control of experimental Escherichia coli septicemia and meningitis in chickens and calves. *Clinical and Diagnostic Laboratory Immunology, 5*(3),294-298

- Begum, Y. A., Chakraborty, S., Chowdhury, A., Ghosh, A. N., Nair, G. B., Sack, R. B., ... & Qadri, F. (2010). Isolation of a bacteriophage specific for CS7-expressing strains of enterotoxigenic Escherichia coli. *Journal of medical microbiology*, *59*(3), 266-272.
- Bentley, R. Meganathan, R. (1982). Biosynthesis of vitamin K (melaquinone) in bacteria. *Microbiological Reviews 46*(3): 241-90.
- Beodeker, E.C. (2005). Vaccines for enterotoxigenic Escherichia coli: current status. *Current Opinion in gastroenterology 21*(1), 15-19
- Berger, C.N., Sodha, S.V., Shaw, R.K., Griffin, P.M., Pink, D., Hand, P., and Frankel, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ Microbiol *12*, 2385-2397.
- Bernet-Carmard, M.F. Coconnier, M.H., Hudault, S., Servin, A.L. (1996). Pathogenicty of the diffusely adhering strain Escherichia coli C1845: F1845 adhesin-decay accelerating factor interaction, brush border microvillus injury, and actin dissasembly in cultured human intestinal epitelial cells. *Infection and immunity 64*(6), 1918-1928
- Bettelheim, K.A. (1994). Biochemical characteristics of Escherichia coli, p. 3-30. In Glydes, C.L. Escherichia coli in domestic animals and humans. CAB international, *Oxon OX10 8DE, UK, Wallingford*.
- Black, R. E., Levine, M. M., Clements, M. L., Cisneros, L., & Daya, V. (1982). Treatment of experimentally induced enterotoxigenic Escherichia coli diarrhea with trimethoprim, trimethoprim-sulfamethoxazole, or placebo. *Review of Infectious Diseases*, *4*(2), 540-545.
- Black, R.E., Brown, K.H., Becker, S., Alim, A.A., Merson, M.H. (1982). Contamination of weaning foods and transmission of enterotoxigenic Escherichia coli diarrhea in children in rural Bangladesh. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *76*(2), 259-264.
- Black, R.E., Merson, M.H., Rowe, B., Taylor, P.R., Alim, A.A., Gross, R.J., Sack, D.A. (1981). Enterotoxigenic Escherichia coli diarrhea: acquired immunity and transmission in an endemic area. *Bulletin of the World Health Organization*, *59*(2), 263.
- Borodovsky, M, Lomsadze, A. (2011). Gene identification in prokaryotic genomes, phages, metagenomes, and EST sequences with GeneMarkS suite. *Current protocols in microbiology*, . Chapter 4:Unit 4.5.1-17
- Bruttin, A., and Brussow, H. (2005). Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. *Antimicrobial agents and chemotherapy*, *49*(7), 2874-2878.
- Campbell, A. (2003). The future of bacteriophage biology. *Nature Reviews Genetics*, *4*(6), 471-477.
- Casjens, S. R. (2005). Comparative genomics and evolution of the tailed-bacteriophages. *Current opinion in microbiology*, *8*(4), 451-458.

- Casjens, S. R., Gilcrease, E. B., Winn-Stapley, D. A., Schicklmaier, P., Schmieger, H., Pedulla, M. L., Ford, M.E., Houtz, M., Hatfull, G.F. & Hendrix, R. W. (2005). The generalized transducing Salmonella bacteriophage ES18: complete genome sequence and DNA packaging strategy. *Journal of bacteriology*, *187*(3), 1091-1104.
- Cassels, F. J., & Wolf, M. K. (1995). Colonization factors of diarrheagenic E. coli and their intestinal receptors. *Journal of industrial microbiology*, *15*(3), 214-226.
- Cassels, F.J., Pannell L.K., Boedeker E.C. 1993. Absolute molecular weight determination of *E. coli* fimbrial major subunits. In *93rd General Meeting of the American Society for Microbiology. Presented* (p. 80)
- Cassels, F.J., Wolf M.K. 1995. Colonization factors of diarrheagenic E. coli and their intestinal receptors. *Journal of industrial microbiology*, *15*(3), 214-226.
- Catalano, C. E. (2005). Viral Genome Packaging Machines: Genetics, Structure, and Mechanism. Landes Bioscience/Eurekah. com and Kluwer Academic.
- Chakraborty, S., Deokule, J. S., Garg, P., Bhattacharya, S. K., Nandy, R. K., Nair, G. B., Yamasaki S., Takeda Y., Ramamurthy, T. (2001). Concomitant Infection of Enterotoxigenic Escherichia coli in an Outbreak of Cholera Caused by Vibrio cholera O1 and O139 in Ahmedabad, India. *Journal of clinical microbiology*, *39*(9), 3241-3246.
- Chibani-Chennoufi, S., Sidoti, J., Bruttin, A., Dillmann, M.L., Kutter, E., Qadri, F., Sarker, S.A., Brussow, H. (2004). Isolation of Escherichia coli bacteriophages from the stool of pediatric diarrhea patients in Bangladesh. *Journal of bacteriology*, *186*(24), 8287-8294
- Clark, C. A., Heuzenroeder, M. W., & Manning, P. A. (1992). Colonization factor antigen CFA/IV (PCF8775) of human enterotoxigenic Escherichia coli: nucleotide sequence of the CS5 determinant. *Infection and immunity*, *60*(3), 1254-1257.
- Clements, A., Young, J. C., Constantinou, N., & Frankel, G. (2012). Infection strategies of enteric pathogenic Escherichia coli. *Gut microbes*, *3*(2), 71-87.
- Cravioto, A., Reyes, R. E., Ortega, R., Fernández, G., Hernández, R., López, D. (1988). Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. *Epidemiology and infection*, *101*(01), 123-134.
- d'Herelle F. (1917). Sur un microbe invisible antagoniste des bacilles dysent´eriques. *C. R. Acad. Sci. Paris* 165:373–75
- De Graaf, F.K., Gaastra, W. (1994). Fimbriae of Enterotoxigenic Escherichia coli, p. 58-83. In Klemm, P. Fimbriae Adhesion, Genetics, Biogenesis, and Vaccines. CRC Press

- De Sauvage, F. J., Horuk, R., Bennett, G., Quan, C., Burnier, J. P., & Goeddel, D. V. (1992). Characterization of the recombinant human receptor for Escherichia coli heat-stable enterotoxin. *Journal of Biological Chemistry*,*267*(10), 6479-6482.
- Denou, E., Bruttin, A., Barretto, C., Ngom-Bru, C., Brüssow, H., & Zuber, S. (2009). T4 phages against Escherichia coli diarrhea: potential and problems.*Virology*, *388*(1), 21-30.
- Deresinski, S. (2009). Bacteriophage therapy: exploiting smaller fleas. *Clinical Infectious Diseases*, *48*(8), 1096-1101.
- DeVito, J. A. (2008). Recombineering with tolC as a selectable/counter-selectable marker: remodeling the rRNA operons of Escherichia coli. *Nucleic acids research*, *36*(1), e4-e4.
- Douglas, J. (1975). Bacteriophages. London New York, Chapman and Hall
- Drasar, B.S., Hill M.J. (1974). Human intestinal flora p. 36-43. Academic press, Ltd. London, United Kingdom
- Duckworth, D. H. (1976). Who discovered bacteriophage?. *Bacteriological reviews*, *40*(4), 793.
- DuPont, H. L., Formal, S. B., Hornick, R. B., Snyder, M. J., Libonati, J. P., Sheahan, D. G., Eugene, H., LaBrec, Kalas, J. P. (1971). Pathogenesis of Escherichia coli diarrhea. *New England journal of medicine*, *285*(1), 1-9.
- DuPont, H. L., Formal, S. B., Hornick, R. B., Snyder, M. J., Libonati, J. P., Sheahan, D. G.,LaBrec E.H., Kalas, J. P. (1971). Pathogenesis of Escherichia coli diarrhea. *New England journal of medicine*, *285*(1), 1-9.
- DuPont, H.L. (2009) Systematic review: the epidemiology and clinical features of travellers' diarrhoea. *Alimentary Pharmacology & Therapeutics* 30(3):187-196
- Eaton, M.D., & Bayne-Jones S. (1934). Bacteriophage therapy: Review of the principles and results of the use of bacteriophage in the treatment of infections. *Journal of the American Medical Association* 103:1769–76, 1847–53, 1934–39
- Echeverria, P., Seriwatana, J., Taylor, D. N., Changchawalit, S., Smyth, C. J., Twohig, J., & Rowe, B. (1986). Plasmids coding for colonization factor antigens I and II, heat-labile enterotoxin, and heat-stable enterotoxin A2 in Escherichia coli. *Infection and immunity*, *51*(2), 626-630.
- Estrada-García, T., Cerna, J. F., Paheco-Gil, L., Velázquez, R. F., Ochoa, T. J., Torres, J., & DuPont, H. L. (2005). Drug-resistant diarrheogenic Escherichia coli, Mexico. *Emerging infectious diseases*, *11*(8), 1306.
- Estrada-Garcia, T., Lopez-Saucedo, C., Thompson-Bonilla, R., Abonce, M., Lopez-Hernandez, D., Santos, J. I., Rosado J.L., DuPont H.L., Long, K. Z. (2009). Association of diarrheagenic Escherichia coli pathotypes with infection and diarrhea among Mexican children and association of

atypical enteropathogenic E. coli with acute diarrhea. *Journal of clinical microbiology*, *47*(1), 93-98.

- Evans, D. G., Evans, D. J., Clegg, S., Pauley, J. A. (1979). Purification and characterization of the CFA/I antigen of enterotoxigenic Escherichia coli. *Infection and immunity*, *25*(2), 738-748.
- Evans, D. G., Evans, D. J., Tjoa, W. S., & DuPont, H. L. (1978). Detection and characterization of colonization factor of enterotoxigenic Escherichia coli isolated from adults with diarrhea. *Infection and immunity*, *19*(2), 727-736.
- Evans, D. G., Silver, R. P., Evans, D. J., Chase, D. G., & Gorbach, S. L. (1975). Plasmid-controlled colonization factor associated with virulence in Esherichia coli enterotoxigenic for humans. *Infection and Immunity*, *12*(3), 656-667.
- Field, M., Graf, L. H., Laird, W. J., & Smith, P. L. (1978). Heat-stable enterotoxin of Escherichia coli: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. *Proceedings of the National Academy of Sciences*, *75*(6), 2800-2804.
- Finlay, B. B., & McFadden, G. (2006). Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell*, *124*(4), 767-782.
- Flaherty, J. E., Somodi, G. C., Jones, J. B., Harbaugh, B. K., & Jackson, L. E. (2000). Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. *HortScience*, *35*(5), 882-884.
- Flores-Abuxapqui, J. J., Suarez-Itoil, G. D. J., Heredia-Navarrete, M. R., Puc-Franco, M. A., & Franco-Monsreal, J. (1993). Frequency of enterotoxigenic Escherichia coli in infants during the first three months of life. *Archives of medical research*, *25*(3), 303-306.
- Fralick, J. A. (1996). Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of Escherichia coli. *Journal of Bacteriology*, *178*(19), 5803-5805.
- Freire-Moran, L., Aronsson, B., Manz, C., Gyssens, I.C., So, A.D., Monnet, D.L., and Cars, O. (2011). Critical shortage of new antibiotics in development against multidrug-resistant bacteria-Time to react is now. *Drug resistance updates*, *14*(2), 118-124.
- Fukuta, S. H. I. N. J. I., Magnani, J. L., Twiddy, E. M., Holmes, R. K., & Ginsburg, V. (1988). Comparison of the carbohydrate-binding specificities of cholera toxin and Escherichia coli heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. *Infection and immunity*, *56*(7), 1748-1753.
- Gaastra, W., & Svennerholm, A. M. (1996). Colonization factors of human enterotoxigenic Escherichia coli (ETEC). *Trends in microbiology*, *4*(11), 444-452.

- German, G. J., & Misra, R. (2001). The TolC protein of Escherichia coli serves as a cell-surface receptor for the newly characterized TLS bacteriophage.*Journal of molecular biology*, *308*(4), 579-585.
- Giamarellou, H. (2010). Multidrug-resistant Gram-negative bacteria: how to treat and for how long. *International journal of antimicrobial agents*, *36*, S50-S54.
- Gill D.M., Richardson S.H. (1980). Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of *Escherichia coli*: comparison with cholera toxin. *J. Infect. Dis*. 141:64-70.
- Gorski, A., Miedzybrodzki, R., Borysowski, J., Weber-Dabrowska, B., Lobocka, M., Fortuna, W., Letkiewicz, S., Zimecki, M., and Filby, G. (2009). Bacteriophage therapy for the treatment of infections. *Current opinion in investigational drugs (London, England: 2000)*, *10*(8), 766-774.
- Gyles, C. L., & Barnum, D. A. (1969). A heat-labile enterotoxin from strains of Escherichia coli enteropathogenic for pigs. *The Journal of infectious diseases*, 419-426.
- Gyles, C., & Boerlin, P. (2013). Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. *Veterinary Pathology Online*.
- Hadas, H., Einav, M., Fishov, I., & Zaritsky, A. (1997). Bacteriophage T4 development depends on the physiology of its host Escherichia coli.*Microbiology*, *143*(1), 179-185.
- Hendrix, R. W. (2003). Bacteriophage genomics. *Current opinion in microbiology*, *6*(5), 506-511.
- Hendrix, R. W., & Duda, R. L. (1992). Bacteriophage lambda PaPa: not the mother of all lambda phages. *Science*, *258*(5085), 1145-1148.
- Hirayama, T., Ito, H., & Takeda, Y. (1989). Inhibition by the protein kinase inhibitors, isoquinolinesulfonamides, of fluid accumulation induced by Escherichia coli heat-stable enterotoxin, 8-bromo-cGMP and 8-bromo-cAMP in suckling mice. *Microbial pathogenesis*, *7*(4), 255-261.
- Hirst, T. R., & Holmgren, J. (1987). Conformation of protein secreted across bacterial outer membranes: a study of enterotoxin translocation from Vibrio cholerae. *Proceedings of the National Academy of Sciences*, *84*(21), 7418-7422.
- Huilan, S., Zhen, L.G., Mathan, M.M., Mathew, M.M., Olarte, J., Espejo, R., Khin Maung, U., Ghafoor, M.A., Khan, M.A., Sami, Z.*, et al.* (1991). Etiology of acute diarrhoea among children in developing countries: a multicentre study in five countries. *Bulletin of the World Health Organization*, *69*(5), 549.
- Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn,

A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.Y., Lopez, R., Hunter, S. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics*. 2014. 30(9):1236-1240.

- Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H., & Krogh, A. (2003). Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Science*, *12*(8), 1652-1662.
- Kaper, J.B, Nataro, J.P., Mobley, L.T. (2004). Pathogenic Escherichia coli. *Nature reviews microbiology, 2*(2):123-140.
- Kingsford, C. L., Ayanbule, K., & Salzberg, S. L. (2007). Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. *Genome biology*, *8*(2), R22.
- Knutton S., McConnell M.M., Rowe B., McNeish A.S. 1989. Adhesion and ultraestructural properties of human enterotoxigenic Escherichia coli producing colonization factor antigens III and IV. *Infection and immunity*, *57*(11), 3364-3371.
- Koronakis, V., Eswaran, J., & Hughes, C. (2004). Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annual review of biochemistry*, *73*(1), 467-489.
- Kotloff, K. L., Nataro, J. P., Blackwelder, W. C., Nasrin, D., Farag, T. H., Panchalingam, S., Levine, M. M., et al. (2013). Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet*, *382*(9888), 209-222.
- Kusters J.G., Gaastra W. (1994). Fimbrial operons and evolution. P. 179-196. In In Klemm, P. Fimbriae Adhesion, Genetics, Biogenesis, and Vaccines. CRC Press.
- Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S., & Abedon, S. T. (2010). Phage therapy in clinical practice: treatment of human infections. *Current pharmaceutical biotechnology*, *11*(1), 69-86.
- Labrie, S. J., Samson, J. E., & Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nature Reviews Microbiology*, *8*(5), 317-327
- Lee, D. J., Bingle, L. E., Heurlier, K., Pallen, M. J., Penn, C. W., Busby, S. J., & Hobman, J. L. (2009). Gene doctoring: a method for recombineering in laboratory and pathogenic Escherichia coli strains. *BMC microbiology*, *9*(1), 252.
- Lee, L. K., & Lerner, B. W. (2003). World of microbiology and immunology.*Detroit, Gale*, 699.
- Lencer, W.I., Constable, C., Moe, S., Rufo, P.A., Wolf, A., Jobling, M.G., Ruston, S.P., Madara, J.L., Holmes, R.K., Hirst, T.R. (1997). Proteolytic activiation of cholera toxin and *Escherichia coli* labile toxin by entry into host epithelial cells. Signal transduction by a protease-resistant toxin variant. *Journal of Biological Chemistry*, *272*(24), 15562-15568.

- Levine, M. M. (1987). Escherichia coli that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent.*Journal of infectious Diseases*, *155*(3), 377-389.
- Levine, M. M., Kotloff, K. L., Nataro, J. P., & Muhsen, K. (2012). The Global Enteric Multicenter Study (GEMS): impetus, rationale, and genesis. *Clinical infectious diseases*, *55*(suppl 4), S215-S224.
- Levine, M.M., Ristaino, P., Marley, G., Smyth, C., Knutton, S., Boedeker, E., Back R., Young, C., Clements, M.L., Cheney, C., Patnaik, R. (1984). Coli surface antigens 1 and 3 colonization factor antigen II- positive enterotoxigenic *Escherichia coli*: morphology, purification, and immune responses in humans. *Infection and Immunity*, *44*(2), 409-420
- Liu, L., Johnson, H. L., Cousens, S., et al. (2012). Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet*, *379*(9832), 2151-2161.
- López-Saucedo, C., Cerna, J.F., Villegas-Sepulveda, N., Thompson, R., Velazquez, F.R., Torres, J., Tarr, P.I., Estrada-García, T. 2009. Single Multiplex Polymerase Chain Reaction to detec diverse loci associated with diarrheagenic Escherichia coli. *Emerging infectious diseases*, 9(1), 127.
- Lowe, T. M., & Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic acids research*,*25*(5), 0955-964
- Lv, M., Wang, S., Yan, G., Sun, C., Feng, X., Gu, J. & Lei, L. (2015). Genome sequencing and analysis of an Escherichia coli phage vB_EcoM-ep3 with a novel lysin, Lysep3. *Virus genes*, 1-11
- Madigan, M.T., Martinko, J.M., and Brock, T.D. (2006). Brock biology of microorganisms, 11th edn (Upper Saddle River, NJ, Pearson Prentice Hall).
- Martin, A., Yeats, S., Janekovic, D., Reiter, W. D., Aicher, W., & Zillig, W. (1984). SAV 1, a temperate uv-inducible DNA virus-like particle from the archaebacterium Sulfolobus acidocaldarius isolate B12. *The EMBO journal*,*3*(9), 2165.
- Matsushita, S., Kawamura, M., Takahashi, M., Yokoyama, K., Konishi, N., Hatakeyama, K., Kai A., Morozumi K., Morita K., Watanabe N., Kanamori M., Kudoh Y. (2001). Increasing fluoroquinolone low-sensitivity in enterotoxigenic Escherichia coli isolated from diarrhea of overseas travelers in Tokyo. *Kansenshogaku zasshi. The Journal of the Japanese Association for Infectious Diseases*, *75*(9), 785-791.
- Mattick, J. S. (2002). Type IV pili and twitching motility. *Annual Reviews in Microbiology*, *56*(1), 289-314.

- Maura, D., & Debarbieux, L. (2011). Bacteriophages as twenty-first century antibacterial tools for food and medicine. *Applied microbiology and biotechnology*, *90*(3), 851-859.
- Maura, D., Morello, E., du Merle, L., Bomme, P., Le Bouguénec, C., & Debarbieux, L. (2012). Intestinal colonization by enteroaggregative Escherichia coli supports long-term bacteriophage replication in mice. *Environmental microbiology*, *14*(8), 1844-1854.
- Mayans, O., Scott, M., Connerton, I., Gravesen, T., Benen, J., Visser, J., Pickersgill R., Jenkins, J. (1997). Two crystal structures of pectin lyase A from Aspergillus reveal a pH driven conformational change and striking divergence in the substrate-binding clefts of pectin and pectate lyases. *Structure*, *5*(5), 677-689.
- McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S., & Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Sciences*,*92*(5), 1664-1668.
- Mims CA, Nash A & Stephen J. 2001. Mims' pathogenesis of infectious disease, Academic Press, London.
- Nair, G.B., Takeda, Y. (1998). The heat-stable enterotoxins. *Microb Pathog 24*(2), 123-131.
- Nakai, T., Park, S.C. (2002). Bacteriophage therapy for infectious diseases in aquiculture. *Res Microbiol.* 153(1),13-8.
- Nakao, H., & Takeda, T. (2000). Escherichia coli Shiga toxin. *Journal of natural toxins*, *9*(3), 299-313.
- Nataro, J.P., and Kaper, J.B. (1998). Diarrheagenic Escherichia coli. *Clin Microbiol Rev 11*(1), 142-201
- Nataro, J.P., Steiner, T.S., Guerrant, R.L. (1998). Enteroaggregative Echerichia coli. *Emerg Infect. Dis*. 4(2), 251-261
- Neeser, J.R., Chambaz, A., Golliard, M., Link-Amster, H., Fryder, V., Kolodziejczyk, E. (1989). Adhesion of colonization factor antigen II-positive enterotoxigenic Escherichia coli strains to human enterocytelike differentiated HT-29 cells: a basis for host-pathogen interactions in the gut. *Infection and immunity*, *57*(12), 3727-3734.
- O'Flaherty, S., Ross, R. P., & Coffey, A. (2009). Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS microbiology reviews*, *33*(4), 801-819.
- Okamoto, K., & Takahara, M. (1990). Synthesis of Escherichia coli heat-stable enterotoxin STp as a pre-pro form and role of the pro sequence in secretion. *Journal of bacteriology*, *172*(9), 5260-5265.
- Okusu, H., Ma, D., & Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. *Journal of Bacteriology*, *178*(1), 306-308.

- Oppenheim, A. B., Kobiler, O., Stavans, J., Court, D. L., & Adhya, S. (2005). Switches in bacteriophage lambda development. *Annu. Rev. Genet.*, *39*, 409-429.
- Park, S. C., & Nakai, T. (2003). Bacteriophage control of Pseudomonas plecoglossicida infection in ayu Plecoglossus altivelis. *Diseases of aquatic organisms*, *53*(1), 33-39.
- Peabody, C. R., Chung, Y. J., Yen, M. R., Vidal-Ingigliardi, D., Pugsley, A. P., Saier, M. H. (2003). Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology*, *149*(11), 3051-3072.
- Perez-Casal, J. O. S. E., Swartley, J. S., & Scott, J. R. (1990). Gene encoding the major subunit of CS1 pili of human enterotoxigenic Escherichia coli.*Infection and immunity*, *58*(11), 3594-3600.
- Perna, N. T., Plunkett, G., Burland, V., Mau, B., Glasner, J. D., Rose, D. J., ... & Blattner, F. R. (2001). Genome sequence of enterohaemorrhagic Escherichia coli O157: H7. *Nature*, *409*(6819), 529-533.
- Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature methods*,*8*(10), 785-786.
- Peterson, J. W., & Whipp, S. C. (1995). Comparison of the mechanisms of action of cholera toxin and the heat-stable enterotoxins of Escherichia coli.*Infection and immunity*, *63*(4), 1452-1461.
- Pirnay, J.P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I.*, et al.* (2011). The phage therapy paradigm: pret-a-porter or sur-mesure?. *Pharmaceutical research*, *28*(4), 934-937.
- Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. *Microbiological reviews*, *57*(1), 50-108
- Qadri, F., Svennerholm, A. M., Faruque, A. S. G., & Sack, R. B. (2005). Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clinical microbiology reviews*, *18*(3), 465-483.
- Quadri F., Das S.K., Faruque G.J., Fuch G.J., Albert M.J., Sack R.B., Svennerholm A.M. (2000). Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a 2-year period from diarrheal patients in Bangladesh. J. Clin. Microbiol. 38:27-31.
- Rao, M. R., Abu-Elyazeed, R., Savarino, S. J., Naficy, A. B., Wierzba, T. F., Abdel-Messih, I., Shaheen H., Frenck R.W., Svennerholm A.M., Clemens, J. D. (2003). High disease burden of diarrhea due to enterotoxigenic Escherichia coli among rural Egyptian infants and young children. *Journal of clinical microbiology*, *41*(10), 4862-4864.

- Rasheed, J. K., Guzmán-Verduzco, L. M., & Kupersztoch, Y. M. (1990). Two precursors of the heat-stable enterotoxin of Escherichia coli: evidence of extracellular processing. *Molecular microbiology*, *4*(2), 265-273.
- Ripp, S., & Miller, R. V. (1997). The role of pseudolysogeny in bacteriophage-host interactions in a natural freshwater environment. *Microbiology*, *143*(6), 2065-2070.
- Russel, M., Model, P. (2006). Filamentous phage. p:146-160. In Calendar, R. The bacteriophages, 2nd edn. New York: Oxford University Press.
- Sansonetti, P. (2002). Host-pathogen interactions: the seduction of molecular cross talk. *Gut* 50, Suppl. 3 S2-S8
- Sears, C., Kaper, J.(1996). Enteric Bacterial Toxins: Mechanisms of Action and Linkage to Intestinal Secretion. *Microbiological Reviews*, 167-215.
- Sixma, T.K., Kalk, K.H., van Zanten, A., Dauter, Z., Kingma, J., Witholt, B., Hol, W.G. (1993). Refined structure of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol.* 230(3), 890-918.
- Smith, H. W., & Halls, S. (1967). Observations by the ligated intestinal segment and oral inoculation methods on Escherichia coli infections in pigs, calves, lambs and rabbits. *The Journal of pathology and bacteriology*, *93*(2), 499-529.
- Smith, H. W., & Huggins, M. B. (1982). Successful treatment of experimental Escherichia coli infections in mice using phage: its general superiority over antibiotics. *Journal of General Microbiology*, *128*(2), 307-318.
- Smith, H. W., & Huggins, M. B. (1983). Effectiveness of phages in treating experimental Escherichia coli diarrhoea in calves, piglets and lambs. *Journal of general microbiology*, *129*(8), 2659-2675.
- So, M., & McCarthy, B. J. (1980). Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic Escherichia coli strains. *Proceedings of the National Academy of Sciences*, *77*(7), 4011-4015.
- Soothill, J.S. (1992). Treatment of experimental infections of mice with bacteriophages. *J. Med. Microbiol. 37*(4):258-61.
- Spangler, B.D. (1992). Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. *Microbiol Rev* 56: 1954-1963.
- Steinsland, H., Valentiner-Branth, P., Perch, M., Dias, F., Fischer, T.K., Aaby, P., Molbak, K., Sommerfelt, H. (2002). Enterotoxigenic *Escherichia coli* infections and diarrhea in a cohort of young children in Guinea-Bissau. *Journal of Infectious Diseases*, *186*(12), 1740-1747.

- Stent, G.S. (1963). Phage therapy and prophylaxis. In: Molecular Biology of Bcterial Viruses. San Francisco: W.H. Freeman, pp- 6-9.
- Streatfield S.J., Sandkvist M., Sixma T.K., Bagdasariam M., Hol W.G.J., Hirst T.R. (1992). Intermolecular interactions between A and B subunits of heat-labile enterotoxin from Escherichia coli promote holotoxin assembly and stability in vivo. *Proceedings of the National Academy of Sciences*, *89*(24), 12140-12144.
- Sulakvelidze, A., E. Kutter. (2005). Bacteriophage therapy in humans, p. 381-436. *In* E. Kutter and A. Sulakvelidze. Bacteriophages: biology and application. CRC Press, Boca Raton, FL
- Summer, E. J., Berry, J., Tran, T. A. T., Niu, L., Struck, D. K., & Young, R. (2007). Rz/Rz1 lysis gene equivalents in phages of Gram-negative hosts. *.Journal of molecular biology*, *373*(5), 1098-1112
- Summers, W. C. (2001). Bacteriophage therapy. *Annual Reviews in Microbiology*, *55*(1), 437-451.
- Summers, W.C. (2006). Phage and the early development of molecular biology. Pp 3-7. In J.P. Lederberg, Encyclopedia of Micriobiology. 2nd edn, vol. 1. Academic Press, New York.
- Suzek, B. E., Ermolaeva, M. D., Schreiber, M., & Salzberg, S. L. (2001). A probabilistic method for identifying start codons in bacterial genomes.*Bioinformatics*, *17*(12), 1123-1130.
- Tanji, Y., Shimada, T., Fukudomi, H., Miyanaga, K., Nakai, Y., and Unno, H. (2005). Therapeutic use of phage cocktail for controlling Escherichia coli O157:H7 in gastrointestinal tract of mice. *Journal of bioscience and bioengineering*,*100*(3), 280-287.
- Tauschek, M., Gorrell, R. J., Strugnell, R. A., & Robins-Browne, R. M. (2002). Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of Escherichia coli. *Proceedings of the National Academy of Sciences*, *99*(10), 7066-7071.
- Tauschek, M., Gorrell, R. J., Strugnell, R. A., & Robins-Browne, R. M. (2002). Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of Escherichia coli. *Proceedings of the National Academy of Sciences*, *99*(10), 7066-7071.
- Teneberg, S., Hirst, T. R., Ångström, J., & Karlsson, K. A. (1994). Comparison of the glycolipid-binding specificities of cholera toxin and porcineEscherichia coli heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine. *Glycoconjugate journal*, *11*(6), 533-540.
- Thomason, L. C., Costantino, N., & Court, D. L. (2007). E. coli genome manipulation by P1 transduction. *Current Protocols in Molecular Biology*, 1-17

- Turner S.M., Scott-Tucker A., Cooper M.L., Henderson, I.R. 2006. Weapons of mass destruction: virulence factors for the global killer Enterotoxigenic Escherichia coli. *FEMS Microbiology Letters* 263:10-20
- Van Regenmortel, M. H. (1990). Virus species, a much overlooked but essential concept in virus classification. *Intervirology*, *31*(5), 241-254.
- van Regenmortel, M.H., Mayo, M.A., Fauquet, C.M., and Maniloff, J. (2000). Virus nomenclature: consensus versus chaos. *Archives of virology*,*145*(10), 2227-2232.
- Voegele, K., Sakellaris, H., & Scott, J. R. (1997). CooB plays a chaperone-like role for the proteins involved in formation of CS1 pili of enterotoxigenic Escherichia coli. *Proceedings of the National Academy of Sciences*, *94*(24), 13257-13261.
- Watson, J.D. (2003). Molecular biology of the gene, 5th edn. San Francisco, Pearson.
- Wolf, M. K. (1997). Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic Escherichia coli. *Clinical microbiology reviews*, *10*(4), 569-584.
- World Health Organization. (1999). New frontiers in the development of vaccines against enterotoxigenic (ETEC) and enterohaemorrhagic (EHEC) E. coli infections. *Weekly Epidemiol. Rec*, *13*, 98-100.
- Yamamoto, T., Yokota, T. (1983) Plasmids of enterotoxigenic Escherichia coli H10407: evidence for two heat-stable enterotoxin genes and a conjugal transfer system. *Journal of bacteriology*,*153*(3), 1352-1360.
- Yamanaka, H., Kameyama, M., Baba, T., Fujii, Y., & Okamoto, K. (1994). Maturation pathway of Escherichia coli heat-stable enterotoxin I: requirement of DsbA for disulfide bond formation. *Journal of bacteriology*, *176*(10), 2906-2913.
- Yamanaka, H., Nomura, T., Fujii, Y., & Okamoto, K. (1998). Need for TolC, anEscherichia coliouter membrane protein, in the secretion of heat-stable enterotoxin I across the outer membrane. *Microbial pathogenesis*, *25*(3), 111-120.
- Young, R., Wang, I. (2006). Phage lysis. pp:104-125. In Calendar, R. The bacteriophages, 2nd edn. New York: Oxford University Press.
- Yu, J., Webb, H., & Hirst, T. R. (1992). A homologue of the Escherichia coli DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in Vibrio cholerae. *Molecular microbiology*, *6*(14), 1949-1958.