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THESIS

Escherichia coli SPECIES PRESENT IN BIOFILMS IN STREAM WATER IN SAN PEDRO RIVER AT AGUASCALIENTES STATE

BY

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TO OBTAIN THE DOCTOR DEGREE IN BIOLOGICAL SCIENCES

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CENTRO DE CIENCIAS BÁSICAS

TESIS

TIPOS DE *Escherichia coli* PRESENTES EN BIOFILMS DE FUENTES DE AGUA NATURAL EN AGUASCALIENTES, MÉXICO

PRESENTA

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Por medio de este conducto hacemos constar el que la Ingeniero Bioquímico Flor Yazmin Ramirez Castillo ha cumplido con todos los requisitos de escritura y revisión de tesis para optar por el grado de Doctor en Ciencias Biológicas con la tesis "Tipos de *Escherichia coli* presentes en biofilms de fuentes de agua natural en Aguascalientes".

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Sin otro particular me permito saludarle muy afectuosamente.

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To my husband and family.

For all the patience, understanding and support.

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LIST OF ABBREVIATIONS

AMR: Antimicrobial resistance.

ATCC: American Type Culture Collection.

BOD: Biological oxygen demand.

CLSI: Clinical and Laboratory Standards Institute.

CFU: Colony forming units.

COD: Chemical oxygen demand.

CV: Cristal violet.

DAEC: Diffusely adherent *E. coli*.

DEC: Diahorreagenic *E. coli*.

EAEC: Enteroagregative *E. coli*.

EHEC: Enterohemorragic *E. coli*.

EIEC: Enteroinvasive *E. coli*.

EPEC: Enteropathogenic *E. coli*.

ETEC: Enterotoxigenic *E. coli*.

ExPEC: Extra-intestinal pathogenic *E. coli*.

FISH: Fluorescence *in situ* hibridation.

InPEC: Intestinal pathogenic *E. coli*.

LB: Luria Bertani Broth.

MBAS: Methylene blue active substances.

Mm^3: Thousands of cubic meters.

OD: Optical density.

UPEC: Uropathogenic *E. coli*.

UTI: Urinary tract infection.

VBNC: Viable but not culturable bacteria.

WWTP: Wastewater treatment plant.

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RESUMEN

La contaminación en agua superficial en países en desarrollo continua siendo de gran preocupación. Las aguas residuales tratadas y sin tratar se descargan continuamente dentro de ríos y agua superficial, llevando a posibles brotes de infección y puede ser un mecanismo significativo de diseminación de genes de resistencia a antimicrobianos en el ambiente. En este estudio, la calidad del agua del río San Pedro, el principal río y colector fluvial del Estado de Aguascalientes, México, fue analizada. Treinta locaciones diferentes fueron examinadas. Los resultados muestran altos niveles de contaminación fecal así como alto contenido en materia orgánica e inorgánica suficientes para soportar el crecimiento heterotrófico de microorganismos, indicando baja calidad del agua. Por otra parte, en este trabajo se estudiaron las características de virulencia y perfiles de resistencia a antimicrobianos de 150 cepas de *E. coli* aisladas del río San Pedro. Se estudiaron sus propiedades fenotípicas de susceptibilidad antimicrobiana y formación de biopelículas, así como sus propiedades genotípicas incluyendo grupos filogenéticos, factores de virulencia, genes de resistencia y mutaciones cromosomales. Los aislados fueron clasificados como patógenos (n = 91) o comensales (n = 59). El método de difusión en disco fue empleado para determinar la susceptibilidad antimicrobiana frente a 13 antibióticos distintos. Cincuenta y dos por ciento de los aislados fueron resistentes al menos a un agente antimicrobiano y 30.6% fueron multi-drogo resistentes. Dieciocho aislados de *E. coli* mostraron resistencia a quinolonas, 16 de ellos fueron multi-resistentes. Genes de resistencia a quinolonas mediada por plásmidos (PMQR) fueron detectados en 12 aislados. Mutaciones en las posiciones Ser-83→ Leu y /o Asp-87 → Asn en el gen *gyrA* fueron detectados así como mutaciones en la posición Ser-80 →Ile en *parC*. Un microarreglo de *E. coli* (Maxivirulence V 3.1) fue utilizado para caracterizar los genes de virulencia y resistencia de las cepas fluoroquinolona resistentes. Genes de resistencia a antimicrobianos tales como bla_{TEM}*, sulI, sulII, dhfrIX, aph3(strA), tet(B)* y la presencia de integrones fueron detectados en las cepas. Por otra parte, las aguas residuales provenientes de los rastros municipales, efluentes industriales y la escorrentía urbana que desemboca en el río pueden contribuir a la diseminación de la resistencia a los antibióticos y las bacterias patógenas en el medio ambiente acuático.

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Asimismo, se encontró evidencia de la presencia de *E. coli* en un estado viable pero no cultivable (VPNC) y la formación de biopelículas de *E. coli* directamente en el agua del río, lo que sugiere que las biopelículas pueden actuar como reservorio de patógenos bacterianos en los ríos contaminados además de que las bacterias no cultivables también pueden proporcionar un riesgo adicional a la salud pública, ya que estas bacterias conservan su virulencia. En conclusión la presencia de *E. coli* potencialmente patógenas y resistentes a antibióticos en el río San Pedro tales como fluoroquinolona resistentes pueden poseer un riesgo potencial para la salud humana y animal.

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ABSTRACT

Contamination of surface waters in developing countries is a great concern. Treated and untreated wastewaters have been discharged into rivers and streams, leading to possible waterborne infection outbreaks and may represent a significant dissemination mechanism of antibiotic resistance genes. In this study, the water quality of San Pedro River, the main river and pluvial collector of the Aguascalientes State, Mexico was assessed. Thirty sample locations were tested throughout the River. The main physicochemical parameters of water were evaluated. Results showed high levels of fecal pollution as well as inorganic and organic matter abundant enough to support the heterotrophic growth of microorganisms. These results indicate poor water quality in samples from different locations. One hundred and fifty *Escherichia coli* isolates were collected and screened by PCR for several virulence genes. Isolates were classified as either pathogenic (n = 91) or commensal (n = 59). The disc diffusion method was used to determine antimicrobial susceptibility to 13 antibiotics. Fifty-two percent of the isolates were resistant to at least one antimicrobial agent and 30.6% were multidrug resistant. Eighteen *E. coli* strains were quinolone resistant of which 16 were multidrug resistant. Plasmid-mediated quinolone resistance (PMQR) genes were detected in 12 isolates. Mutations at the Ser-83→Leu and/or Asp-87→Asn in the *gyrA* gene were detected as well as mutations at the Ser-80→Ile in *parC*. An *E. coli* microarray (Maxivirulence V 3.1) was used to characterize the virulence and antimicrobial resistance genes profiles of the fluoroquinolone-resistant isolates. Antimicrobial resistance genes such as bla_{TEM}, *sulI, sulII, dhfrIX, aph3(strA),* and *tet(B)* as well as integrons were found in fluoroquinolone (FQ) resistance *E. coli* strains. Furthermore, the discharge of slaughterhouses, industrial sewage and urban runoff may contribute to the dissemination of antibiotic resistance and pathogenic bacteria in the aquatic environment. We also found evidence of the presence of viable but not culturable (VBNC) *E. coli* in water sources as well as biofilm-forming *E. coli* directly from river water, suggesting that these biofilms may act as a reservoir for bacterial pathogens in polluted rivers and non-culturability bacteria may also provide an additional risk from

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public health since these bacteria conserve their virulence. In conclusion, the presence of potential pathogenic *E. coli* and antibiotic resistance in San Pedro River such as FQ resistant *E. coli* could pose a potential threat to human and animal health.

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1. INTRODUCTION

1. *Escherichia coli.*

Escherichia coli are Gram-negative, rod-shaped, facultative anaerobic bacterium from the *Enterobacteriaceae* family (Ewing 1998). Most *E. coli* strains harmlessly colonize the gastrointestinal tract of humans and blod-warm animals a few hours after the birth as normal flora. Nevertheless, with over 250 serotypes, *E. coli* is a highly versatile bacterium; it could acquire virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands (Lim *et al*., 2010), ranging from harmless gut commensal to intra- or extra-intestinal pathogens (Beloin *et al*., 2008; Kaper *et al*., 2004), causing disease in both, human and animals; producing different clinical pictures in them.

In humans, *E. coli* causes 630 millions of diarrhea cases worldwide an approximately 775,000 deaths per year, mainly affecting the pediatric population of developing countries. In addition, these bacterial is one of the most frequently opportunistic pathogens associated with urinary tract infections and sepsis in humans (Blanco 2002).

This bacterium is capable to survive and persist in many kinds of environments, such as water, soil, food and animals. It had been showed that some strains such as enterohemorrhagic *E. coli* are able to survive in water for long time periods; over eight months (LeJeune *et al*., 2001). In fact, it has been found in low nutrient aquatic environments, such as surface waters or man-made water treatment systems; providing a source of infection.

The ability of *E. coli* to survive and grow in the environment is likely due to its versatility in energy acquisition; *E. coli* is a heterotrophic bacterium, requiring only simple carbon and nitrogen sources, plus phosphorus, sulfur and other trace elements for their growth (Ishii and Sadowsky, 2008). In addition, strains within each subgroup occupy various ecological niches, and can be broadly characterized by either commensal or pathogenic. In some cases, genomic islands can be pinpointed to enable the latter

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behaviour. Thus, genomic islands, on one hand, broad environmental significance, and on the other hand, virulence, are highlighted in the context of *E. coli* survival in its niches (van Elsas *et al*., 2011).

E. coli also can be found as a planktonic bacteria or developing a structured and heterogeneous, matrix-encased bacterial communities known as *biofilms*, that in most ecological niches due to bacterial interactions with a surface, this behavior is promoted (Beloin *et al*., 2008). The biofilms physiology is characterized by increased tolerance to stress as lack of food, presence of heavy metals, chlorination, and others (Pereira *et al*., 2010), as well as biocides, antibiotics, and host immunological defenses (Beloin *et al*., 2008).

Interestingly, Kell *et al.,* showed that *E. coli*, depending of the environmental conditions (such as stress, temperature, pH, starvation, etc), can be enter in a viable-but-non-culturable (VBNC) state, in which the bacteria cannot form colonies in nutrient-rich media in which often the bacteria are cultivated and counted, resulting in rejection of the number of viable cells (Wu *et al.* 2009), furthermore these bacterial could be either commensal of pathogenic. The presence of VBNC bacteria, it could be a great factor to the sanitary control, attributable to, as suggested some authors (Pommepuy *et al.,* 1996; Grimes and Colwell, 1986; Colwell *et al.,* 1985) pathogenic bacteria VBNC keep their virulence becoming a potential outbreaks reservoir.

Given its ubiquity in fecal material and its relatively short persistence in environmental matrices, *E. coli* is the gold standard for detection of fecal pollution in water, and the presence and density of *E. coli* in water is widely used to measure and regulate water quality (Chen *et al*., 2011; McLellan *et al.,* 2004).

Strains of *E. coli* are divided phylogenetically into four major groups: A, B1, B2, and D. They can be distinguished upon the presence of *chuA* and *yjaA* genes, and the non-coding region Tspe4.C2 (Clermont *et al.,* 2000). Although virulence determinants are considered to be mobile, a link between strain phylogeny and virulence has been reported. Usually, virulent extra-intestinal strains mainly belong to groups B2, and to a lesser extent D; commensal strains belong mostly to groups A and B1, and diarrheagenic *E. coli* are related with groups A, B1 and B2 (Mokracka *et al.,* 2011; Clermont *et al.,* 2000). The predominance of different phylogroups may also vary due to geographical

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location, the site of infection, and the level of antibiotic resistance (Bukh *et al.,* 2009; Duriez *et al.,* 2001). Recently, new phylogroups has been added, including the groups C, E, F. Among them, phylogroup C has been related with commensal or diarrheagenic *E. coli* since are similar to groups A and B1; phylogroup E has been related to diarrheagenic *E. coli* since the main strains of this group is enterohemorragic *E. coli* O157:H7; and finally, phylogroup F which has been related to the groups D and B2 (Clermont *et al.,* 2013).

1.1 PATHOGENIC *E. coli.*

There are several highly adapted *E. coli* clones that have acquired specific virulence attributes which confers an increased ability to adapt to new niches and allows them to cause a broad spectrum of disease. These virulence attributes are frequently encoded on genetic elements that can be mobilized into different strains to create novel combinations of virulence factors, or on genetic elements that might once have been mobile, but have now evolved to become “locked” into the genome. Only the most successful combinations of virulence factors have persisted to become specific “pathotypes” of *E. coli* that are capable of causing disease in healthy individuals (Kaper *et al*., 2004).

E. coli strains are categorized on pathotypes according to their different virulence trails, serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors (Kaper, 2004; Nataro & Kaper, 1998; Muhldorfer *et al*., 1996). There are many virulence factors evolved the pathogenicity mechanisms of *E. coli*, which include: secretion systems, adhesins, invasins, toxins, and, modification factors of the host cell surface (Bekal *et al.,* 2003).

E. coli can cause enteric diarrheal diseases and extra-intestinal disease (Hamelin *el al.,* 2006; Kaper, 2004; Nataro & Kaper, 1998). The diarrheagenic *E. coli* strains include: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), shiga toxin-producing *E. coli* (STEC) enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli*, necrotoxic *E. coli*, and cell-detaching *E. coli*. While extra-intestinal infections are caused by three separate *E. coli*

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pathotypes: uropathogenic (UPEC) strains that cause urinary tract infections, strains involved in neonatal meningitis (MENEC), and strains that cause septicemia in humans and animals (Hamelin *et al.,* 2007; Bekal *et al.* 2003; Nataro & Kaper, 1998).

Even though pathogenic *E. coli* is primarily associated with food-borne diseases, contamination of drinking or recreational waters with some pathotypes has resulted in waterborne disease outbreaks and associated mortality (Vital *et al*., 2010; Servais *et al*., 2009; Liu *et al*., 2008; Hamelin *et al*., 2007; Juhna *et al*., 2007; Hamelin *et al*., 2006). Indeed, new kinds of highly *E. coli* pathogenic as enterohemorrhagic-enteroaggregative *E. coli* O104:H4, has been emerging in last years causing outbreaks (Mellmann *et al.,* 2011).

1.1.1 INTRAINTESTINAL PATHOGENIC *E. coli.*

Diarrheagenic strains of *E. coli* represent the most common cause of pediatric diarrhea worldwide (Nataro and Kaper, 1998). Several distinct clinical syndromes accompany infections with DEC categories, including traveler`s diarrhea (ETEC), hemorrhagic colitis and hemolytic-uremic syndrome (EHEC), persistent diarrhea (EAEC), and watery diarrhea of infants (EPEC, Figure 1, Kaper, 2004).

Several diarrheagenic *E. coli* have recognized as major causes of childhood diarrhea in developing countries, enteropathogenic *E. coli* (EPEC) is one of them (Afset *et al.,* 2006; Rodríguez-Ángeles, 2002; Nataro & Kaper, 1998). EPEC generates a histopathological lesion referred to as attaching-and-effacing (A/E) lesion, which begins when the bacterium attaches intimately to the enterocyte and induces the effacement of microvilli and the bacterial adherence to the epithelial cell membrane. Rearrangements of the actin cytoskeleton are doing to form a pedestal-like structure where bacterium tightly cups the cells, leading to degeneration of brush border microvilli and the elimination of the function of the small bowel (Rodríguez-Ángeles, 2002; Scaletsky *et al*., 2002; Nataro & Kaper, 1998). The genes responsible for A/E lesions have been called the locus of enterocyte effacement (LEE, Lim *et al.,* 2010). These EPEC strains can also harbor the EPEC adherence plasmid (EAF) that comprises the cluster of genes encoding the bundle-forming pilus (BFP), that allowing the formation of colonies of

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bacterium and their aggregation (Nataro & Kaper, 1998).

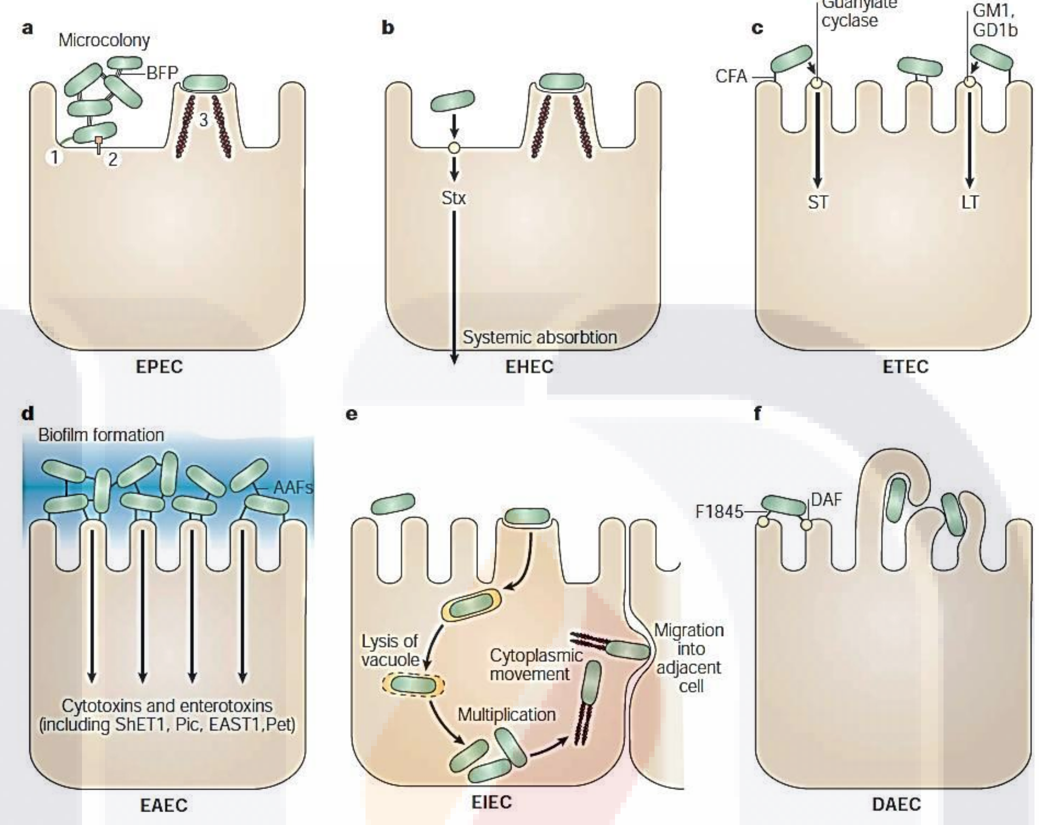

Figure 1. Pathogenic scheme of diarrhoeagenic *E. coli*. The six recognized categories of diarrhoeagenic *E. coli* and their interaction with eukaryotic cells. a) EPEC adheres to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion. Cytoskeletal derangements are accompanied by an inflammatory response and diarrhea. b) EHEC also induce the attaching and effacing lesion, but in the colon. EHEC produce Shiga toxin (Stx). c) ETEC adheres to small bowel enterocytes and induce watery diarrhea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins. d) EAEC adheres to small and large bowel epithelia in a thick biofilm and elaborates secretory enterotoxins and cytotoxins. e) EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments. f) DAEC elicits a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections, which wrap around the bacteria (Kaper *et al.,* 2004).

Another DEC *E. coli* comprise enterohemorrhagic *E. coli* (EHEC), (Lim *et al.,* 2010; Estrada-Garcia *et al.,* 2009), this pathotype has been considered as a major public health concern worldwide since has been defined as pathogenic *E. coli* strains that produce Shiga toxins (Stxs) and cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans and animals (Lim *et al.,* 2010; Yoon *et al.,* 2008; Nataro & Kaper, 1998).

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Strains as enterotoxigenic *E. coli* (ETEC) producing heat labile (LT) and/or heat-stable (ST) enterotoxins are one of the main causes of childhood diarrhea in worldwide, since the microorganisms colonize the surface of the small bowel mucosa and elaborate their enterotoxins, giving rise to a net secretory state (Nataro and Kaper, 1998; Cassels *et al*., 1995).

Enteroinvasive *E. coli* (EIEC) strains are closely related to *Shigella spp*., which could made and invasion of colonic epithelium and the entorotoxins production (Nataro & Kaper, 1998), resulting in watery diarrhea with blood and mucus as clinical symptoms.

Enteroaggregative *E. coli* has been recognized as an emerging pathogen mainly associated with persistent infantile diarrhea in middle-income countries. EAEC is defined by its adhesion to cultured epithelial cells in a stacked brick-like formation aggregative adherence (AA), and produce fimbrial colonization factors called aggregative adherence factors (AAFs) encoded by plasmids (pAA) and other afimbrial factors (Pereira *et al.,* 2010). EAEC strain can elaborate the enterotoxins EAST1 and ShET1 (Huang *et al*., 2007; Harrington *et al.,* 2006), but the transcriptional activator termed AggR has been described as the major EAEC-virulence regulator (Pereira *et al.,* 2010; Harrington *et al.,* 2006).

Diffusely adherent *E. coli* (DAEC) strains are defined by the presence of the specific characteristic of diffuse pattern of adherence to HEp-2 cell monolayers. These strains cause diarrhea, particularly in children under one year. DAEC strains induce a cytopathic effect that is characterized by the development of long cellular extensions, which wraps around the adherent bacteria. This strain produce a fimbrial adhesin called F1845 involve in the adherence diffuse phenomenon and which belongs to the Dr family of adhesions (Nataro and Kaper, 1998).

1.1.2 EXTRAINTESTINAL PATHOGENIC *E. coli.*

Extra intestinal pathogenic *E. coli* (ExPEC) possesses virulence traits that allow it to invade, colonize, and induce disease in bodily sites outside of the gastrointestinal tract. Human diseases caused by ExPEC include urinary tract infections (UTI), neonatal

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meningitis, sepsis, pneumonia, surgical site infections, as well as infections in other extra intestinal locations (Kaper 2004). ExPEC-induced diseases represent a large burden in terms of medical costs and productivity losses (Russo *et al*., 2003; Stamm *et al*., 2001; Foxman *et al*., 2000). In addition to human illnesses, ExPEC strains also cause extra intestinal infections in domestic animals and pets (Murray *et al*., 2004).

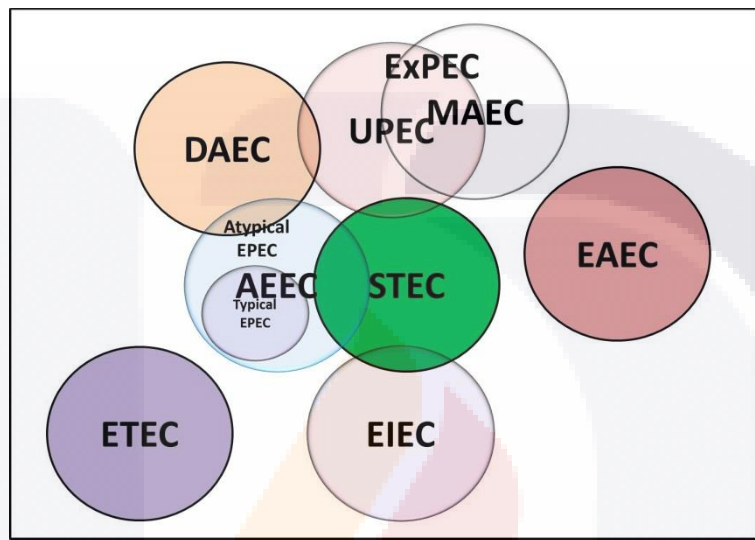

Figure 2. Schematic illustrating the complex relationships between different pathotypes of *E. coli* that cause disease in humans. *AEEC, attaching and effacing *E. coli*; DAEC, diffuse-adhering *E. coli*; EAEC, enteroagregative *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enterophatogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; MAEC, meningitis-associated *E. coli*; STEC, shiga-toxigenic *E. coli*; UPEC, uropathogenic *E. coli*.

Uropathogenic *E. coli* (UPEC), one of the members of the ExPEC strains, are predominant pathogens causing UTIs (Engel and Schaeffer, 1998). Urinary tract infections are one of the most common diseases worldwide. Although these infections are treatable, the increasingly accelerated rate of global trafficking of multi-drug resistant (MDR) bacteria may lead to complication and treatment failure and increase the cost of the treatment. UPEC causes 80-90% of community-acquired UTIs and 30-50% of nosocomially-acquired UTIs (Kucheria *et al*., 2005). UPEC harbor a variety of virulence factors including adhesions, toxins host defense avoidance mechanisms and multiple iron acquisition systems that allow their successful transition from the intestinal tract to the urinary tract, facilitate colonization of host surfaces, overcome host defenses and establish infection. Generally, UPEC harbor specific adhesions, including P (Pap),

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type 1 fimbriae and others (such as F1C, S, M and Dr), that allows the adherence to uroepithelial cells; and toxins as autotransporte toxins (Pic), RTX toxins (HlyA) which causes cell lysis, cytotoxic necrotizing factors (CNF -1, -2), that altered the cytoskeleton and causes necrosis, as well as iron acquisition systems. These virulence factors are found in differing percentages among various subgroups of UPEC. UPEC strains carry large and small pathogenicity islands containing gene loci that are not found in the chromosome of faecal strains (Kaper *et al*., 2004).

1.1.3 VIRULENCE FACTORS

The ability of pathogenic strains of *E. coli* to cause different intestinal and extra intestinal diseases types derived from the expression multiple virulence factors contributing to increased efficiency in the colonization of specific host surfaces, evasion of immune defenses, or direct to their cell and tissue damage resulting in the establishment of disease (Table 1, Johnson, 2002). Pathotypes of *E. coli* can cause a common disease using a common set of virulence factors (Figure 2). This virulence factors are grouped into adhesions and colonization factors and toxins (Mainil 2013; Kaper *et al*., 2004).

At least three different general classes of *E. coli* adhesions can be defined: different appendices like fimbriae and curli, the afimbrial family (Afa), and specific outer membrane proteins (OMPs) like the intimin (Mainil 2013; Mainil 2005a; Kaper *et al*., 2004).

More numerous than surface structures that trigger signal transduction pathways are secreted toxins and other effectors proteins that affect an astonishing variety of fundamental eukaryotic processes (Kaper *et al*., 2004).

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Table 1. Definition, host range and main virulence properties of intestinal and urinary tract pathogenic *E. coli* in animals and humans (Mainil, 2013).

Name	Host range	Disease	Virulence	Genetics
Enteroinvasive (EIEC)	Humans, primates	Dysentery	Invasion and multiplication in the enterocytes	Plasmid
Enteropathogenic (EPEC)	Humans, all mammals	Diarrhea	Attaching and effacing (AE) lesion; type 4 BFP fimbriae by typical (t) EPEC of humans (dogs, cats)	Pathogenicity island (AE lesion); plasmid (BFP fimbriae)
Verotoxigenic (VTEC)	Human, piglets	Hemolytic-uremic syndrome (HUS) in humans; oedema disease in piglets	Verotoxins (VTx); afimbrial (Saa by human or AIDA by porcine VTEC) and fimbrial (F18 by porcine VTEC) adhesins	Phages (VTx); chromosome or plasmids (adhesins)
Enterohemorrhagic (EHEC)	Humans (cattle)	(Hemorrhagic) colitis and HUS in humans; diarrhea in young calves	VTx and AE lesion	Phages (VTx); pathogenicity island (AE lesion)
Enteroadherent or enteroaggregative (EAEC or EAggEC)	Humans	Diarrhea	Small fimbrial adhesins (AAF/Hda); toxins (Pet, EAST1, ShET1); transcriptional activator gene (aggR)	Plasmids (AAF, Hda, Pet, EAST1, AggR); pathogenicity island (ShET1)
Diffusely adherent (DAEC)	Humans, animals	Diarrhea, urinary tract infections, septicemia	Adhesins of the Afimbrial Adhesin (AFA) family; AIDA adhesin	Chromosome (Afa, AIDA); plasmids (Afa)
Necrotoxigenic (NTEC)	Humans, animals (NTEC1); ruminants (NTEC2)	Diarrhea, urinary tract infections, septicemia	Cytotoxic Necrotizing Factors (CNF) 1 or 2 and α haemolysin (αHly); fimbrial (Pap/Prs, Sfa/F1C and/or F17) and/or afimbrial adhesins (AFA family); siderophores; resistance to complement	Chromosome, including pathogenicity islands (CNF1, αHly, Pap/Prs, Sfa/F1C, F17, Afa, siderophores); plasmids (CNF2, F17, Afa, siderophores, resistance to complement)
Uropathogenic (UPEC)	Humans, animals (especially dogs and cats)	Cystitis, pyelonephritis, bacteraemia, septicemia	NTEC: CNF 1 or CNF2, αHly; fimbrial (Pap/Prs, Sfa/F1C and/or F17) and/or afimbrial adhesins (AFA family); siderophores, resistance to complement	Chromosome, including pathogenicity islands (CNF1,αHly, Pap/Prs, Sfa, F17, Afa, siderophores); plasmids (CNF2, F17, Afa, siderophores, resistance to complement)
			Others: αHly; fimbrial (Pap/Prs, Sfa/F1C and/or F17) and/or afimbrial adhesins (AFA family); siderophores, resistance to complement	

1.2 ANTIMICROBIAL RESISTANCE.

Antimicrobial resistance remains a serious global health concern and solutions to address this fact are urgently required (da Costa *et al*., 2013). Antibiotics are a class of naturally-occurring, semi-synthetic and/or chemically synthesis compounds with antimicrobial activity. They are widely used in human and veterinary medicine to treat

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and prevent diseases and as growth promoters in animal intensive industries (Jury *et al.,* 2010). Unfortunately, resistance to all classes of antibiotics has emerged and antimicrobial resistance (AMR) bacteria could lead to treatment failure. In fact, bacterial resistance to antibiotics is currently directly responsible for 15 times as many deaths as acquired immunodeficiency syndrome (AIDS) every year in Europe (Gonzalez-Zorn *et al*., 2012).

AMR is given by natural antibiotic producer's bacteria or by developing or acquiring antibiotic resistance mechanisms. Genetic mechanisms involved in horizontal gene transfer (HGT) of antimicrobial resistance genes among environmental bacteria may include: a) conjugative transfer by mobile elements including plasmids, transposons, and integrons on plasmids or transposons (direct cell-to-cell transfer); b) transduction (phage-assisted transfer); and c) transformation by naked DNA (DNA-to-cell transfer, Figure 3, Gaze *et al*., 2013; Heuer *et al*., 2012; Colomer-Lluch *et al*., 2011).

There are two mechanisms of resistance in bacteria, the acquired resistance and the intrinsic mechanisms, which are inherent resistance that confers resistance to one or more families of antibiotics to some microorganisms. Nevertheless, the acquired resistance plays the principal role which is explained by mutations or horizontal gene transfer (HGT, Alekshun & Levy, 2007; Tenover, 2006; Canton *et al.,* 2003; Livermore, 2003). Thus, resistance genes could be transferred among bacteria of different taxonomic and ecological groups by mobile genetic elements such as bacteriophages, plasmids, naked DNA or transposons (Levy, 2002). These genes are generally directed against a single family or type of antibiotic, although multiple genes, each bearing a single drug resistance trait, can accumulate in the same organism (Levy & Marshall *et al.,* 2004). Furthermore, efflux pumps for causing multple might be present.

In the absence of plasmids and transposons (which generally mediate high-level resistance), a step-wise progression from low-level to high-level resistance occurs in bacteria through sequential mutations in chromosomes (Levy & Marshall *et al.,* 2004; Schneiders *et al.,* 2003; Levy 2002; Wang *et al.,* 2001). Thus, the organism later acquired transposons bearing genes with high-level resistance to these drugs, like

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fluoroquinolones resistance bacteria (Levy & Marshall *et al.,* 2004; Levy 2002b; Wang *et al.,* 2001).

Some of the prominent means of resistance include: altered permeability barriers across bacterial outer membranes, preventing uptake of the compound by inhibiting its corresponding transport carrier, modifying the targets binding sites so that it no longer recognizes the antibiotics, and the ability to chemically and/or enzymatically degrade the antibiotic (Figure 3, Jury *et al.,* 2010).

Antibiotic resistance in most environmental bacteria is due to the acquisition of new genes, often associated with the mobile elements (Zhang *et al.,* 2009; Gaze *et al*., 2005; Frost *et al.,* 2005). Furthermore, virulence factors are expressed proteins encoded by genes located in the chromosome or in plasmids, thus the location of virulence factors in genetic mobile elements is may facilitate the spread of virulence within bacterial communities and the acquisition of resistance or virulent traits which might represent an advantage for the survival of the microorganism (Da Silva & Mendoça 2012). *E. coli* strains, such as other Gram-negative bacteria are inherently resistant to antimicrobial hydrophobic, such as macrolides, novobiocins, rifamycins, actinomycin D and fusidic acid (Scheutz and Strockbine, 2005).

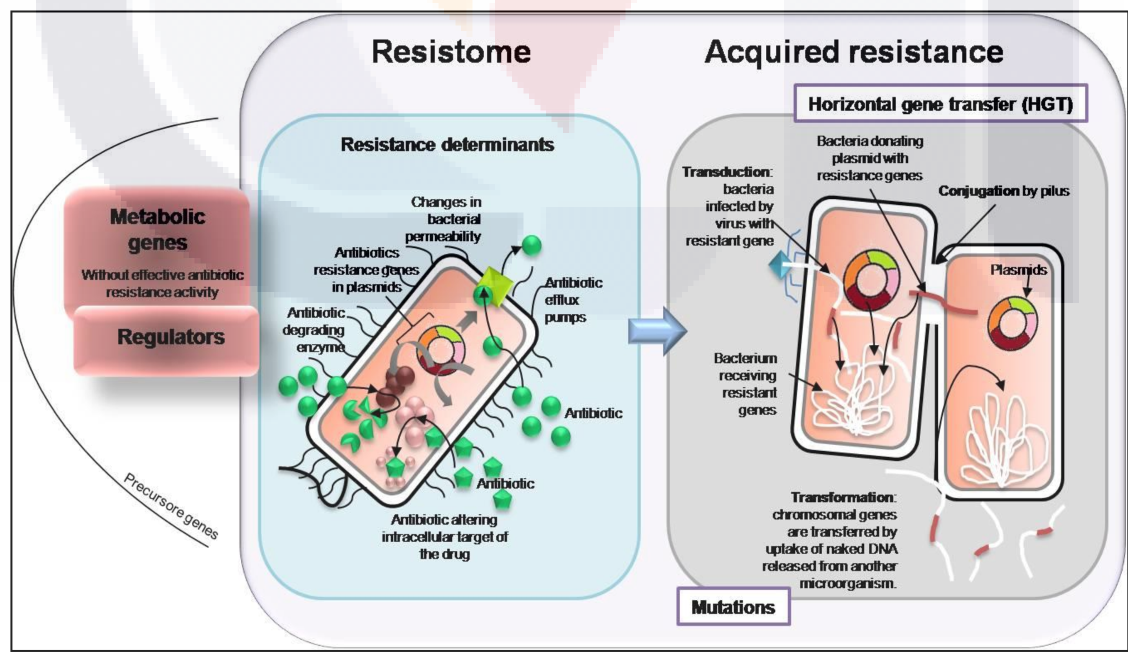

Figure 3. Microbial resistome: resistance mechanisms and spread of drug resistance (figure based in Levy & Marshall 2004 and Olivares *et al*., 2013 with modifications).

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Table 2. Mechanisms of action and genetic determinants of resistance.

Antibiotic class	Phenotype	Genotype	Resistance Mechanisms	Location
Aminoglycoside	Streptomycin, kanamycine, gentamicine, spectinomycine, streptothricine, neomycin, tobramycin, apramycin.	*strA/strB, aac(3)-Ia, aac(3)-IVa, aac(6')-le-aph(2'')-la, aph(3')-Ia, aph(3')-IIa, aph(3')-IIIa, aadE, aadA, sat(4), aad9,aadB.*	Enzymatic modification (adenyl-, phosphor-acetyltransferase).	Plasmid, integron, transposon.
Quinolone	Ciprofloxacin, nalidix acid.	*gyrA, gyrB, parC, parE, qnr, aac(6')-Ib-cr, qepA, oqxAB.*	Mutation (DNA gyrase, topoisomerase IV), efflux pomps (*marR, acrR*), aquisicion plasmid (*qnrA, qnrB, qnrC, qnrS*), enzymatic modification (*aac(6')-Ib-cr)*. Over expression of major porin (OmpF).	Chromosome, plasmid.
Tetracycline	Tetracycline, oxytetracycline.	GroupI *tet* (B), *tet* (C), *tet* (D); Group II *tet* (A),*tet* (E), *tet* (G); Group III *tet* (K), *tet* (L), *tet* (M), *tet*(O), *tet* (S); Group IV *tetA* (P), *tet* (Q), *tet* (X).	Export membrane protein, protection and target altered (ribosome binding site modification).	Chromosome, plasmid.
Phenicol	Chloramphenicol, florfenicol.	*catI, catII, catIII, floR, cmlA. pp-flo.*	Enzymatic degradation (chloramphenicol acetyltransferase, CAT), Export membrane protein.	Chromosome, plasmid.
Beta-lactam	Amoxicillin-clavulanic acid, ampicillin-sulbactam, or piperacillin-tazobactam cefepime, amikacin.	SHV-1, TEM-1, CTX-M family, OXA β-lactamase genes (*blaOXA-1* group, *blaOXA-2* group, *blaOXA-10* group, *blaOXA-9, blaOXA-20, blaOXA-18*), IMP-1, *pse-4*.	Carbapenem-hydrolyzing activity, plasmid-encoded AmpC enzymes, o hydrolyze ring β-lactam.	Chromosome, plasmid, transposon, Integron.
Sulfonamide	Sulfonamide.	*sul1, sul2, and sul3.*	Variant cycle enzyme dihydropteroate insensitive to sulfonamides.	Plasmid, transposon, Integron.
Trimethoprim	Trimethoprim.	*dfrA genes (dfrA1, dfrA5, dfrA6, dfrA7, dfrA12, dfrA13, dfrA14, dfrA15, dfrA16, dfrA17, dfrA21, dfrA22 and dfrA25, dfrA27), dhfr genes (dhfrI, dhfrV, dhfrVII, dhfrIX, dhfrXIII).*	Variant of the target enzyme dihydrofolate insensitive to trimethoprim (dihidrofolate reductase).	Chromosome, plasmid, transposon, Integron.

From Maynard *et al*. 2004 with modification.

The antimicrobial more frequently used in the *E. coli* infection treatment are ampicillin, trimethoprim-sulfamethoxazol, aminoglycosides, and cephalosporins and quinolones as "last resource". Nevertheless, *E. coli* has the ability to acquire resistance genes to make it able to resist against several types of antibiotics including fluoro/quinolone. High proportions (40 to 90%) of *E. coli* strains are resistant to ampicillin, streptomycin, sulfonamides and tetracyclines. They are many strains (15-30%) resistant to 1st generation cephalosporins, neomycin, kanamycin, chloramphenicol

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and quinolones. Among the antibiotics that have lower rates of resistance are available amoxicillin-clavulanate, cephalosporins of 2nd and 3rd generation, gentamicin, tobramycin, amikacin, colistin and polymyxin B (Blanco *et al*., 2002).

1.2.1 ANTIMICROBIAL RESISTANCE IN AQUATIC ENVIRONMENTS.

The global occurrence of antibiotic resistance genes in bacteria in water environments is raising concerns since the majority of antibiotics are excreted unchanged into the environment (Zhang *et al.,* 2009).

Most antibiotics used for treating infections are produced by environmental microorganisms, including bacteria within soils and water (Finley *et al*., 2013), meaning that genes for antibiotic resistance must have emerged in non-clinical habitats (Martínez, 2009). Antibiotic resistance bacteria can also enter into aquatic environments by direct discharging of untreated wastewater through wastewater collection systems and subsequently into the environments with effluents and discharged sludge (Auerbach *et al.,* 2007), runoff, and sewage. In addition, after the administrations, antibiotics are excreted unchanged and/or not fully metabolized into the environment (Kümmerer, 2003). Therefore, a mixture of pharmaceuticals and their metabolites will enter municipal sewage and wastewater treatment plants (WWTP) (Kümmerer, 2004), and could reach the environment (Figure 4). In recent years, antibiotics contamination is recognized as an emerging environmental pollution in aquatic environments, because of their potential adverse effects on the ecosystem and human health (Kümmerer, 2009a; Huang *et al*., 2001).

The anthropogenic activities (including sewage discharge, wastewater treatment, and drug manufacturing and intensive agricultural livestock) are changing environmental reservoirs of resistance genes and their precursors: "*the resistome*" (D`Costa *et al*., 2006; Finley *et al*., 2013). Thus, probability of recruitment of resistance genes into clinically relevant pathogens will increase (Finley *et al*., 2013; Knapp *et al*., 2011; Peak *et al*., 2007) due to environmental bacteria act as an unlimited source of genes that might act as resistance genes when entering in pathogenic organisms (Baquero *et al*., 2008). Furthermore, as pharmaceuticals are constantly released into the environment, organisms

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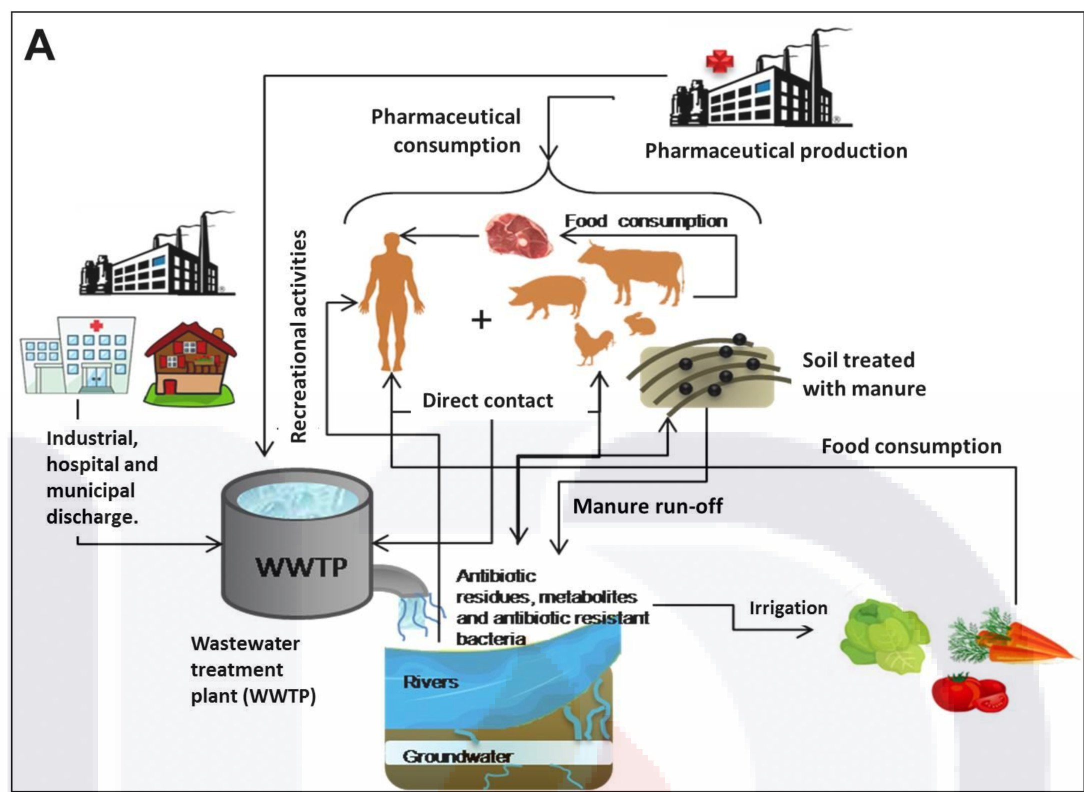

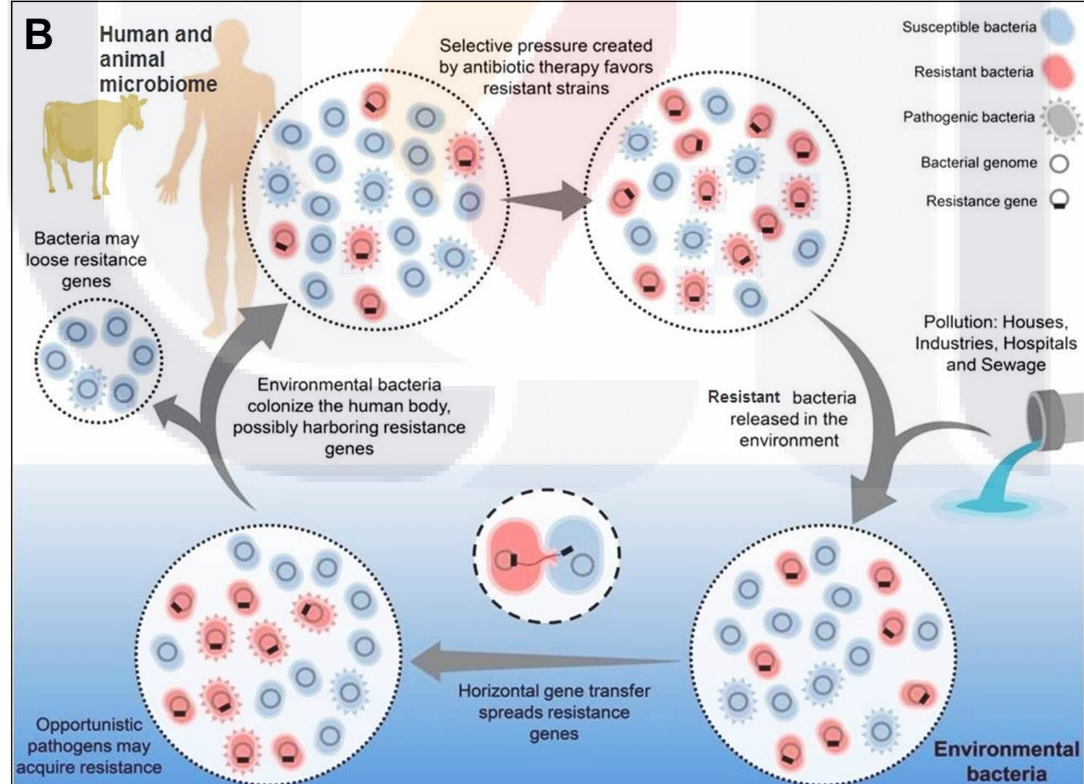

Figure 4. A) Occurrence and fate of pharmaceuticals in the environment, and B) Schematic representation of the interactions between pollution, resistant bacteria and aquatic environments (Coutinho *et al*., 2013, with modifications).

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will be exposed to many of these compounds for their entire lifetime which could cause effects on endogenous bacteria of the environment (Boxall, 2004).

Stress conditions, which are usually found in polluted environments, have also the potential to increase recombination and HGT favoring the dissemination of antibiotic resistance genes (Martínez, 2009; Beaber *et al*., 2004). Other types of contaminations such as heavy metal pollutions (McArthur and Tuckfield, 2000), quaternary ammonium compounds (QAC) (Hegstad *et al*., 2010) and personal care products might result in the presence of antimicrobial resistant bacteria.

Furthermore, WWTPs receive complex mixtures of chemicals and bacteria from multiple origins, creating hotspots for selection and transfer of adaptive genes. Indeed, several studies have been found opportunistic pathogens in river sources that carry class 1 integron, a mobil genetic element which cause resistance to several antibiotic classes (Wellington *et al.,* 2013; Chen *et al*., 2011, Mokracka *et al*., 2011). Microorganisms that carry genes encoding resistance to a broad range of antibiotics have been found in hospital wastewater and animal production wastewater as well as in sewage, surface water, groundwater and drinking water (Kümmerer, 2004). Thus, aquatic environments are described as natural reservoirs of antibiotic-resistant bacteria and wastewater treatment plants are among the leading water reservoirs of these microorganisms, and greatly points to gene transfer and to spread resistance among related bacteria (Jury *et al,*. 2010; Kümmerer, 2004).

One of the main routes of dissemination of pathogenic and/or antibiotic resistant bacteria is the environment, including water, soil and air. Consumption and handling of water, whether treated or untreated, can lead to colonization of the gastrointestinal tract of humans and animals by bacteria containing resistance genes and exchange genes with bacteria already present in the intestinal tract (Finley *et al*., 2013; Coleman *et al*., 2012; Baquero *et al*., 2008).

Humans and animals can be exposed to antibiotics, antimicrobial resistance genes or resistant bacteria in the environment by different pathways (Figure 4): a) cultures exposed to activated and/or sludge, animals (chicken, cattle, pig, etc.,) that have been treated with antibiotics as drugs or growth promoters, b) groundwater and surface water containing residues of pharmaceuticals and used as drinking water or irrigation, c)

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inhalation of dust emitted by livestock facilities and food crops (Wellington *et al*., 2013; Benotti *et al*., 2009; Farkas *et al*., 2007; Boxall *et al*., 2006; Kumar *et al*., 2005).

1.3 BIOFILM.

Biofilms are a survival microorganism strategy that allows the colonization of hostile environments, host tissues or inert surfaces, even under changing conditions. The biofilm can be defined as communities of microorganisms irreversibly adhered to a surface to produce extracellular polymeric substance (EPS) and exhibiting an altered metabolic state compared to the corresponding planktonic growth, especially with respect to the transcription and interactions between cells (Lindsay and Holy, 2006). Biofilm formation are depending of genes encoding appendages consisting of oligomerized subunits responsible for motility (type IV pili or TFP, flagella) or with other functions (fimbriae, other types of pili, curli), the matrix of exopolysaccharides (EPS), surface adhesions, or other secreted elements (Bordi *et al.,* 2011).

The development of a biofilm involves planktonic cells (individual cells of free movement) reversibly adhering to a surface. The next step involves the irreversible binding to the surface, the multiplication of bacteria and the formation of a microcolony, as well as the production of a polymer matrix (ECM) surrounding the microcolony. The biofilm becomes a little thicker (about 50 µm) and obtained a similar way to a fungus or a pillar is often seen as a main feature of a mature biofilm. Subsequent steps include dispersing the biofilm focus, releasing bacterial cells that can be disseminated to other locations and to form a new biofilm. The release process can be caused by bacteriophage activity in the biofilm (Figure 5). Mature biofilms may contain water channels. Motile bacteria can use the type IV pili to attach to a biofilm formed by other bacteria that colonize the top of the structure (Burmølle *et al*., 2010; Beloin *et al*., 2008).

It is known that virulence factors of *E. coli* such as fimbriae, curli, flagella, and antigen 43, and molecules of the extracellular matrix including colonic acid and poli-β—1,6-N-acetil-D-glucosamine contribute to the biofilm development, adhesion cell to cell and different surface, and establish the structure of the biofilm (Jacobsen *et al*., 2008; Beloin *et al*., 2008).

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In the post-exponential growth phase of biofilm formation, in which nutrients concentration are no longer optimal but not yet completely exhausted, *E. coli* cells produce flagella and become highly motile. Morphologically, cells become successively shorter but are still rod-shaped and continue to divide. However, when resources are further declining, cells stop producing flagella, they become smaller and ovoid and they eventually cease to grow and divide and enter into stationary phase and concentrate on survival. In parallel, they begin to produce and excrete autoaggregative curli fibers, a process which leads to cellular aggregation by the generation of amyloid curli fibers (Serra *et al*., 2013; Burmølle *et al*., 2010). Biofilm formation in *E. coli* requires a set of gene expressions facilitating its initiation, attachment and subsequent maturation; for example, a variety of virulence factors such as haemolysin, fimbriae, lipopolysaccharide (LPS), secreted proteins, capsules and iron-acquisition systems (Wingender and Flemming 2010; Stoodley *et al.,* 2002; O'Toole *et al.,* 2000).

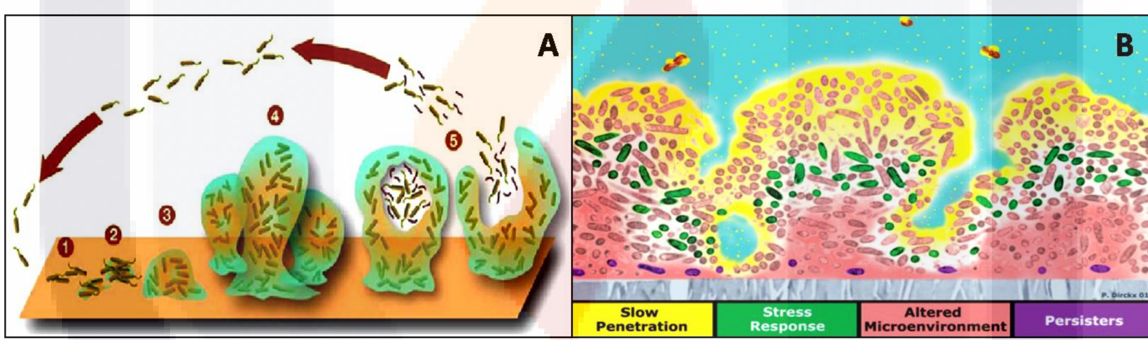

Figure 5. A) Temporal evolution of biofilm. Schematization of the four-stage universal growth cycle of a biofilm with common characteristics, including initiation (1), maturation (2 and 3), maintenance (4), and dissolution (5). B) Four possible mechanisms of biofilm antibiotic resistance. The image is a cross section of a biofilm with the attachment surface (gray) at the bottom and the aqueous phase containing the antibiotic (yellow) at the top. In zones where there is nutrient depletion (red), antibiotic action may be antagonized. Some bacteria may activate stress responses (green), while others may differentiate into a protected phenotype (purple, Chambless *et al*., 2006).

Since bacterial concentrations tend to be higher in biofilms than in the water, the growth of opportunistic pathogens within biofilms is of particular interest to public health protection. Biofilms allow some pathogens to proliferate in the external environment outside the body of the host and increase their numbers to what might be an infective dose (Barbeau *et al*., 1998). Pathogen association with biofilms in drinking

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water distribution systems and potable water sources has been demonstrated with the survivalof *Helicobacter pylori*, a bacterium which causes gastritis (Gião *et al*., 2010). Flanders and Yildiz (2004) pointed out that the protected, nutrient-rich environment of the biofilm might be ideal for pathogen retention. Pathogens, such as *E. coli, Campylobacter jejuni* and *Pseudomonas aeruginosa* have been shown to survive and proliferate in biofilms (Wingender 2011b).

As for studies in natural aquatic systems, a study by Muirhead *et al.* (2004) simulated an artificial in-stream flood in the absence of rainfall and compared the contribution made by river rock biofilms and sediments to the loading of *E. coli* in a stream under dry conditions and found that sediment made the major contribution (Figure 6, Maal-Bared, 2013).

Biofilms have a role in up to 60% of human infections and they are difficult to eradicate with antimicrobial treatment. Additionally, biofilm infections of indwelling medical devices are of particular concern, as once the device is colonized infection is virtually impossible to eradicate. In vitro susceptibility tests have shown considerable increase in resistance of biofilm cells to disinfection (Spoering *et al*., 2001). Many characteristics of biofilms, such as resistance to antimicrobials and increased opportunities for genetic exchange, could increase the pathogenic potential of a biofilm community while promoting survival of the organisms in the environment. Since bacteria in biofilms can hardly be eradicated, they cause massive damage in medical as well as technical environments (Hall-Stoodley *et al*., 2004).

Bacteria grown as a biofilm are protected against a variety of environmental stresses such as lack of food, presence of heavy metals, chlorination biocides antibiotics, disinfectants and host immunological defenses (Pereira *et al*., 2010; Bridier *et al*., 2011; Hoiby *et al*., 2010; Hall-Stoodley and Stoodley 2009; Beloin *et al*., 2008). The difference between free-floating cells and biofilm-embedded cells is that the biofilm bacteria are sufficiently protected by other defenses, such as retarded antimicrobial penetration or slow growth, which allows the biofilm cells to respond to an antimicrobial challenge that overwhelms planktonic cells (Figure 5, Chambless *et al*., 2006).

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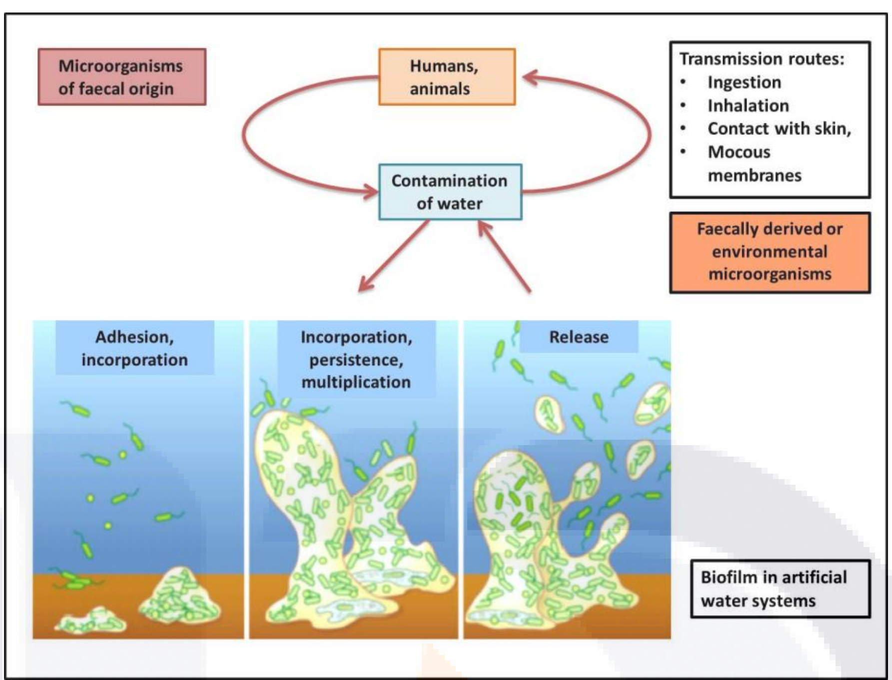

Figure 6. Role of biofilms as environmental reservoirs of hygienically relevant microorganisms and as sources of contamination and infection in drinking water systems (taken from Wingender and Flemming 2011).

1.4 WATER POLLUTION.

Access to clean and safe water, adequate sanitation, and improved hygiene are critical to sustaining human health and life. Although water is essential for life, it also causes injuries and can spread illness when it is contaminated by disease-causing organisms (CDC, 2013). Surface waters must undergo careful quality assessment before being used as drinking water or reclaimed water. Contamination with wastewaters, whether treated, untreated or partially treated, is a risk to public health.

Most waterborne infections are caused by ingestion of contaminated water since contaminated water acts as the passive carrier of the infectious agent. Direct contact or inhalation of contaminated water also represents a risk (Leclerc *et al*., 2002). Industrialized countries, epidemic episodes are mainly due to the spread of new agents or newly identified infections (Aulicino *et al*., 2005). It is estimated that 3.2% of deaths globally are attributable to unsafe water caused by poor sanitation and hygiene, a problem that is particularly acute in rural areas in the developing countries (WHO, 2010).

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Eighty million people do not have access to safe water, and an estimated 2.5 billion people (50% of the developing world) lack access to adequate sanitation worldwide. Diarrheal diseases are the second leading cause of death among children under 5 years. Approximately 88% of deaths due to diarrheal illness are attributable to unsafe water, poor sanitation and inadequate hygiene, making waterborne infections a global health problem (CDC, 2013).

Between 2007 and 2009, 134 recreational water-associated outbreaks (pools, interactive fountains, parts of rivers used for swimming) were reported by 38 States in United States and Puerto Rico. These resulted in at least 13,966 cases including 84% outbreaks of acute gastrointestinal illness, which were associated to the presence of *E. coli* and *Shigella* spp. (CDC, 2013). In 2000, and outbreak in Walkerton, Ontario was related with the presence of *E. coli* O157:H7 in the Great Lakes area resulted in 2,300 cases of illness (Hamelin *et al*., 2006). The global burden of infectious waterborne disease is considerable. Reported numbers highly underestimate the real incidence of waterborne diseases (Leclerc *et al*., 2002).

Aquatic environments such as rivers and lakes possess ecological and economical relevance. Unfortunately, these sites are subjected to high levels of anthropogenic impact worldwide and increases over the years. Urban, industrial and farming activities are responsible for the discharge of pollutants. Fertilizers, sludge, organic compounds, heavy metals and all sources of wastewater are released into water bodies often without appropriate treatment. A few possible outcomes from these contaminations are: eutrophication, hypoxia, toxicity, bioaccumulation, and dissemination of pathogens (Nogales *et al*., 2011). These pollutants can spread across long distances.

Waterborne outbreaks of bacterial origin in the developing countries have declined dramatically from 1900s. Nevertheless, some early bacterial agents are still prevalent, including *Shigella sonnei*, *Campylobacter jejuni* and *E.coli* O157:H7, which may contaminate pristine waters through wildlife or domestic animal feces (Leclerc *et al*., 2002). Important waterborne bacterial pathogens which can infect the gastrointestinal tract of humans and warm-blooded animals and are excreted with the faeces into the environment include *Salmonella enterica*, *Shigella* spp., *Vibrio cholera*,

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pathogenic *E. coli* variants, *Yersinia enterocolitica*, *Campylobacter* spp., and *Helicobacter pylori*. These pathogens have in common that they are transmitted by ingestion of faecally contaminated water and primarily cause gastrointestinal (diarrheal) diseases. Furthermore, all of them may have the potential to become components of microbial communities in biofilms (Wingender 2011; Wingender and Flemming 2011). Others waterborne bacterial pathogens include: *Aeromonas* spp., *Pseudomonas aeruginosa*, *Legionella* spp., and *Mycobacterium avium*, these last two, could transmit through direct inhalation of aerosols (Leclerc *et al*., 2002).

Surface waters are most vulnerable to pollution due to their availability for the disposal of wastewaters. Both natural processes, such as precipitation inputs, erosion, weathering of crystal materials, and anthropogenic influences urban, industrial and agricultural activities, increasing exploitation of water resources, together determine the water quality of surface water in a region.

1.4.1 WATER QUALITY PARAMETERS.

Water-quality monitoring is the process of sampling and analyzing water conditions and characteristics. Water characteristics, such as dissolved oxygen, pH, nutrients, and temperature, are known as parameters. Parameters can be physical, chemical or biological in nature. Some of the physical characteristics of water quality include, but are not limited to, temperature, dissolved oxygen, conductivity, turbidity and suspended solids. Chemical parameters are a measure of substances, such as nutrients, heavy metals and pesticides, which are dissolved in the water or are in particulate form. Biological parameters generally include total coliforms and fecal coliforms which are a measure of faecal contamination in the water. Under guidance from the U.S. Environmental Protection Agency (USEPA), states develop water-quality standards that designate levels of each parameter that are acceptable for designated uses such as drinking water, irrigation, swimming, and aquatic life.

Monitoring different aspects of water quality over time enables changes to the aquatic environment to be detected and understanding of the ecosystem. If only physical or chemical parameters are measured, it is difficult to gauge the impact they have on the life in or using the water. Similarly, measuring biological parameters can tell you if the

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ecosystem is under stress, but not necessarily what is causing the stress. The combined data can be used to generate information essential for those managing and protecting natural resources, allowing them to determine if the conditions of the water resource are improving or worsening with time and human use.

Table 3. Parameters to water quality monitoring including the desirable levels to use as irrigation water.

	Parameters	**Desirable levels**		**Parameters**	**Desirable levels**
In field	pH	6.5-8.5	Nutrients	Phosphorus	0.1 mg/L
	[a]DO	5.5-8-0 mg/L		Nitrogen	1 mg/L
	Conductivity	2000 uS/cm	Organic toxics	[d]MBAS	0.5 mg/L
Organic matter	[b]BOD	15 mg/L		Phenols	0.010 mg/L
	[c]COD	20 mg/L		Anilines	0.002 mg/L
	Fats and Oils	10 mg/L	Fecal contamination	Total coliforms	1000 MPN/100mL
				Fecal coliforms	100 MPN/100mL

Table taken from Guzmán-Colis *et al.*, 2011 with modifications. [a]DO: dissolve oxygen; [b]BOD: biochemical oxygen demand; [c]COD: chemical oxygen demand; [d]MBAS: methylene blue active substances.

1.5 CASE OF STUDY: SAN PEDRO RIVER AT AGUASCALIENTES, MÉXICO

Aguascalientes State is located in the central region of Mexico; its geographical coordinates extreme are from 22º21' to 21º38' north and from 101º53' to 102º52' west (INEGI, 2006). The State falls mostly within the Lerma-Santiago-Pacific hydrologic region in the Pacific slope, which drains an approximate area of 77,000 km^2, of which 7,2% (5,544 km^2) are located in the State of Aguascalientes (Santos-Medrano *et al.,* 2007). Only 2% of the State (73.17 km^2) corresponds to hydrologic region 37, El Salado (CONAGUA, 2007).

In the State, the only natural recharge is from surface sources precipitation. In general, the estimated volume of rainfall of 531 mm^3/year, and varies from 381 mm^3 in the northern portion, to 538 mm^3 in the west. The rainy season occurs between June and September, during which precipitation is about 75% of the total annual average, of which approximately 248,8 mm^3 slip surface, 200 mm^3 infiltrate into the ground and taken up to 2195,4 mm^3 atmosphere by evaporation (Guzmán-Colis *et al.,* 2011). The average annual availability of surface water in the state is about 238,8 mm^3, that are assigned almost entirely to agricultural and livestock sectors, covering 35% of the needs of these (Guzmán-Colis *et al.,* 2011; CONABIO, 2008).

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In Aguascalientes, lies the San Pedro River, also called Aguascalientes River; important affluent of the Santiago River, major riverbed of the state and main pluvial collector. This comes at 40 km south of the city of Zacatecas and crosses the entity from north to south, a straight-line distance of about 90 km and 2,821 km^2 of area, covering directly 7 of the 11 municipalities that conforms the state, and leaves through the Chilarillo town in Jalisco State (Guzmán-Colis *et al.* 2011; Santos Medrano *et al.,* 2007).

In its course, the San Pedro River is joined by the rivers: Pabellón, Santiago, Morcinique, Chicalote and San Francisco; and the brooks: El Saucillo, Rincón, El Salto, El Pedernal, Escobas, El Xoconoxtle, Jesús María, Milpillas, El Pastor and La Virgen. Another streams from the San Pedro River in the east and from north to south directions are; the rivers: El Chicalote and San Francisco; and the brooks: Chiquihuite, Las Pilas, Caldera, El Molino, Los Arellanos, Ojo Zarco, San Nicolás, El Cedazo, Peñuelas and Las Ventas (CONABIO, 2008).

The river lies on a fault line with average depth of 50 cm, presents mainly clay type sediments and has an area of 2821 km^2 (Guzmán-Colis *et al.,* 2011). Currently no river base flow, receives input from 24 streams and about 96% (about 120 Mm^3/year) of raw and treated wastewater generated by the various sectors of the State. Currently, the river is an intermittent stream with little flow.

1.5.1 WATER POLLUTION IN SAN PEDRO RIVER.

Due to the land area that forms the natural water stream, the main productive activities for economic development of the Aguascalientes State, such as agricultural production, industrial production, commercial sector and urban development, coupled with increasing pollution and exploitation immoderate, have led to changes water quality in the State (Guzmán-Colis *et al.,* 2011; Carrazco-Rosales *et al.,* 2008, unpublished data).

For decades, in the main water bodies it has been deposited large pollution loads, generating a severe impairment to rivers, streams, dams and levees. In the late nineteenth century the contamination of San Pedro River began, since become a collector of massive sewage discharges, garbage, debris, feces, and various pollutants. In

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the last 30 years have been proliferated a lot of housing developments located on the banks of the river, including the stone materials extraction of the stream (Guzmán-Colis *et al.,* 2011).

In general, highlights as major sources of pollution: the discharge of wastewater from domestic population centers, the wastewater from industrial activity, discharges from agricultural activities and the existence of areas around the stream used as landfill, debris and defecation outdoors (Carrazco-Rosales *et al.,* 2008, unpublished data).

Currently, are considered contaminated the following water bodies: the rivers San Pedro and Chicalote; the brooks: La Salud, El Molino, Los Arellano, El Cedazo and San Francisco; and the dams: El Niágara, Media Luna, Plutarco Elías Calles, Abelardo Rodríguez and San Jerónimo (Carrazco-Rosales *et al.,* 2008).

It is important to mention that in the plains and valleys of the central region where it joins the main hydrological aspects, are located the greatest potential productive land and settle the major population centers and industries; highlighting the industrial shifts metal-mechanics, electronics, textile, electroplating, cement and mining, as well as agricultural (CONAGUA, 2003).

In the State, the wastewater generation is an order to 117.7 millions of cubic meters per year. Aguascalientes have diverse wastewater treatment plants which trated 114mm^3 of wastewater volume from municipality, 84mm^3 from service companies and 45mm^3 from industrial sector (CONABIO, 2008). However, in some cases, municipal treatment infrastructure and the industrial sector, are working at lower operation levels than their capacity that could be insufficient to the wastewater volume that must receive a treatment.

In addition, the systems based on oxidation ponds, septic tanks and wetland installed at the State, do not satisfy particular conditions required of the wastewater discharges, especially in references to the fecal coliforms; this is mostly due to some wastewater treatment plants that do not properly perform the installations maintenance. Thus, they are not in optimal conditions to operate, working over their capacity or with an inadequate design (CONABIO, 2008).

Aguascalientes State has been using different water streams as a vehicle to conduce the wastewaters, which in general are dumped without any previous treatment,

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thus, treated wastewater volumes are mixed with no treatment wastewater volumes, giving an infection source. Indeed, in the industrial sector and service companies, approximately 37.7% of their wastewater discharges volumes are pulled out to the agricultural land and green areas without any treatment, while the rest are dumped on the rivers, streams and brooks. It stands out that at municipal sector, the wastewater that are treated are re-used in the agricultural irrigation, green areas irrigation and fodder crop (CONAGUA, 2003).

Due to low pluvial precipitation and the increasing wastewaters discharge into the stream, the San Pedro River, an intermittent stream with almost no self-purification capacyti has become a collector of sewage (Figure 7), exceeding the limit of absorption of pollutants (Mora-Silva *et al.* 2007, unpublished data), causing the existence of toxic levels, a significant pollution load of organic matter and a high level of fecal contamination (Carrazco-Rosales *et al.,* 2008, unpublished data), including the detection of pathogenic bacteria such as *E. coli*, *Salmonella* spp, *Shigella* spp, and *Proteus* spp, and *Pseudomonas* spp., in almost all surface water bodies (Carrazco-Rosales 2008; unpublished data), constituting a serious health hazard and a latent focus of infection.

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Photography 1. Direct discharge from FREASA to San Pedro river

Photography 2. Direct discharge from FREASA to San Pedro river.

Photography 3. Direct discharge from FREASA to San Pedro river.

Photography 4. Las Ánimas community.

Photography 5. Los Vázquez- Los Ramírez community.

Photography 6. Jesús María municipal trail.

Photography 7. Valle de Aguascalientes Industrial park junction to Chicalote river to San Pedro river.

Photography 8. Valle de Aguascalientes Industrial park junction to Chicalote river to San Pedro river.

Photography 9. Valle de Aguascalientes Industrial park junction to Chicalote river to San Pedro river after the bridge.

Photography 10. Las Flores I community.

Photography 11. Gómez-Portugal community.

Photography 12. Jaltomate community.

Figure 7. Actual conditions of San Pedro River at Aguascalientes State, Mexico (pictures taken by the present study).

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2. JUSTIFICATION

In many developing countries including Mexico, availability of water has become a critical and urgent problem. In fact, the growing levels of pollution and overconsumption or resources require some sort of solution. Therefore high quality water sources may be required not only from drinking purposes, while the quality of water for other domestic uses can be quite variable (Salem *et al*., 2011).

The need to preserve water has resulted in an increase in the use of treated sewage effluent for many non-drinking purposes such as irrigation. Therefore, it is very important to implement water conservation. However, reclaimed water used for irrigation often contains parasites, bacteria, and disease-causing viruses which are cause of diarrhea and extraintestinal infections. Thus, create a potential health hazard for the exposed human population (WHO, 2006).

The problem with water resources of the State of Aguascalientes is mainly generated by the low percentage of surface water available to meet the total demand. Therefore, the highest percentage of the demand is covered by groundwater sources, causing a systematic exploitation of aquifers (Guzmán-Colis *et al*., 2011).

The contamination of the San Pedro River, the main surface water in the State includes the influx of wastewater from several industries and municipal discharges that carrying high organic charges, textile dyes, phenols, anilines, detergents and heavy metals. Agricultural, livestock and human activities in the vicinity of the Rio San Pedro, imply a constant risk of contamination, which can be recorded by the presence of pathogens in its riverbed including the enteropatogens *E. coli, Salmonella, Shigella*, *Proteus mirabilis, Morganella, Providencia, Citrobacter freundii, Klebsiella pneumoniae, Enterobacter,* among others (Carrazco-Rosales, 2008, unpublished data).

Moreover, it is important to note that in the methodology of sampling and microbiological testing of the water quality, the sample is limited to only water, even when the bacterium are able to adhere at almost any surface forming biofilms, which enhances their resilience and persistence in the environment. However, this capacity is neglected in the traditional view of the methodology. In addition, pathogenic *E. coli* can exist in two states; in viable state or viable non-culturable state (Kell *et al*., 1998),

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making difficult to detect pathogenic bacteria, and hence it is not evaluate by microbiological testing of the water quality.

Furthermore, pollution by antibiotic and their determinants can enhance the possibilities of human pathogens for acquiring resistance. Aquatic environments are one of the principal ways to spread and evolution of antimicrobial resistant bacteria since antimicrobials and antimicrobial resistant bacteria are often directly released in the environment (Lupo *et al*., 2012). In water, especially with high density of microorganisms, bacteria of different species and from diverse origins are mixed and given arise to new antimicrobial resistant strains due to the genetic exchange between species. The discharge of antibiotics, quaternary ammonium compounds, health care products and heavy metals into the stream water could favor the selection of antimicrobial resistant bacteria and reduce the susceptible microbiota in the environment. Indeed, use of antibiotics for agricultural purposes, particularly for growth enhancement, has come under much scrutiny worldwide, as it has been shown to contribute to the increased prevalence of antibiotic-resistant bacteria of public health significance (Finley *et al*., 2013). This highlights the importance of the environment as a reservoir of resistance genes and dispersal vector.

The present research is designed to detect the presence *Escherichia coli* in stream water in San Pedro river as planktonic bacteria, biofilm forming-bacteria, viable but not culturable state, and the occurrence of the different pathotypes and antimicrobial resistance profiles of these strains, such as primary evidence of the potential risk that can cause this contamination into aquatic ecosystems, as well as human and animal health. This study will generate necessary data on the current condition of the quality of surface water in the State so that it can determine the impact of pollution on the generation of pathogenic bacteria and antimicrobial resistance dissemination and can serve as a basis for risk analysis of the potential damage that could cause the river to the public health at the State.

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3. OBJECTIVE

3.1 GENERAL OBJECTIVE.

To detect and characterize the different pathogenic potential, antibiotic resistance and biofilm formation of *Escherichia coli* present in the San Pedro River in Aguascalientes State, Mexico.

3.1 PARTICULAR OBJECTIVES.

1. To evaluate if the river water could support the growth of bacteria as *E. coli* by microbiological and physico-chemical assess.
2. To conduct a study of the occurrence and distribution of *E. coli* in its viable culturable (VC) and viable but non-culturable state (VBNC).
3. To establish the antimicrobial resistance profiles by phenotype in the *E. coli* isolates from the river.
4. To conduct a study of the prevalence of *E. coli* pathotypes in river water.
5. To characterize the *E. coli* by the ability to form biofilms.
6. To establish the relationship among multidrug resistant strains, pathogenic bacteria and water quality.

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4. HYPOTHESIS

The San Pedro River in the State of Aguascalientes harbors *Escherichia coli* with different pathogenic potential, antibiotic resistance, which could be a cause of outbreaks in humans and animals, being a potential health problem.

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5. MATERIALS AND METHODS.

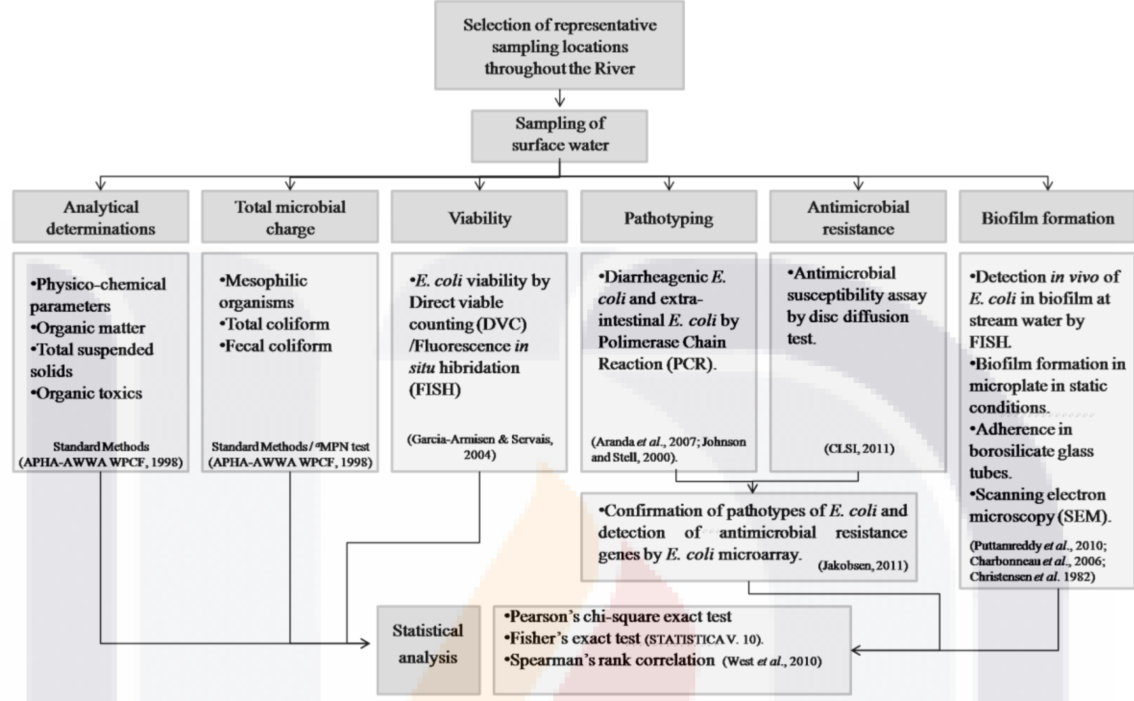

Figure 8. General methodological approach of the study. [a]MPN: most probable number, CLSI: Clinical Laboratory Standard Institute.

5.1 SELECTION OF SAMPLING LOCATIONS AND SAMPLING.

Thirty sample locations were selected throughout San Pedro River and its main tributaries (Figure 9). All locations were sampled once and selected due to the presence of important discharges of treated or untreated wastewater into the river (Table 4). Sampling was performed from June to November according to procedure described by the American Public Health Association (APHA, 1998), the World Health Organization (WHO, 2006) and the Mexican Norm NMX-AA-003. Water samples were collected in 180 mL sterile glass bottles in triplicates. The samples were stored at 4°C until analysis, which was done within 24 h of the sample collection.

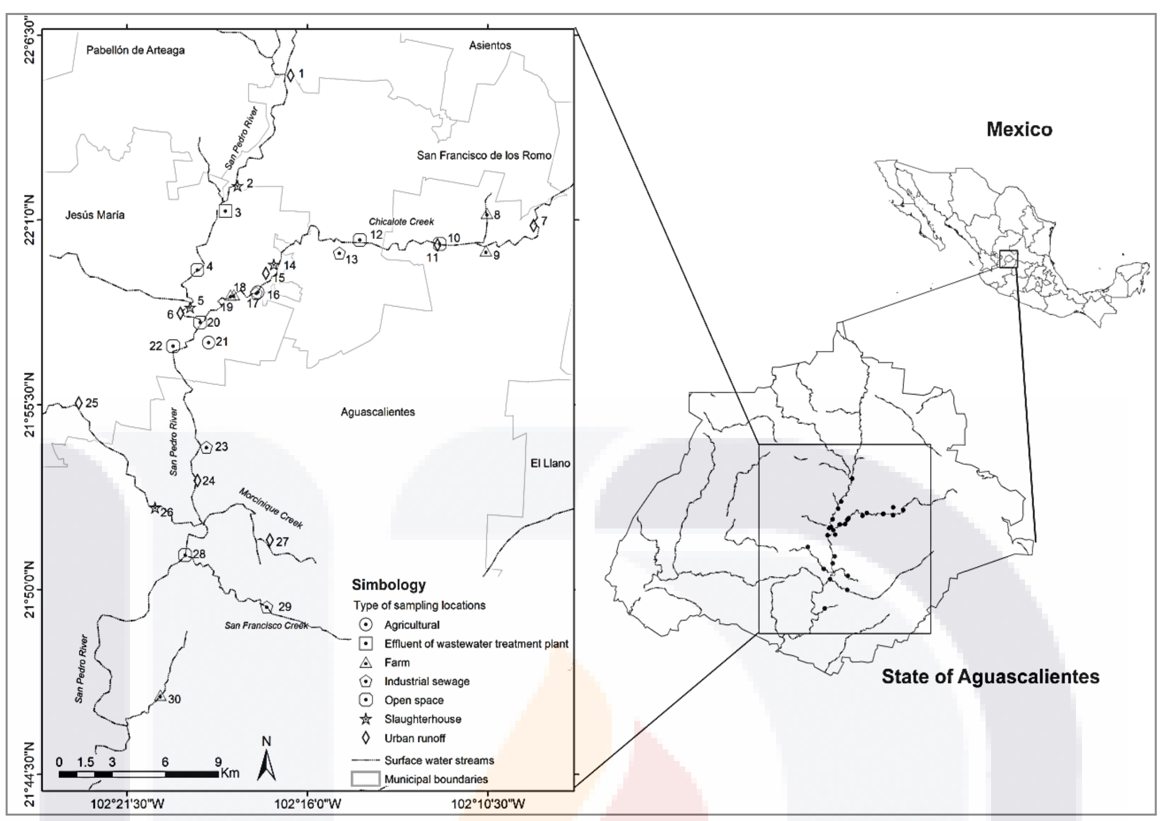

Figure 9. Diagram of sample locations along the San Pedro River. Chicalote, Morcinique, and San Francisco Creek are the main tributaries of the San Pedro River.

The number and location of sampling stations were determined bases on the amount of total and fecal coliform expressed en previous studies (Guzmán-Colis *et al*., 2011; Carrazco-Rosales, 2008, unpublished data; Mora-Silva, 2007, unpublished data). The sample size was calculated by taking into account the size of the population as the number of locations sampled in previous studies (Guzmán-Colis *et al*., 2011; Carrazco-Rosales, 2008).

Prior to the sampling a field visit was carried out in each sampling station. In addition, it was consulting the hydrological location at the National Institute of Statistics, Geography and Informatics (INEGI). To locate the sampling stations were taken in latitude (N) and longitude (W) at each sampling site using a GPS Garmin GPS model map 60c. Locations of the sampling stations are shown in Table 4 and Figure 9).

The samples were taken in agreement with the Official Mexican Norm NMX-AA-003 and APHA-AWWA-WEF,1998. Samples were taken in triplicate in sterile glass

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bottles of 175 ml with tight lid; approximately 150 mL were put them and kept from their transport until analysis at 4 ^{o}C ± 1 ^{o}C, in order to inhibit bacterial activity. The bacteriological analysis of samples was performed within 24 h.

Table 4. Location and main sources of pollution of the 30 sampling locations studied.

Sample No.	Sample locations	^{a}Type of sampling location	Latitude W	Longitude N
1	San Pedro River. Las Ánimas.	UR	22^{0}5'17.6"	102^{0}16'30"
2	San Pedro River. FREASA	S	22^{0}03'45"	102^{0}18'58.8"
3	San Pedro River. FREASA-PT.	WWTP-E	22^{0}03'52.5"	102^{0}17'0.2"
4	San Pedro River. San Antonio de los Horcones.	OS	22^{0}03'53.0"	102^{0}17'1.0"
5	San Pedro River. Rastro municipal de Jesús María.	S	21^{0}56'13.26"	102^{0}19'51.8"
6	San Pedro River. Los Ramees-Los Vásquez.	UR	21^{0}59'30.2"	102^{0}18'20.9"
7	Chicalote Creek. Jaltomate.	UR	22°00'49.5"	102°09'5.8"
8	Chicalote Creek. El Becerro II.	F	22^{0}00'04.5"	102^{0}10'34.1"
9	Chicalote Creek. El Becerro I.	F	22^{0}00'04.5"	102^{0}10'34.1"
10	Chicalote Creek. Cañada Honda I.	UR	22^{0}00'15.5"	102^{0}12'1.4"
11	Chicalote Creek. Cañada Honda II.	OS	22^{0}00'16.1"	102^{0}11'59.4"
12	Chicalote Creek. Loretito.	OS	22°00'23.8"	102°14'29.01"
13	San Pedro- Chicalote River. Confluencia PIVA-Chicalote-Gómez Portugal.	IS	21°59'24.4"	102°17'15.4"
14	Chicalote Creek. Gómez Portugal - Cremería. Area oeste.	UR	21^{0}59.403'	102^{0}17.093"
15	Chicalote Creek. Gomez Portugal.	S	21^{0}58'50'	102^{0}17'24.3"
16	Chicalote Creek. Reserva Brandy.	AR	21^{0}58'50.6"	102^{0}17'30.1"
17	San Pedro- Chicalote River. Confluencia PIVA-Chicalote.	IS	21^{0}58'49'	102^{0}17'32.7"
18	Chicalote Creek. La Florida I.	F	21^{0}58'45.8"	102^{0}18'15.5"
19	Chicalote Creek. La Florida II.	F	21^{0}58'45.7"	102^{0}18'21.4"
20	San Pedro River-Chicalote Creek.	OS	21^{0}57'56.6"	102^{0}19'15.7"
21	San Pedro River. Paso Blanco.	AR	21^{0}52'25.2'	102^{0}20'36.2"
22	San Pedro River. Tepetate-San Miguelito.	OS	21^{0}57'17.9"	102^{0}20'2.6"
23	San Pedro River. Canal interceptor.	IS	21^{0}53'14.4"	102^{0}19'20.5"
24	San Pedro River. Puente Curtidores.	UR	21^{0}54'13.7"	102^{0}19'04.1"
25	Morcinique Creek. Los Arquitos.	UR	21^{0}52'26.2"	102^{0}20'38.2"
26	Morcinique. La Negrita primera sección.	S	21^{0}55'32.6"	102^{0}22'57.6"
27	San Pedro River-Morcinique Creek. Aguascalientes López Portillo.	UR	21^{0}51'28.2"	102^{0}19'8.1"
28	San Pedro River-San Francisco Creek.	OS	21^{0}51'0.9"	102^{0}19'43.2"
29	San Pedro River-San Francisco Creek. Puente Bonaterra.	IS	21^{0}49'28.7"	102^{0}17'13.7"
30	San Pedro River. Fatima.	F	21^{0}46'51.1"	102^{0}20'29.8"

^{a}AR, agriculturally impaired; F, farm; IS, industrial sewage; OS, open spaces; S, slaughterhouse; UR, urban runoff; WWTP-E, wastewater treatment plant effluent.

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5.2 ANALITICAL DETERMINATIONS.

The following parameters were determined in all samples: organic matter (BOD and COD), solids (total suspended), nutrients (total nitrogen and total phosphorus), toxic organic (detergents, dyes and phenols), microbial load (mesophilic, total coliforms and fecal coliforms), *E.coli* VBNC and biofilm *E. coli* in the river. In field, the following physic-chemical parameters were measured: pH, temperature, conductivity and dissolved oxygen were measured. The temperature, pH, dissolved oxygen (DO) and conductivity were measured *in situ* by electrometric methods at each sampling stations.

5.3 PHYSICO-CHEMICAL PARAMETERS.

Physico-chemical parameters were measured as water quality indicators. All the determinations were performed according to Standard Methods for the water and wastewater analysis (APHA-AWWA-WPCF, 1998). The organic matter content was obtained by the BOD and COD. Biological oxygen demand (BOD) determination of the wastewater samples in mg/L was carried out using standard method 5210 B, BOD was calculated after the incubation period of five days. Chemical oxygen demand (COD) was carried out using the method of closed reflux and colorimetric (5220 D). Total nitrogen (Nt) was carrying out by micro-Kjeldahl method (4500-N). The inorganic nutrients included determination of total nitrogen and total phosphorus. Total phosphorus was determinate by ascorbic acid method (4500–P). For quantification of persistent organic compounds detergents and methylene blue active substances were determinate by (SAAM, 5540 C) method. The determination of phenols was carried out by 4-aminoantipyrine and chloroform extraction method (5530 C). The anilines determination was carried out by colorimetric method described by Hess *et al*., 1993.

5.4 TOTAL MICROBIAL CHARGE.

In order to determine the number of viable organisms in water samples, analysis was performed for mesophilic organisms (growth at 35 ± 2°C) aerobic and anaerobic

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according to Official Mexican Norm NOM-092-SSA1-1994. All microbiological tests were performed up to 24 h after taking the simple. Decimal dilutions with phosphate buffer as diluant (NOM-110-SSA1-1994) were used.

5.5 TOTAL COLIFORMS, FECAL COLIFORMS AND ESCHERICHIA COLI BY MOST PROBABLE NUMBER (MPN).

Total coliforms (TC) and fecal coliforms (FC) were determinate by the most probable number (MPN) following the Standard Methods 9221 C and Mexican Standard NMX-AA-042-1987, as indicators of fecal pollution in water. Coliforms organisms are water and drinking water fecal contamination indicator. They are characterized by rod-shaped bacteria, Gram negative, aerobic and facultative anaerobes, which ferment lactose at 35 °C with gas production within 48 h. Coliforms are indicators of sanitary quality of the water (APHA, 1992).

5.6 *E. coli* VIABLE CULTURABLE.

In order to confirm the presence of *E. coli* in water samples, it was carried out the procedure in tube using EC medium with MUG (4-methylumbelliferyl-β-D-glucoronide). Serial dilution of water samples was transferred to EC broth media containing MUG. All the tubes were incubated in water bath at 44.5°C ± 0.2°C for 24 h. After the time, the tubes were observed under UV light of 366 nm. The presence of clear blue fluorescent light with or without gas production is an indicator of the presence of *E. coli*. The MPN can be quantified and determined from the number of positive tubes (Feng *et al*., 2002).

5.7 *E. coli* VIABLE BUT NO CULTURABLE (VBNC).

The determination of *E. coli* viable but not culturable (VBNC) was carried out by the direct count method of fluorescent in situ hybridization (DVC-FISH), based on Garcia-Armisen and Servais (2004). For this procedure was used the colinsitu probe for

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hybridization of *E. coli* (5'-GAG ACT CAA GAT TGC CAG TAT CAG-3'), 5'-labeled with Cy3. The probe is targeting 637-660 position in *E. coli* 16S rRNA. The filters were mounted on slides to see the epifluorescence microscope. It was analyzed 10 fields per filter under a microscope for enumeration. The number of cells per volume of extract is calculated as follows: Number of cells / µl = (cells per field * area of filter) / (area of the field * volume of extract applied).

5.8 *E. coli* ISOLATION.

All the *E. coli* strains were isolated from serial dilution of the water samples and put them in eosin methylene blue agar plate and MacConkey agar. The plates were incubated 24 h at 37°C. Five colonies, including lactose-fermenting and lactose non-fermenting, likely to be *E. coli* were taken and kept them in glycerol 20%, -80°C for the biochemical test (Gram-negative, IMViC (Indol production, Voges-Proskauer, Methyl red-reactive compounds, citrate; ++-- or -+-- , Feng *et al*., 2002). Isolates meeting the *E. coli* test profile were confirmed by detecting the *uidA* gene using primers listed in supplementary data Table 4:S. In total, one hundred and fifty strains were isolated.

5.9 ANTIMICROBIAL SUSCEPTIBILITY ASSAY.

Antimicrobial susceptibility test of 150 *E. coli* isolated from stream water was performed using a disc diffusion assay according to CLSI standards, 2010. Isolates that were resistant to three or more antimicrobial agents were defined to have a multiple drug resistant (MDR) phenotype. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, United States), was included in each assay as a negative control. Antimicrobial agents were tested using the following antimicrobial susceptibility test multidisc for Gram–negative bacteria with the following antimicrobial agents: amikacin-30 µg, ampicillin-10 µg, cephalothine-30 µg, cephotaxim-30 µg, ceftriaxone-30 µg, chloramphenicol-30 µg, gentamicin- 10 µg, netilmicin- 30 µg, nitrofurantoin- 300 µg, pefloxacin-5 µg, carbenicillin-30 µg, trimethoprim-sulfamethoxazole-1.25/23.75 µg. The quinolone levofloxacin-5 µg was tested separately using a Bio-Rad Sensi-Disc.

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5.10 PHYLOGENETIC GROUPS.

The DNA extraction was performed by the boiling method (Hamelin *et al*., 2006). The phylogenetic groups A, B1, B2, C, D, E, and F were determined with the quadruplex PCR method (Clermont *et al*., 2013).

5.11 PATHOTYPE AND VIRULENCE GENES DETERMINATION BY PCR.

Detection of intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC) virulence genes were performed by PCR with primers described in Table 4:S. InPEC isolates were defined according to criteria established by (Aranda *et al*., 2007) with modifications. ExPEC isolates were defined by criteria described by (Johnson and Stell, 2000). Positive controls are listed in supplementary data Table 5:S. The PCR products were visualized by agarose gel electrophoresis stained with 1.5% ethidium bromide.

5.12 CHROMOSOMAL-ENCODED AND ACQUIRED QUINOLONE RESISTANCE GENES.

Eighteen quinolone resistant and intermediate resistant isolates were selected to investigate mutations in the quinolone resistance determining region (QRDR) of *gyrA* and *parC* genes, as well as the presence of the acquired genes *qnrA, qnrB, qnrS,* and *aac(6')-Ib cr*. The oligonucleotides and PCR conditions used in this study are listed in supplementary data Table 4:S. The quinolone resistance determining regions of *gyrA* and *parC* genes were amplified and sequenced as described by (Nambodiri *et al*., 2011). Amplicons were sequenced on both strands and predicted peptide sequences were compared to the corresponding gene from the MG1655 genome using BLAST program in Geneious R6 software (v. 6.0., Biomatters Ltd, New Zealand). The *aac(6')-Ib cr* genes was detected as described by Park *et al*., 2006. The *aac(6')-Ib cr* variant was identified by sequencing the PCR products (Park *et al*., 2006).

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5.13 DNA MICROARRAY ANALYSIS.

Microarray hybridizations were performed using the *E. coli* maxivirulence version 3.1 microarray as previously described by (Jakobsen *et al*., 2011). It allows the detection of 348 virulence genes and 98 antibiotic resistance genes and variants. DNA extraction and hybridizations were performed as described previously (Bruant *et al*., 2006). Each isolate was assigned to a specific *E. coli* pathotype according to its virulence gene profile and based on classification described previously (Jakobsen *et al*., 2011; Bonnet *et al*., 2009). *E. coli* isolates were also assigned to a phylogenetic group based on the presence of *chuA, TspE4.C2,* and *yjaA* as described previously (Clermont *et al*., 2000). Twenty *E. coli* strains were chosen based on results for antimicrobial resistance phenotypes and the results of pathotyping PCRs to be tested by the microarray technology in order to determinate their pathogenic potential according to their virulence and virulence-related gene content as well as their antimicrobial resistance genes profile.

5.13.1 CLASSIFICATION OF PATHOTYPES BY MICROARRAY.

Each *E. coli* isolate are assigned a particular pathotype according to the set of virulence genes or markers in base of Hamelin *et al*., 2006. For ETEC, LT and/or STa and/or STb; for STEC subgroup EHEC, *stx1* and/or *stx2*, *espA*, *espB*, *tir,* and *eae*; for STEC, *stx1* and/or *stx2*; for EAEC, *capU*, *shf,* and *virK*, aggregative adherence fimbria encoding genes (*aafA*); for atypical EPEC, *espA*, *espB*, *tir*, *eae*, and its variants and the absence of *bfpA*; for ExPEC, the uropathogenic subgroup (UPEC), gene coding for P pili, *hlyA*, S fimbria genes encoding, *chuA*, *fepC*, *cnf1*, *irp1*, *irp2*, *fyuA*, *iroN*, and *usp*; for ExPEC, the subgroup associated with septicemia (SEPEC), *cdtB-3*, *cdtB-2*, *cdtB-1*, *cdtB-4*, *cnf2*, F17A, f165(1)A, *iucD*, and *gafD*; for ExPEC, the subgroup associated with meningitis (MNEC), *ibeA*, *neuA*, and *neuC*; and for “other ExPEC,” *kpsMII*, *iutA*, *iucD*, *traT*, *malX*, *irp1*, *irp2*, *fyuA*, *chuA*, *fepC*, *iss*, and *kfiB*; for ExPEC-like the presence of several genes of the previous gene list of “other ExPEC”. The lack of one or more of the genes identified for each given set of genes is considered a non-pathogenic

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strain. The isolates of *E. coli* are assigned a phylogenetic group based on the presence of genes *chuA*, TspE4.C2, and *yjaA* described by Clermont *et al*. (2000).

5.14 BIOFILM ASSAY.

5.14.1 DETECTION OF *E. coli* IN BIOFILMS IN STREAM WATER.

For development of the biofilm in the stream water, glass slides were placed in sterile plastic tubes directly in the water in counter current of the river. After 7 days, the tubes were collected from the water and put at 4°C until analysis. The slides were removed using tweezers sterilized stainless steel flame. To analyze the biofilm formation on glass slides, hybridization was carried out according to Lehtola *et al.,* (2006) with modifications. Briefly, the slides are taken out from the plate and dried. The biofilms were subjected to a pre-treatment with sodium citrate (1 mM) at 95°C for 5 min. The samples were washed with distilled water (50°C) for 5 minutes. Cover the sample with hybridization buffer and a coverslip (180 µl of 5 M sodium chloride, 20 ml of 1M Tris-HCl, 1 ml of 10% SDS, 1 ml of Triton X-100, 350 µl of formamide, 449 µl of distilled water and 1.5 µM of oligonucleotide colinsitu-probe labeled with Cy3 (Garcia-Armisen and Servais, 2004). The hybridization was carried out for 28 hours at 50°C in the absence of light. After the incubation time are subjected to washing by immersion in 400 ml of washing solution (0.35 g NaCl, 0.242 Tris Base and 400 µl Triton X-100), for 25 minutes in darkness at 50°C, and washed with distilled water. Mount the samples with Prolong Gold and cover with a coverslip and analyzed at epifluorescence microscope.

5.14.2 BIOFILM FORMATION BY MICROTITER ASSAY.

Biofilm formation assays were performed following the method of Puttamreddy *et al*. 2010 and Charbonneau *et al*. 2006 with modifications. Briefly, the strains were grown overnight in Luria–Bertani (LB) liquid media at 37°C. Following the incubation, dilutions 1:100 (vol/vol) were made in M9 media supplemented with 0.45% glucose and were grown overnight as describe above. The wells of a sterile 96-well microtiter plate

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were filled in triplicate with a dilution 1:100 (vol/vol) of each overnight bacterial culture. Following an incubation of 24 h at 30°C, the wells were washed with water and allowed to dry for 5 min at 30°C. The wells were then stained with 150 μL of 0.1% crystal violet (CV) for 2 min at room temperature; the colorant was then discarded and the wells rinsed with distilled water. The plate was dried at 30°C for 15 min and the CV was solubilized in 150 μL of absolute ethanol:acetone 80:20 (vol/vol). The optical densities (ODs) of the wells were read at 595 nm. The resulting absorbance is directly related to the amount of cells adhered to the polystyrene and thus is indicative of the isolate's biofilm-forming ability. The assays were performed in triplicate in three different times. The *E. coli* strain ATCC 25922 was used as positive controls and non inoculated broth as negative control. Isolates were considered positive for biofilm formation if the crystal violet-stain biofilm had an OD_{595} equal to or greater than 3-fold the value obtained in the well containing bacteria-free medium.

5.14.3 ADHERENCE TO BOROSILICATE TEST TUBES.

The investigation of the biofilm production was done on the bases of the adherence of the biofilms to borosilicate test tubes, as was previously done (Christensen *et al*., 1982). The suspensions of the tested strains were incubated in glass tubes which contained Brain Heart Infusion Broth (BHI) at 37°C for 48h. Then, the supernatants were discarded and the glass tubes were stained with 0.1% safranin solution washed with distilled water 3 times and dried. A positive result was defined as the presence of a layer of the stained material which adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid-air interfaces was considered negative (Niveditha *et al*., 2012). The *E. coli* strain ATCC 25922 and *Staphylococcus epidermidis* ATCC 35984 were used as positive controls and non inoculated broth as negative control.

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5.14.4 SCANNING ELECTRON MICROSCOPY.

The sample preparation was carried out under an high vacuum process. Briefly, five samples of *E. coli* biofilm previously identified as pathogen or commensal, including the *E. coli* ATCC 25933, were fixed with 4% formaldehyde. The biofilms were taken in round slides (1cm of diameter) and placed 1.5 mL vials. Subsequently, the sample vials were placed in graded alcohols (70%, 80%, 90%, and 96 %) for 10 minutes at each level of alcohol to achieve dehydration of the sample. Subsequently, the vials were placed in the critical point dyer chamber (Tousimis) to remove through carbon dioxide, pressure and temperature in the sample moisture. The samples were placed in stubs (aluminum cylinder 1 cm high by 1.2 cm diameter) and coated with a gold layer of approximately 2 nm thickness. For the examination of the samples, the stubs were placed in the Scanning Electron Microscope (SEM) JEOL LV-5900. Electron micrographs were taken with a professional camera that uses black MAMIYA 120 mm.

5.15 STATISTICAL ANALYSIS.

Comparisons of associations between resistant phenotypes in *E. coli* isolated from stream were performed separately by using Pearson's chi-square exact test and Fisher's exact test (STATISTICA V. 10, Stat Soft, United States). Continuous variables were compared using Mann Whitney U test (STATISTICA V. 10, Stat Soft, United States). All P values were 2-tailed; P values less than 0.05 were considered statistically significant. Spearman's rank correlation (West *et al*., 2010) was used to examine the relationships among temperature, pH, conductivity, dissolved oxygen, chemical oxygen demand, biological oxygen demand, total phosphorus, nitrogen, total solid suspended, mesophilic bacteria and total and fecal coliform density across all sample locations. For biofilm analysis and comparison between biofilm formations by different pathotypes the two-way ANOVA was performed (Graph Pad Prism V 5.0).

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6. RESULTS

6.1 PHYSICOCHEMICAL ANALYSES OF WATER OF SAN PEDRO RIVER.

The physicochemical properties of each sample locations of the San Pedro River were characterized using parameters such as temperature, DO, pH, biological oxygen demand (BOD) and chemical oxygen demand (COD). These parameters were included given that they have a major influence on bacterial growth (Salem *et al*., 2011; Zamxaka *et al*., 2004). Results of physicochemical parameters are shown in the Table 5. The mean value for water temperature was 24.7^{o}C, which is usually considered a favorable temperature for the growth of microorganisms as well as for a wide range of human and animal pathogens. The pH values of water samples varied from 7.4 to 7.7 units, with a mean value of 7.5 units. The pH values were within the permissible limits (6.0 to 9.0) established by the WHO for wastewater discharge into the sea or the environment. Furthermore, this pH range value is optimal for bacterial growth.

Table 5. Physico-chemical parameters from San Pedro River.

Variable	Mean (*n* =30)	Interval
Temperature (^{o}C)	24.7	(22.6-25.8)
pH	7.5	(7.4-7.7)
Conductivity (µS/cm)	1639	(1520-2547)
[a]DO (mg/L)	0.3	(0.38-2.1)

[a]DO: dissolved oxygen.

The dissolved oxygen concentrations (DO) for almost all the sample locations were lower than 1 mg/L suggesting high organic matter levels. These DO concentrations are near anoxygenic conditions, which allow the growth of a broad spectrum of both aerobic and anaerobic microorganisms. For conductivity, 96.6% of the samples were above the WHO guideline value of 1000 µS/cm for wastewater discharging into a stream. These conductivity levels imply high concentrations of dissolved inorganic matter suggesting that high concentrations of inorganic nutrients available for microbial

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proliferation. Significant differences in conductivity were observed between location sites close to farms and slaughterhouses ($p<0.001$) compared to other types of sample locations.

The indicators of organic matter, COD and BOD, were generally high along sample locations (Figure 10). This confirms the discharge of raw wastewater of urban runoff including municipal origin into the river. The mean values were 722 mg/L and 1723 mg/L for BOD and COD, respectively, representing a high organic load in the river. Sample locations with highest levels of BOD and COD were related to farms, urban runoff, industrial sewage and slaughterhouses. All water samples were above the maximum permissible limit of organic matter (BOD < 200 mg/L) set by Mexican Norms (NOM-001-ECOL-1996) for streams discharged into rivers used for agricultural irrigation. These results showed abundant carbon and energy sources to support the heterotrophic growth of microorganisms.

Total suspended solids (TSS) were correlated with concentration of COD, BOD and mesophilic bacteria (TSS–COD, $r_s=0.79$, $p=0.05$; TSS–BOD, $r_s=0.79$, $p=0.05$; TSS–mesophilic bacteria density, $r_s=0.89$, $p=0.012$). The measured concentrations of COD and BOD showed a strong correlation ($r_s=1$, $p>0.001$). All the sample locations exceeded the total phosphorus threshold of 0.1 mg/L as well as the nitrogen threshold (1 mg/L). Temperature and nitrogen concentration were negatively correlated ($r_s=-0.79$, $p=0.048$). Total phosphorus concentration was strongly negatively correlated with dissolved oxygen concentration ($r_s=-0,96$, $p=0,003$).

Literature classifies wastewater TSS as follows: TSS less than 100 mg/L as weak (best quality of water), TSS greater than 100 mg/L but less than 220 mg/L as medium and TSS greater than 220 mg/L as strong wastewater (Salemn *et al*., 2011). Results of this study classified the sample locations located near to WWTP effluent as weakly contaminated wastewater, which reflects the efficiency of WWTP. Less contaminated sample locations close to open space were classified as medium wastewater. In total, 27 sample locations were classified as polluted sites and only three as less polluted. Locations number three (wastewater treatment plant effluent), fifteen (slaughterhouse) and twenty five (urban runoff) were categorized as less polluted sites in base of their physico-chemical results (Figure 10).

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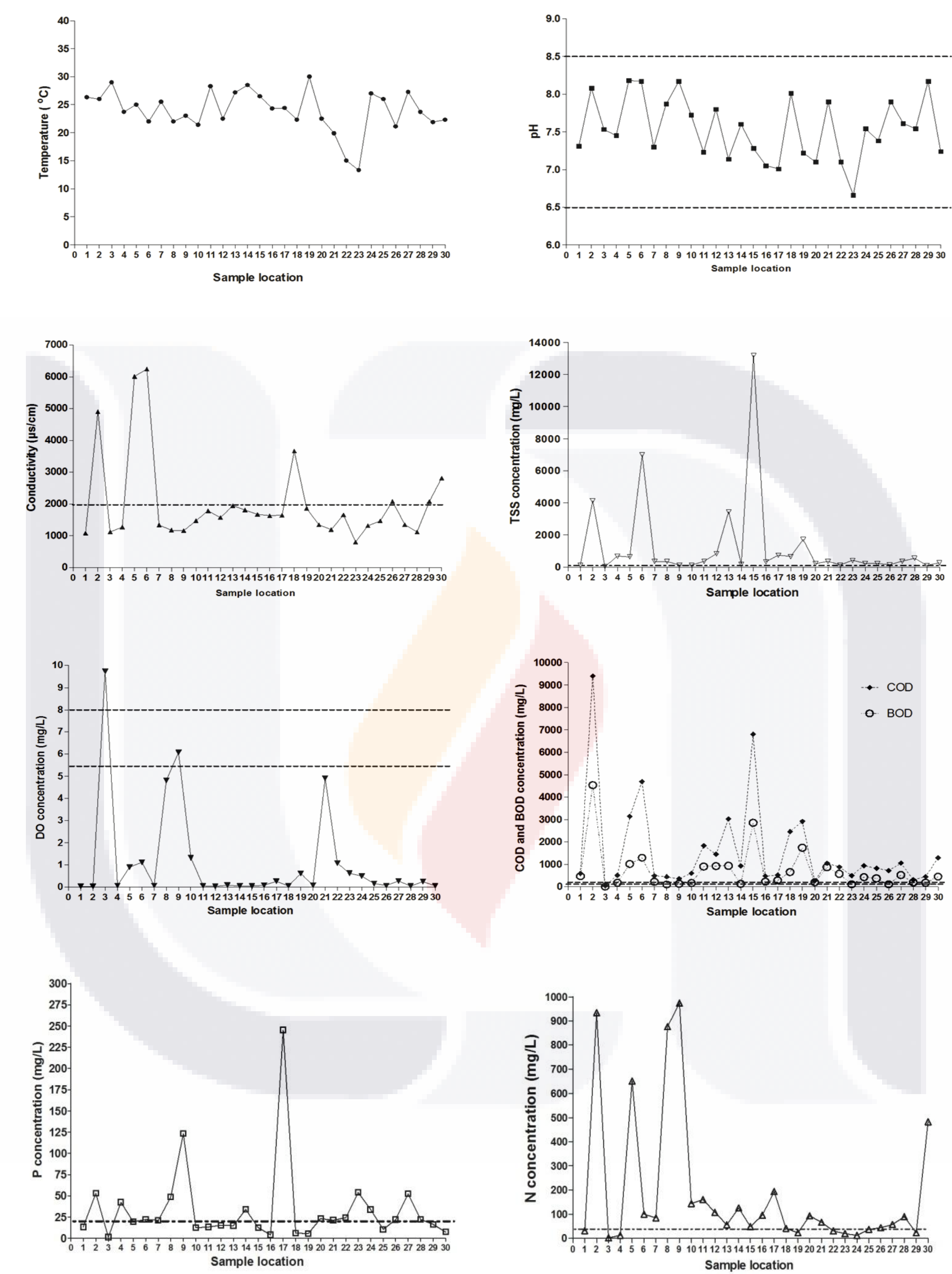

Figure 10. Spatial variation of physico-chemical parameters from San Pedro River per sample location. Permissible maximum limits are shown in dotted lines. pH values between 6.5 and 8.5 units are considered as good quality in potable water (NOM-127-SSA1-1994). Conductivity values above 2000µS/cm are exceeding the permissible limits. Nitrogen values above 40 mg/L are exceeding the permissible limits. Phosphorus levels rating higher than 20 mg/L is considered as poor water quality. Total solid suspended has a permissible value of 120 mg/L. Dissolved oxygen concentration limits are between

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5.5 and 8.0 mg/L (Guzmán-Colis *et al*., 2011). BOD and COD concentrations limits are 150 mg/L and 100 mg/L respectively according to Mexican Norms NOM-003-ECOL-1997 and NOM-001-ECOL-1996.

High nitrate concentrations are frequently encountered in treated wastewater, as a result of ammonium nitrogen. High nitrate levels in wastewater could also contribute to eutrophication effects, particularly in freshwater (OECD, 1982). The levels of nitrate exceeded the WHO limits of 45 mg/L for nitrate in wastewater discharged into river. Total nitrogen shown a mean value of 373 mg/L, with a maximum value of 5935 mg/L (point related with discharges from slaughterhouses into the river) and a minimum value of 3 mg/L (point related with wastewater treatment plant effluent into the river). Furthermore, values of ammonia nitrogen shown a mean value of 55.9 mg/L, a minimum value of 18.3 mg/L and a maximum value of 371.0 mg/L; which are far lower that the values of total nitrogen but also exceeds the WHO limits. In general, the sample points with highest nitrogen levels derived from human sewages and farming effluents.

The levels of phosphate in the ninety two percent of the samples were higher than the WHO limit of 5 mg/L for the discharged of wastewater into river. The site 11 belonging to the confluence of San Pedro´s Rivers and creeks showed the greatest phosphate level with maximum value of 245.20 mg/L. The site 16 belonging to water used for irrigation, and site 3 belonging to water plant treatment effluent showed the minimal levels of phosphate. The levels of nitrate in addition to phosphate levels can cause eutrophication and may pose a problem for human health.

Regarding to organic toxics as phenols, anilines and methylene blue active substances (MBAS), all the samples showed values exceeding the desirable levels for irrigation water (Figure 11) with values of 0.010 mg/L, 0.002 mg/L and 0.5 mg/L, respectively (Guzmán-Colis *et al*., 2011). The mean values were 52.55 mg/L for anilines, 0.535 mg/L for phenols and 27.70 mg/L for MBAS. The highest level of anilines was of 169.7 mg/L in sample 14, related to human sewages. For MBAS, the maximum value was of 170 mg/L, sample related from slaughterhouse effluent. For last, maximum value for phenols was 2.8 mg/L for site 13, related of industrial effluents.

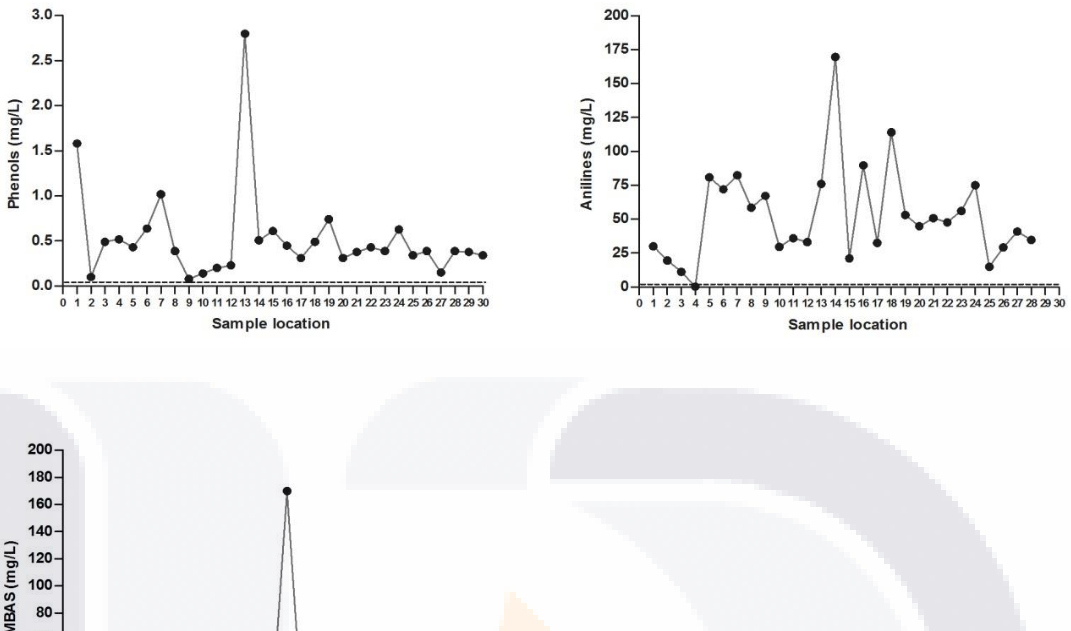

Figure 11. Spatial variation of persistent organic pollutants on San Pedro River per sample location. Permissible maximum limits are shown in dotted lines. Phenols have a permissible value of 0.010 mg/L, 0.002 mg/L for anilines and 0.5 mg/L for MBAS (Guzmán-Colis *et al*., 2011).

6. 2 BACTERIOLOGICAL ANALYSIS

The mesophilic microorganism counts were between 10^4 to 10^6 CFU and these measurements were consistent with the high levels of organic and inorganic nutrients found in the San Pedro River, and the favorable physicochemical conditions for microbial growth found in the river. Although agricultural, farm and industrial sewage site locations tended to have greater counts of mesophilic bacteria than open space, no significative differences were found ($p>0.05$).

Half of the samples exceeded the limit of 1000 MPN/100 mL (WHO, 2006). Some samples were as low as 1 MPN and others as high as $2.4X10^4$ MNP/100 mL. Some samples presented low fecal coliforms counts as low as 0.5 MPN and others were as high as $1 X 10^4$ MNP/100 mL. Statistically significant associations were found between the levels of total and fecal coliforms and water temperature ($p = 0.02$), and

between coliforms and conductivity ($p = 0.03$), suggesting fecal bacteria proliferation due to appropriate conditions in the water environment. Total and fecal coliform were strongly correlated (r_s=0.86, p=0.023). Industrial sewage and urban runoff sites tended to have greater total and fecal coliform densities than the agricultural, farm locations and wastewater treatment plant effluent.

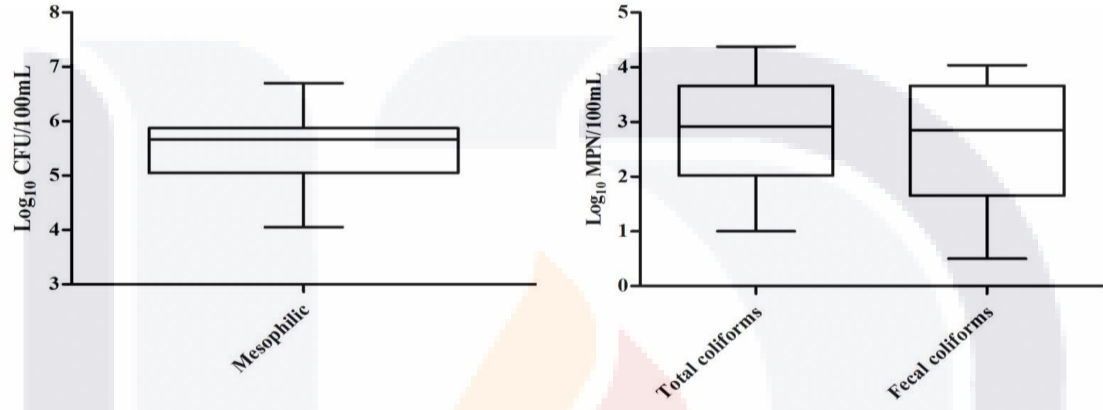

Figure 12. Results of microbiological analyses of San Pedro River in thirty sample location. Bacterial counts values are shown in a $\log_{10}$ scale, a) mesophilic microorganisms b) total and fecal coliforms. Total and fecal coliform density rating above 1000 MPN/100mL and 240 MPN/100mL are considered as poor water quality according to Mexican Norms NOM-003-ECOL-1997 and NOM-001-ECOL-1996.

The results showed that there is a significant correlation between water temperature and the density of aerobic mesophilic organisms ($p = 0.0315$), but not for other physicochemical parameters ($p > 0.05$), although a significant relationship between electrical conductivity and pH was found as expected ($p = 0.011$). As for aerobic mesophilic the water from each sampling site not directly measured physicochemical parameters, except also by water temperature ($p = 0.0029$).

For total and fecal coliforms (Figure 12), the results have shown significant correlation with water temperature parameters ($p = 0.0299$) and conductivity ($p = 0.0348$); as well as water temperature ($p = 0.0136$), conductivity ($p = 0.0322$), and pH ($p = 0.0029$), respectively for both parameters. The results showed that *E. coli* growing is dependent on the water characteristics as temperature ($p = 0.0177$) and pH ($p = 0.0063$).

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6.3 CULTURABLE *E. coli*.

High values of *E. coli* were also found in almost all the sample points (Figure 13) whereas thirthy percent of the samples were above the maximum permissible limit established by WHO, 2006 and Mexican norms. These sample points were related to discharges without treatment from municipal sewage mainly followed by farms and slaughterhouses. The points of sampling or River related with chemical industry discharge did not shown great levels of *E. coli* nevertheless, the shown high levels of microorganisms. For total and fecal coliforms, the results have shown significant correlation with water temperature parameters ($p = 0.0299$) and conductivity ($p = 0.0348$); as well as water temperature ($p = 0.0136$), conductivity ($p = 0.0322$), and pH ($p = 0.0029$), respectively for both parameters. The results showed that *E. coli* growth is dependent on the water characteristics as temperature ($p = 0.0177$) and pH ($p = 0.0063$). The levels of total and fecal coliforms showed the heavy contamination of fecal origin may be a potential source of pathogen transmission. Totals of isolates were assayed for confirmation of *E. coli* by PCR for the *uidA* gene which encodes the beta-glucuronidase enzyme. All the strains were confirmed as *E. coli.*

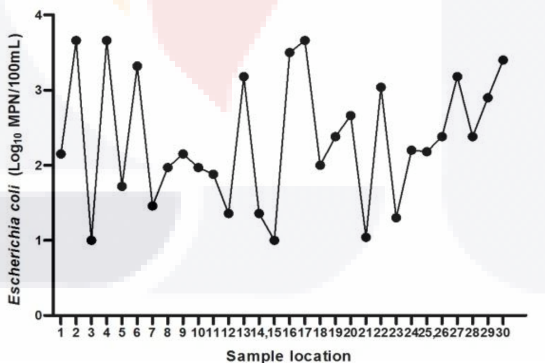

Figure 13. Detection of *E. coli* by the Most Probable Number assay. Confirmation of *E. coli* was done by growing and specific media (*E. coli* is growing in EC + MUG at 37°C per 24 h) as well as by PCR for the gene *uidA* which encodes the beta-glucuronidase enzyme.

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6.4 *E. coli* VIABLE BUT NO CULTURABLE.

The Figure 14 shows the values obtained from *E. coli* in culturable and non culturable state. Pearson correlation indicates no significant differences between the two measurements ($p = 0.096$). However, it is to be noted that in general bacteria had a higher mean VBNC for most points, except for the points 1, 3, 7, 8, 12 and 23, where there was no bacteria labeled with the characteristics of being *E. coli* to be considered as such. This relationship between VC and VBNC in addition for demonstrating the existence of *E. coli* non culturable in aquatic environments such as river water, also assumes the existence of *E. coli* as a pathogen in a VBNC state and could revert to culturable state and be a potential risk to public health, but we need to do further assays in order to confirm such assumption. It also demonstrated a correlation between the charge density of *E. coli* VBNC with pH ($p = 0.0451$) and conductivity ($p = 0.0009$), whereas the remaining parameters did not show any correlation ($p > 0.05$).

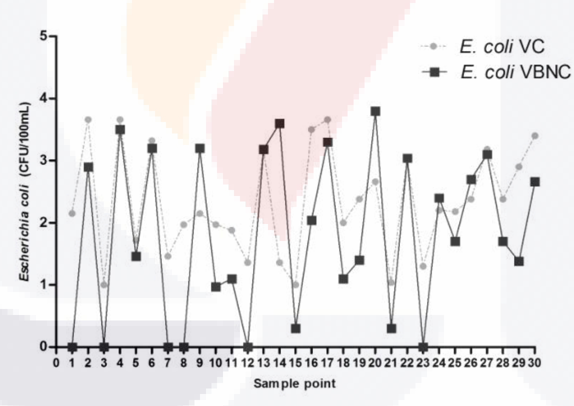

Figure 14. Spatial variation of *E. coli* in viable and culturable state as well as viable but not culturable from San Pedro River water per sample location.

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A B C D
20µm 20µm 20µm 1µm

Figure 15. Images of *E. coli* detected by DVC-FISH procedure using the ''Colinsitu'' probe labeled with the CY3 dye.

6.5 *E. coli* PHYLOGENETIC GROUP.

The most abundant phylogroup among *E. coli* strain isolates tested by phylogroup-typing methods (Clermont *et al*., 2012) from river was the phylogroup A (40.7%), followed by C (20.7%), B1 (14.7%), D (8%), B2 (2%), F (2%), E (0.7%), Clade I or II (0.7%) and undetermined strains (10.7%).

The frequencies of phylogroups were according to those reported previously from environmental and animal samples. Sabaté *et al*. 2008 have shown that phylogroup D was the least abundant in human wastewater isolates of *E. coli* (8%) and not frequent among chicken sewage strains (12%). Majority of the strains belonged to group A, followed by B1. Our results in contrast to those observed in random environmental an

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animal samples of *E. coli* in the study of Higgins *et al*., 2007 in which phylogroup B1 was most abundant (31%), followed by A (26%), D (25%) and B2 (17%). In our study the phylogenetic group A was the most abundant, followed by C, B1 and D, but only three strains were identify as phylogenetic group B2. Nevertheless, we applied a new method by Clermont *et al*., 2012 which integrate the new members of phylogroups. In this metho, phylogroup F consists of strains that form a sister group to phylogroup B2 and D (Jaureguy *et al*., 2008), phylogroup C consists of strains related to phylogroup B1, and E is related to enterohemorragic *E. coli* such as O157:H7 (Tenaillon *et al*., 2010) which might explain the differences in the phylogroups.

6.6 ANTIBIOTIC RESISTANCE.

The antimicrobial susceptibility of 150 *E. coli* isolated from the San Pedro River to 13 antimicrobial agents was measured by the disc diffusion method (CLSI, 2010). Fifty-two percent (79/150) of the isolates were resistant to at least one antimicrobial agent; 37.3% (56/150) were resistant to at least two and 30.6% (46/150) were multi-drug-resistant. Furthermore, 10.7% of the strains were resistant to more than five different antibiotics. A total of 59 isolates (39.3%) were resistant to ampicillin (Figure 16). The second most prevalent antibiotic resistance was towards trimethoprim-sulfamethoxazole (28.6%, 43/150 isolates), followed by carbenicillin (26%, 39/150 isolates), chloramphenicol (22%, 33/150 isolates) and cephalothine (17.3%, 26/150 isolates). Few isolates (1.33%; 2/150) had a resistance towards cephotaxim, netilmicin and amikacin. Interestingly, it was noticed that 7.3% (11/150) of the isolates were resistant to pefloxacin and 4% (6/150 isolates) were resistant to levofloxacin. These antibiotics are second and third-generation quinolones widely used in Mexico against intestinal and urinary tract infections (Guajardo-Lara *et al*., 2009). Most *E. coli* isolates with resistance to fluoroquinolone were found in the sample locations close to farms, agricultural areas, urban runoff and industrial sewage.

Among isolates with a multi-resistant phenotype, 1.3% (2/150) was resistant to seven antimicrobial agents; 3.3% (5/150) were resistant to six antimicrobial agents; 5.3% (8/150) were resistant to five antimicrobial agents, 7.3% (11/150) were resistant to

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four antimicrobial agents and 12% (18/150) were resistant to three antimicrobial agents. A noticeable result shows that a co-resistance to beta-lactams and sulfonamides was frequently observed because most of sulfonamide-resistant isolates were also resistant to beta-lactams (Table 6). Resistance phenotype to quinolones was associated with beta-lactams and sulfonamides resistance ($0.05 \geq p \geq 0.01$), and beta-lactams and phenicols resistance ($0.01 \geq p \geq 0.001$).

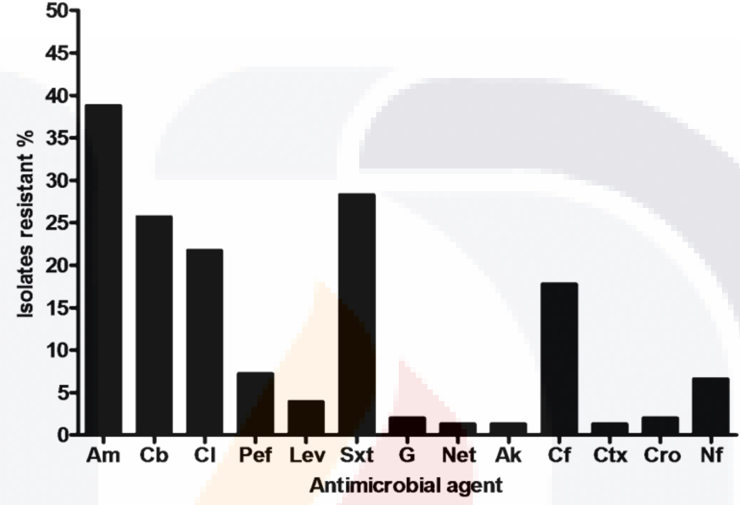

Figure 16. Antimicrobial resistance (%) of *E. coli* isolated from San Pedro River. A total of 150 isolates were characterized by antimicrobial susceptibility assay. [a]Am, ampicillin; Cb, carbenicillin; Cl, chloramphenicol; Pef, pefloxacin; Lev, levofloxacin; Sxt, trimethoprim-sulfamethoxazole; G, gentamicin; Net, netilmicin; Ak, amikacin; Cf, Cephalotine; Ctx, cephatoxim; Cro, ceftriaxone; Nf, nitrofuratoin.

Table 6. Association between antimicrobial resistance phenotypes of *E. coli* isolates from stream water.

Antimicrobial agent		Phenicols	Quinolones		Sulfonamides	Beta-lactams		Cephalosporin
		Chloramphenicol	Pefloxacin	Levofloxacin	Trimethoprim-sulfamethoxazole	Ampicillin	Carbenicillin	Cephalotine
Quinolones	Pefloxacin	++						
	Levofloxacin	-	+					
Sulfonamides	Trimethoprim-sulfamethoxazole	++	++	++				
Beta-lactams	Ampicillin	++	+++	++	-			
	Carbenicillin	++	++	-	+	+		
Nitrofurans	Nitrofuratoin	+	+	-	-	-	+++	
Cephalosporin	Cephalotine	-	+++	++	+++	+++	+++	
	Cephatoxim	-	-	-	-	+	-	-
	Ceftriaxone	++	-	-	-	++	-	+

Only the antimicrobial multi-resistant phenotypes that exhibited an association with another phenotype at the $p < 0.05$ level are shown. The levels of significance of the association (as assessed by the chi-square exact test) were as follows: -, $p > 0.05$; +, $0.05 \geq p \geq 0.01$; ++, $0.01 \geq p \geq 0.001$; +++, $0.001 \geq p$.

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The location sites close to discharges from urban runoff and industrial sewage had a more important proportion of isolates that were resistant to multiple antibiotics (15 and 10 isolates, respectively). WWTP effluent and agricultural locations had the lowest proportion of antibiotic resistant bacteria with only two multi-resistant and one resistant bacteria in each location. Urban runoff locations as well as industrial sewage, open space and slaughterhouse sample locations had the most important counts of antibiotic resistant bacteria and were also the locations with most density of total and fecal coliforms. Additionally, even when farm locations presented low proportion of antibiotic resistant bacteria, these locations presented multidrug resistance patters to more antibiotic classes. Non resistant bacteria were found in samples isolates from farms (site locations No. 8 and 30), urban runoff (site location No. 14) and agricultural (site location No. 21) locations. Furthermore, density of multi-resistant bacteria was negatively-correlated with nitrogen concentration (r_s=-0.78, p=0.04).

WWTP effluent sample, which was considered as less polluted based on tested parameters presented proportion of antibiotic resistant bacteria similar to agricultural sector (30%). Nevertheless, samples from industrial sewage had the highest proportion of multi-drug resistant bacteria (50 %) suggesting that industrial sewage might have an impact on the presence of multidrug resistant bacteria. Unfortunately, no negative controls (sample sites with non pollution) were taken since all the streams of the river were considered polluted. Nevertheless, sites with the best quality were taken into count in order to see if there were differences of antimicrobial and multi-drug resistant bacteria between polluted and less polluted areas. In base of this, a significative difference was found between the presence of antimicrobial resistant bacteria in polluted sites towards unpolluted sites (p=0.0421).

Given the high prevalence of antibiotic resistant commensal bacteria in San Pedro´s River waters, the risk of water recreants being exposed to these bacteria appears to be credible. The magnitude of this risk likely depends on the microbiologic quality of the water as well as the source of the faecal contamination. In addition to recreation, contaminated surface water may also be a source of exposure to antibiotic resistant bacteria if it is used for irrigation of vegetables and fruits that are consumed uncooked.

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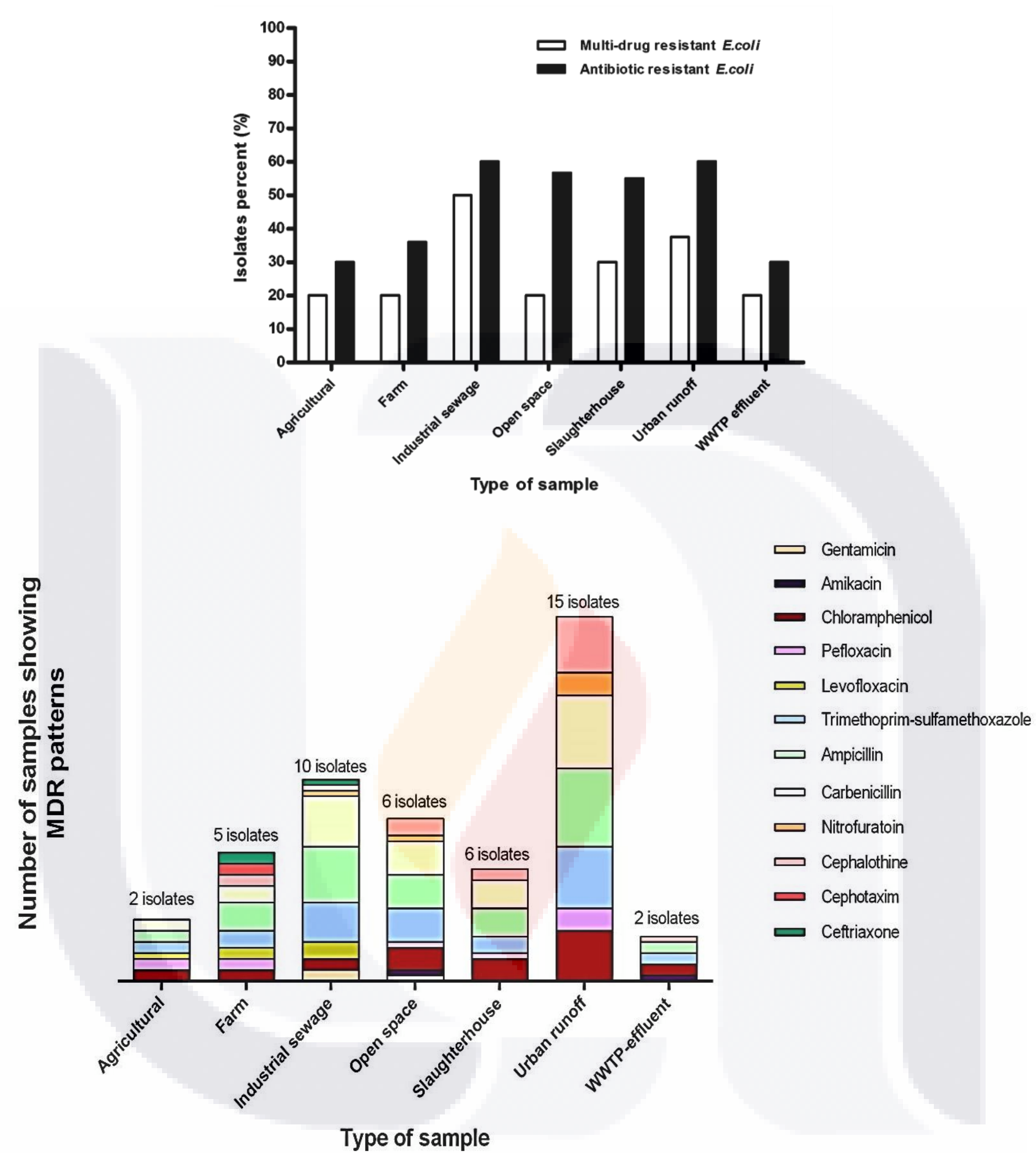

Figure 17. A) Antimicrobial resistant *E. coli* found at different types of polluted sites. B) Multi-drug resistance *E. coli* isolates from the environment. [a]Am, ampicillin; Cb, carbenicillin; Cl, chloramphenicol; Pef, pefloxacin; Lev, levofloxacin; Sxt, trimethoprim-sulfamethoxazole; G, gentamicin; Net, netilmicin; Ak, amikacin; Cf, Cephalotine; Ctx, cephatoxim; Cro, ceftriaxone; Nf, nitrofuratoin. [b]MDR, multi-drug resistance; WWTP-effluent, wastewater treatment plant effluent.

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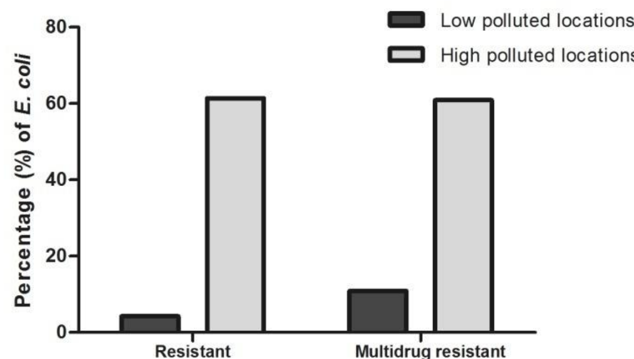

Figure 18. Antimicrobial resistant and multidrug resistant *E. coli* isolates frequency according to the pollution of the sample location. Locations 3-WWTP-E, 20-OS and 28-OS were considered as low polluted areas. Significant different were found among resistant and multi-resistant strains in low and high polluted locations (p=0.0421).

Moreover, exposure to antibiotic resistant bacteria via environmental components may pose additional risks compared to other exposure routes, considering the possibility of exposure to novel mixtures of bacteria and resistance genes.

6.7 PATHOTYPE AND VIRULENCE GENES DETERMINATION OF *E. coli*.

Sixty percent (91/150) of the strains were PCR positive for at least one virulence gene (Aranda *et al*., 2007). Eighty-six isolates were identified as InPEC, including 44.6% (67/150) EAEC, 6.6% (10/150) EPEC, and 6% (9/150) ETEC. Only 5 (3.3%) isolates were identified as incomplete ExPEC because these isolates were positive for the virulence genes *fyuA*, *kpsMII*, *sfa* and *afa/dra* (Johnson and Stell, 2000). EIEC and Shiga-toxigenic *E. coli* were not detected. In addition, slaughterhouse (85%, 17/20 isolates), industrial sewage (80%, 16/22 isolates) and agricultural (60%, 6/10 isolates) sample locations had the most important proportion of pathogenic bacteria.

Furthermore, strains belonging to the pathotypes EPEC (n = 7), ETEC (n = 4), EAEC (n = 37) and incomplete ExPEC (n = 4) were at least resistant to one antimicrobial agent. Among strains with a multi-antimicrobial resistance phenotype, 22 were EAEC, 4 were ETEC isolates, 6 were EPEC, and 1 was an incomplete ExPEC. Most pathogenic *E. coli* that had multi-resistant phenotype were also resistant to beta-lactams and trimethoprim-sulfamethoxazole. Amongst the commensal *E. coli*, multi-resistance was also detected in 22% (13/59) of the isolates.

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Significative differences between number of pathogenic isolates (*p*=0.02) and isolates with resistance to one antimicrobial agent (*p*=0.04) were found when comparing polluted samples with that of less polluted sample sites. Non statistical differences were found compared multidrug resistance (*p*>0.05). This suggests that there are more pathogenic bacteria in the highest polluted water (Figure 21) than in the lowest polluted areas.

Table 7. Antimicrobial resistance found in potentially pathogenic and commensal *E.coli.*

Antimicrobial class	Antimicrobial agent	No. (%)[a] of potentially pathogenic *E. coli* [b]				Commensal (*n* = 59)
		EPEC (*n* = 10)	ETEC (*n* = 9)	EAEC (*n* = 67)	Incomplete ExPEC (*n* = 5)	
Aminoglycosides	Gentamicin	0 (0)	0 (0)	3 (4)	0 (0)	0 (0)
	Netilmicin	0 (0)	0 (0)	1 (1)	0 (0)	1 (2)
	Amikacin	0 (0)	0 (0)	0 (0)	0 (0)	2 (3)
Phenicols	Chloramphenicol	3 (30)	3 (33)	16 (24)	1 (20)	10 (17)
Quinolones	Pefloxacin	1 (10)	1 (11)	3 (4)	0 (0)	7 (12)
	Levofloxacin	1 (10)	1 (11)	3 (4)	1 (20)	0 (0)
Sulfonamides	Trimethoprim-sulfamethoxazole	3 (30)	3 (33)	22 (33)	0 (0)	14 (24)
Beta-lactams	Ampicillin	6 (60)	4 (44)	29 (43)	1 (20)	17 (29)
	Carbenicillin	5 (50)	3 (33)	19 (28)	1 (20)	12 (20)
Nitrofurans	Nitrofuratoin	1 (10)	0 (0)	5 (7)	1 (20)	3 (5)
Cephalosporins	Cephalotine	2 (20)	1 (11)	12 (18)	0 (0)	7 (12)
	Cephatoxim	1 (10)	0 (0)	1 (1)	0 (0)	0 (0)
	Ceftriaxone	1 (10)	0 (0)	2 (3)	0 (0)	0 (0)

[a]Percentages were calculated as follows: number of isolates with resistance phenotype/total number of *E. coli* isolates per pathotype per 100. [b]EPEC, enteropathogenic *E. coli*. ETEC, enterotoxigenic *E. coli*. EAEC, enteroaggregative *E. coli*. Incomplete ExPEC, incomplete extra-intestinal pathogen *E. coli*.

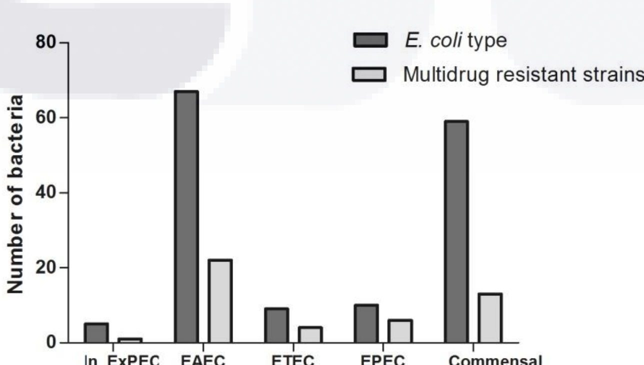

Figure 19. Pathotype and multi-drug resistant *E. coli* isolated from the San Pedro River. *E. coli* isolates were classified as potential pathogens (91/150, 60.6%) or commensal (59/150 isolates, 39.4%). Multidrug resistance patterns were found more frequently in pathogenic strains (33/91 isolates, 36.2%) than in commensal strains (13/59 isolates, 22%).

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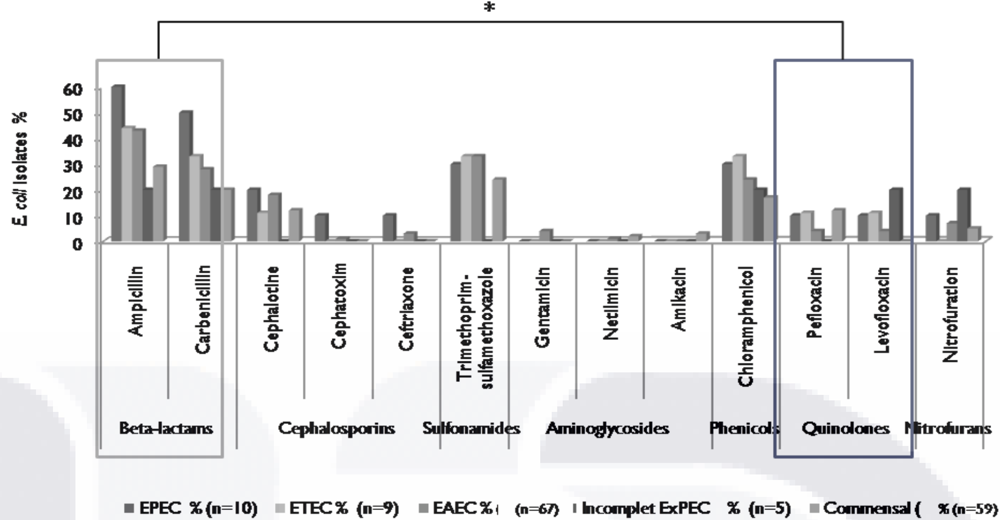

Figure 20. *E. coli* resistance percent per antimicrobial agent (*n* =150). *Antimicrobial multi-resistance phenotype that exhibited an association with another antibiotic resistance phenotype (P <0.05).

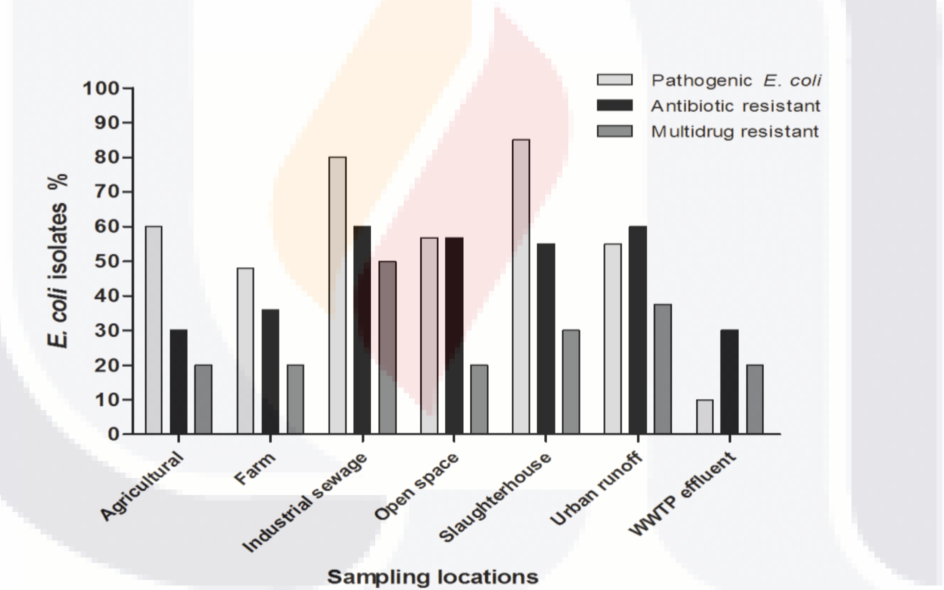

Figure 21. Antimicrobial resistance and potentially pathogenic *E. coli* found by type of sample. Significant differences were found among density of pathogenic and resistant bacteria at WWTP-effluent and the density of pathogenic and antibiotic resistant bacteria at industrial sewage ($P < 0.05$).

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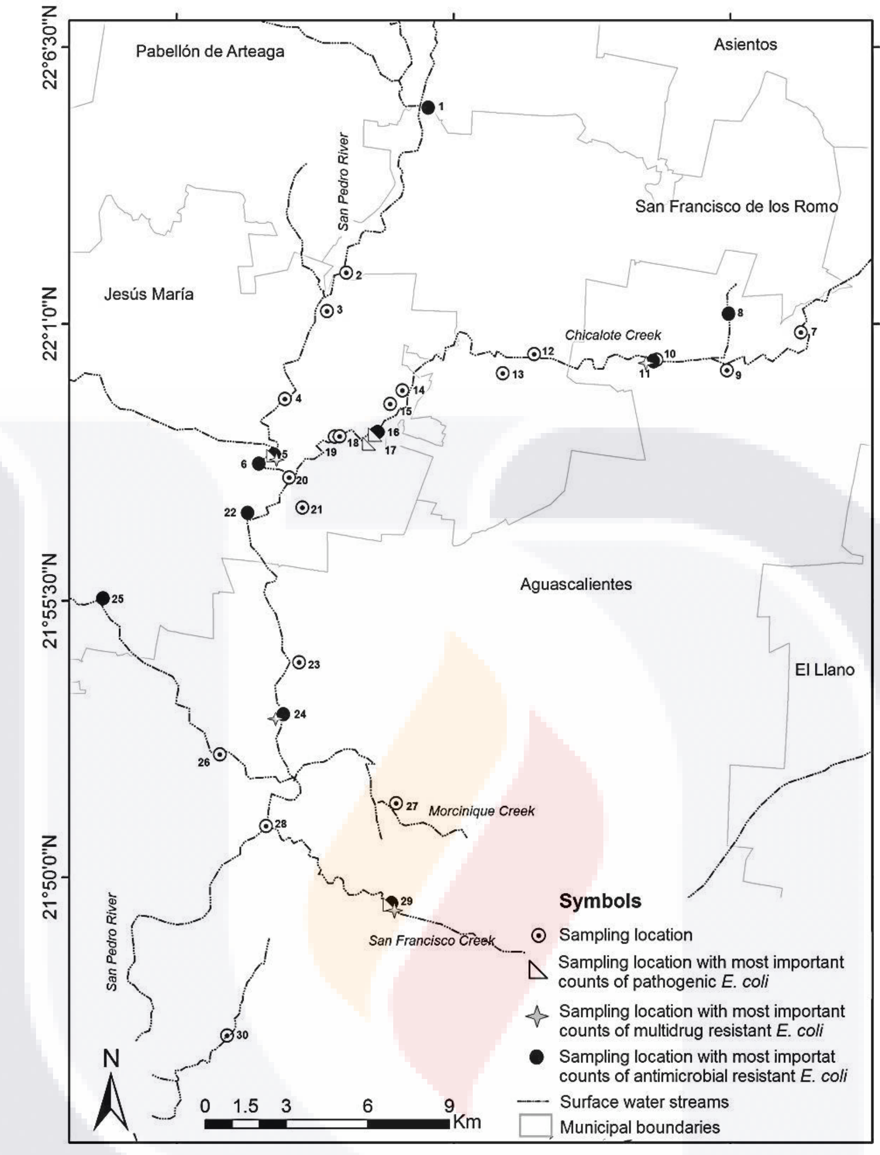

Figure 22. Diagram showing the highest levels of pollution, highest counts of the pathotype and antimicrobial resistant bacteria of the sample locations studied.

6.8 VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES AMONG QUINOLONE RESISTANT *E. coli* ISOLATES.

Microarray analysis was done on seveteen quinolone resistant *E. coli* isolates. Overall, 146 virulence and 40 antimicrobial resistance genes among the 315 and 82 genes and variants investigated were detected at least once in one or more of the isolates. The total number of virulence genes per isolate ranged from 2 to 64 genes. The median

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number of the virulence genes per isolate was 22. Microarray hybridizations demonstrated that all the *E. coli* isolates tested possessed virulence genes related to a pathotype including ExPEC and diarrhoeagenic *E. coli.* The total number of antimicrobial resistance genes per isolate ranged from 0 to 20 genes with a median number of 6.8 genes. Almost all the strains presented different antibiotic resistance genes types, i.e. a strain have genes to resist aminoglycosides (*aph* genes), trimethoprim (*dhfr* genes), tetracyclin (*tet* genes), and beta-lactams (*bla* genes), so it is resistant to 4 different families of antibiotics at the same time, as well as were positive to integron class 1 or 2 which plays an important role in the dissemination of resistance genes among bacteria (Table 10). .

Based on the microarray analysis, nine isolates were classified as commensal and six were classified as InPEC, ExPEC or potentially pathogenic *E. coli*. Commensal *E. coli* showed also the presence of different virulence genes, might acquire in order to better survive (Tables 9 and 10). The isolates belonged to the phylotype group A (15 isolates), group D (1 isolate) and B1 (2 isolate). The most frequent antimicrobial resistance genes were *aph3strA* (10/18), *bla*$_{TEM}$ (10/18) and *tet(B)* (5/18), which code for resistance to streptomycin, ampicillin and tetracycline respectively (Table 10). A *bla*$_{TEM}$ and *aph3(strA)* combination was observed in nine isolates, and a *bla*$_{TEM}$, *aph3(strA)* and *tet(B)* combination was observed in six isolates. In our study, 10 quinolone resistance isolates also carried *bla*$_{TEM}$ gene (Table 10). Among these 10 isolates, nine isolates were positive for the beta-lactamase gene and a plasmid acquired resistance gene. In agreement with Hamelin *et al*., 2006 all multi-resistant *E. coli* isolates (isolates carrying more than three antimicrobial resistance genes) and the majority of *E. coli* isolates with three resistance genes contained a class 1 integron, which is a very efficient genetic mechanism for the diffusion of antimicrobial resistance. Indeed, for the most part virulence and antimicrobial resistance genes are on plasmids, bacteriophages, or pathogenicity islands. These genetic determinants contribute to the rapid evolution of *E. coli* strains and to the creation of new pathogenic variants since they are frequently subject to rearrangement, excision, and horizontal transfer (Hamelin *et al*., 2007, 2006).

Isolates with *qnr* genes and the triple mutation patter in the QRDR were found in sample locations close to farm and agricultural sector. Nevertheless, isolates with

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intermediate-resistance to fluoroquinolone phenotypes were found in sample locations close to urban runoff, industrial sewage, slaughterhouses, open space and wastewater treatment plant. Interestingly, samples with resistance to one or other fluoroquinolone phenotype were sampled in locations close to urban runoff, industrial sewage, open space, farm and agricultural sectors.

Table 8. Virulence genes detected among *E. coli* isolates from stream water.

Function	Gene (s)
Adhesins	*afaD, afaE1, afaE3, afaE5, afrA, aggA, agn43 agn43, aidaI, bfpA(2), bfpA(3), bfpA(4), bfpA. cfaB, clpG, cooA, CS15, CS5, csgA, csgE, csnA, daaE, f165(1)A, F17c-A, cafA, faeGab, fimA, fimF41A, fimH, focG, fotA, gafD, iha, IngA, IpfA (EHEC), IpfA (O113), IpfA (O157) IpfA, nfaA, nfaE111, papA(11), papA(12), papA(13), papA(14), papA(48), papA(8), papC, papGI2, papGI, papGII, papGIV, pilL, pilS, pixA, sfaA*
Colicins and microsins	*caa, cba, cia, cib, cka, cma, cvaC, mcbA, mccB, mchB, mcjA*
Toxins	*astA, astA(2), esta1, stx1B*
Iron acquisition or transport systems	*chuA, fepC, fyuA, iroN, iroN (2), irp1, irp2, iucD, iutA, iutA(2), iutA (UPEC), sitA, sitD*
Capsular and somatic antigens	*kpsM-II, rfbO101, wb(O8), wzy(O15), wzy(O91)*
Haemolysins and haemagglutinins	*ehxA, hlyE, hraI, tsh, vat*
Various functions	*agn43, capU, ccdB, deoK, eibC, eibE, espP, fliC, gad, ibeB, iss, iss(3), malX, ompA, ompT, ompT(2), pic, tibC, traT, tspE4.C2*
Newly recognized or putative *E. coli* virulence genes	*artJ, b1121, etpA, iol, mviM, mviN, shf, ureD, usp, virK, yjaA*
Locus of enterocyte effacement (LEE)	*eae(alpha), eae(beta2), eae(delta), eae(iota), eae(iota2), escJ, escN, espA-1, map-1, map-2, map-3*
Non-LEE elements	*nleA(EHEC), nleA(EPEC), nleG(O103), nleG(O157), nleH*
[a]ETT2 elements	*eivG, eprJ, spaS, z4184*

[a]*E. coli* type III secretion system 2.

Furthermore, microarray analysis also detected the genes often associated with adhesion to host cells, aggregation and biofilm formation such as the autotransporter protein antigen 43 (*agn43* gene), which mediates autoaggregation and biofilm formation and has been reported to be involved in the persistence of *E. coli*; the adhesin of type 1 fimbriae (*fimH* gene) which is associated with colonization of the urinary tract; the type IV pilus lipoprotein, Locus of enterocyte effacement (LEE)-negative STEC pili (*pilL* and *pilS* genes) which also is related with STEC strains; the major fimbrial subunit of aggregative adherence fimbria type I (AAF/I, *aggA* gene) related to the aggregative adherence (AA) of EAEC; the adhesin involved in diffuse adherence (*aidaI* gene) of

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Table 9. Pathotype and commensal *E. coli* isolates from stream water, phylogroup and distribution of virulence factors of interest among each strain.

Strain ID	*E. coli* type	Virulence genes	Others virulence genes
4A	Commensal	-	*aidaI, bfpA(2), lpfA(O157), fedA , fotA, nfaE111 , papA (13)*
18E	Commensal	-	*afrA, aidaI, artJ, ccdB, cma, gad, ibeB, mviN, vat*
17A	Commensal	-	*artJ, cma, csgA, daaE, fliC, gad, hlyE, ibeB, iss(3), iss, vat*
11D	Commensal	-	*artJ, b1121, caa, cka, cma, cooA, cs(5), csgA, daaE, deok, eae(beta2), eae(delta), eae(pi), eivG, eprJ, escJ, fimA, fimH, fliC, fotA, gad, ibeB, mviM, mviN, nleA, nleG, ompA, papG, pilL, pixA, rfb(O101), spas*
20E	Commensal	ND	ND
28C	Commensal	-	*esta1*
23E	EAEC	*aafA, esta1*	-
21A	Commensal	-	*agn43, artJ,astA, asta(2), b1121, cooA, CS5, csgA, csgE, eprJ, fimA, fimH, fliC, gad, hlyE, ibeB, iss, lngA, lpfA, ompA, ompT, ompT(2), pic, wb(O8)*
13D	EAEC	*aggA, capU, shf*	*b1121, ccdB, gad, malX, ompA, ompT, usp, vat*
28E	EAEC	*aggA, aafA, capU, shf*	*artJ, b1121, cib, CS5, csgA, fliC, gad, hlyE, ibeB, mcbA, ompA, pilL, pixA, sitA, sitD*
13A	ETEC/EAEC	*aggA , astA(2), astA, CS5, esta1, capU, shf, virk*	*afaD, aidaI, artJ, ccdB, cib, csgA, csgE, cvaC, daaE, eprJ, fliC, fyuA, gad, hlyE, ibeB, irp(1), irp(2), iss, iucD, iut(A2), iutA, mcbA, mcjA, mviM, mviN, ompA, ompT, ompT(2), rfb(O101), sitA, sitD, spaS, z(4184)*
11C	ETEC	*esta1, st, cooA, CS15, CS5, faeGab, fotA*	*afaD, aidaI, cka, csgA, deok, eae(alfpha 2), eae(jota), escN, F17a-A, fimA, fimH, fliC, focG, IpfA, nfaE111, nleG, papA(11),papA(13), papC, papGIV, sfaA*
14A	ETEC	*cfaB, CS3, cseA, cssA, csvA, cswA, esta1, st, fasA, fedA, lngA*	*artJ, astA, astA(2), b1121 csgA, csgE, eae(delta), eprJ, fimA, fimH, fliC, fyuA, gad, hlyE, ibeB, iol, irp(1), irp(2), iss(3), iss, mcbA, mcc, mviM, mviN, nfaA, ompA, wzy(O91)*
30A	ETEC	*esta1, st*	*ccdV, fimC, ibeV, cvaC, eprJ, fimA, fimH, gad, lpfA, lpfA(O113), mviM, mviN, ompA, ompT, ompT(2), z(4184)*
28E	EPEC	*bfpA, bfpA(2), eae(jota), eae(lamda), espA-1, lpfA*	*artJ, CS5, cib, csgE, fimA, fliC, gad, hlyE, iss, ompA*
14C	EPEC	*afrA, bfpA, bfpA(2), bfpA(3), bfpA(4), bfpAalpha, eae(alpha), eae(beta), eae(delta), eae(epsilon), eae(eta), eae (gamma), eae(jota), eae(lamda), eae(mu), eae(nu), eae, espA-1, lpfA(EHEC), lpfA(O113), lpfA(O157), lpfA, map-1, map-2, map-3, paa, tccP, tir-1*	*afaE(3), afaE(5), aggA, arJ, astA, astA(2), b1121, ce1a, cei, cfa, cib, cka, cma, cna, CS3, csa, cseA, csgA, csgE, csnA, cssA, csvA, cswA, eibG, eivG, eprJ, esta1, f165(1)A, F17a-A, F17b-A, fasA, fedA, fimA, fimF41a, fimH, fliC, fliC(H7), focA, focG, fyuA, gad, gafD, hlyE, ibeB,iha, iol, irp(1), irp(2), iss(3), iss, lngA, mcbA, mcc, mce, mrfS, mviM, mviN, nfaA, nfaE, nleB(O103), nleH, ompA, papA(10), papA(11), papA(12), papA(13), papA(15), papA(16), papA(40), papA(48), papA(8), papA(9), papC, papGI, papGII, papGIII, pilL, pilS, sfaH, spaS, stx1A, ureD, wb(O8), wzy(O91)*
13E	Incomplet ExPEC	*chuA, fepC, fyuA, ifoN(1), iroN(2), kpsM(II), papA(14), papC, pic*	*agn43, artJ, b1121, CS5, csgA, csgE, daaE, deok, eivG, eprJ, fimH, fliC, gad, hlyE, hraI, ibeB, lpfA(O113), mviN, ompA, wzy(O15), z(4184)*
28A	Incomplet ExPEC	*iss, iss(3), iucD, iutA, iut(A2), mccB, mviM, mviN, ompA, sitA, sitD*	*afaE(5), agn43, artJ, asta(2), b1121, cba, ccdB, cia, cka, cma, cooA, csgA, csgE, deok, fimA, fimH, fliC, gad, hlyE, ibeB, rfb(O101)*

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DAEC strains, among others. Thus, suggesting that the isolated bacteria can form biofilms (Table 8).

Since the microarray carries all known virulence factors, numerous virulence genes of ExPEC and InPEC strains which would normally be missed in a PCR-based assay were found. Thus, various unusual gene combinations were discovered, such as ExPEC pathogenic profiles with assorted EPEC genes like the type III secretion system 2 proteins ErpJ and the protein eprS (gene e*prJ* and *spaS*, respectively). These unusual combinations provide evidence of genetic exchange between the various pathotypes (Hamelin *et al*., 2006; Bekal *et al*., 2003).

6.9 CHARACTERIZATION OF QUINOLONE RESISTANCE IN *E. coli*.

The genotypes associated with the quinolone resistance (including intermediate resistance) phenotype were characterized for seven pathogenic *E. coli* and 11 commensal *E. coli*. Three of 18 isolates were resistant to second generation quinolones (levofloxacin and pefloxacin), nine isolates were resistant to one of the two quinolones and six showed intermediate resistance to pefloxacin (6/18 isolates). Resistance to quinolones was usually observed in strains with a multi-drug resistance phenotype (16/18 isolates).

The sequencing results for the QRDR of *gyrA* and *parC* are summarized in Table 8. The Ser-83 → Leu and Asp-87 → Asn substitution in *gyrA* and the Ser-80 → Ile substitution in *parC* were found in isolates resistant to both levofloxacin and pefloxacin (n = 2), to levofloxacin alone (n = 2), and to pefloxacin alone (n = 3). *E. coli* isolates with only the Ser-83 → Leu and Asp-87 → Asn substitution in *gyrA* showed resistance and intermediate resistance to pefloxacin. A single mutation in *gyrA* at Ser-83 → Leu (n =2) was found in one strain resistant to both (levofloxacin and pefloxacin), and one with intermediate resistance to pefloxacin. An isolates with a single mutation (Ser-80 → Ile) in *parC* exhibited an intermediate resistance towards pefloxacine. Three strains had one or more *qnr* genes. Five *E. coli* isolates possessed *qnrA*, seven isolates had *qnrS* and two isolates had *qnrB*. Overall, eight isolates had chromosomal mutations in *gyrA*, *parC* or both as well as horizontally acquired *qnr* genes. The *qnr* genes were found in two strains

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Table 10. Summary of the pathotype, antimicrobial resistance patterns, QRDR mutation and presence of *qnr* resistance genes for the 18 *E. coli* isolates selected for their resistance to fluoroquinolones of second (pefloxacin) and third (levofloxacin) generation.

Strain ID	MDR phenotype[a]	Diffusion disc[b]		QRDR mutation		PMQR genes	Other resistance genes	*E. coli* type[c]
		LEV	PEF	ΔGyrA	ΔParC			
11C	AmCbClSxtLevPef	R	R	S83→L, D87→N	S80→I	*qnrA*	*aph3(strA)*, bla_{TEM}, class 1 integron	InPEC
14A	AmCfCtxCroLevPefStx	R	R	S83→L, D87→N	S80→I	*aac-(6')-lb-cr*, *qnrS*	*aph3(strA)*, *aph6(strB)*, bla_{TEM}, *tet(B)*, *tet(M)*	InPEC
14C	AmCfCtxCroLevPefStx	R	R	S83→L	None	*aac-(6')-lb-cr*, *qnrS*	*aph3(strA)*, *aph6(strB)*, bla_{TEM}, *dhfrVII*, *tet(B)*	InPEC
13A	AmGLevSxt	R	S	S83→L, D87→N	S80→I	None	bla_{TEM}, *aac(3)-IIa(aacC2)*, *aph3(strA)*, *mphA*, *sulII*, class 1 and 2 integron	InPEC
13D	AmCbGLev	R	S	S83→L, D87→N	S80→I	None	*aph3(strA)*, bla_{TEM}, *aac(3)-IIa(aacC2)*, *mphA*, *sulI*, *sulII*, *tet(B)*, class 1 integron	InPEC
13E	AmCbLev	R	S	None	None	None	*aph3(strA)*, bla_{TEM}, *dhfrXII*, *sulII*, *tet(A)*	Incomplete ExPEC
28A	AmCbClCfPefSxt	S	R	S83→L, D87→N	S80→I	*qnrA*	*aph3(strA)*, bla_{TEM}, *dhfrXII*, *mphA*, *sulII*, *tet(B)*, class 1 integron	InPEC Incomplete ExPEC
11D	AmCbClPefSxt	S	R	S83→L, D87→N	S80→I	*qnrA*	*aph3(strA)*, bla_{PSE}, bla_{TEM}, *catI*, *dhfrXII*, *mphA*, *tet(A)*, *tet(B)*, *sulI*, *sulII*, class 1 and 2 integron	Commensal
28C	AmCbClPefSxt	S	R	S83→L, D87→N	S80→I	None	*cmlAI*, *dhfrXII*, class 1 integron	Commensal
23E	AmCbClPefSxt	S	R	S83→L, D87→N	S80→I	None	*dhfrXII*, *cmlAI*	InPEC
4A	CfNfPef	S	R	S83→L, D87→N	None	*qnrS*	*aph3(strA)*	Commensal
20E	Pef	S	R	None	None	*qnrS*	ND	Commensal
17A	AmClStxPef	S	I	None	None	*qnrA*	*aph3(strA)*, bla_{PSE}, *dhfrVII*, *sulII*, *tet(A)*, class 2 integron	Commensal
18A	AmClStxPef	S	I	S83→L, D87→N	None	None	Class 1 and 2 integron.	Commensal
23A	AmCbNfSxtPef	S	I	None	S80→I	*qnrA*, *qnrB*	bla_{PSE}, *dhfrXII*, class 1 integron	InPEC
30A	AmPef	S	I	None	None	*qnrB*, *qnrS*	*aph3(strA)*, bla_{TEM}	InPEC
23E	AmCfCbSxtPef	S	I	None	None	*qnrS*	*aadA (1)*, bla_{TEM}, *dhfrI*, *sulI*, class 1and 2 integron, transposon *Tn21*	InPEC
21A	AmCbPef	S	I	S83→L	None	*qnrB*, *qnrS*	Transposon *Tn21*	Commensal

[a]Multi-drug resistance (MDR) phenotype: Am, ampicillin; Cb, carbenicillin; Cl, chloramphenicol; Pef, pefloxacin; Lev, levofloxacin; Sxt, trimethoprim-sulfamethoxazole; G, gentamicin; Net, netilmicin; Ak, amikacin; Cf, Cephalotine; Ctx, cephatoxim; Cro, ceftriaxone; Nf, nitrofuratoin. ND, not determined. [b]R, resistant; I, intermediate resistance; S, susceptible.

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with a triple mutation profile (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*) that were resistant to both levofloxacin and pefloxacin.Quinolone resistance isolates (*n* = 3) with one mutation Ser-83 → Leu in *gyrA,* or, Ser-80 → Ile in *parC* possessed also *qnr* genes. The three strains with no chromosomal mutation possessed one or two *qnr* genes. One of the isolates did not have either a chromosomal mutations or *qnr* genes. In addition, the ETEC pathotype isolate 18A (Table 10) exhibited a multi-resistance phenotype and had three substitutions (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*), *qnrS* and the newly described *aac (6')-Ib-cr* gene. Additionally, five isolates that had the triple mutation profile (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*) in the QRDR carried also one *qnr* gene (*qnrA* or *qnrS*) and at least one beta-lactamase gene.

6.10 BIOFILM ASSAY

6.10.1 BIOFILM PRODUCTION IN STREAM WATER.

Biofilms in surface waters primarily consist of allochtonous microorganisms. Under conditions of pollution faecally derived bacteria may interact with these biofilms. Most of the studies concerning to water quality and the presence of *E. coli* or any other pathogenic bacteria in the aquatic environment have focused on planktonic bacteria in the water and do not reflect the situation in biofilms (Schwartz *et al*., 2003), which is the preferred pattern of life of many bacteria.

As biofilm formation is associated with stress tolerance, the ability of *E. coli* to form biofilms in the environment may represent a survival strategy. If this was the case, the different pollution showed at sampling locations of the river might apply selective pressures that favor strains capable of forming mature biofilms under these conditions (Moreira *et al*., 2012). In the present study the occurrence of *E. coli* as faecal indicators was investigated in biofilms directly at the stream water of the river. We also tested the biofilm-forming capacity of *E. coli* isolates in polystyrene plates (Puttamreddy *et al.,* 2010; Charbonneau *et al*., 2006) as well as borosilicate tubes (Christensen *et al*., 1982).

In most sampling locations (57%) we were able to observe the presence of biofilm formation *E. coli* in water by fluorescence in situ hibridation (FISH). The presence of *E. coli* biofilms were no detected in sample locations 20-OS and 28-OS considered as less polluted locations, nevertheless, we found the presence of *E. coli* biofilms in the locations 3-WWTP-E, which is a wastewater treatment plant effluent. We also observed the presence of biofilm formation by others bacteria that did not belong to *E. coli* in several locations. However, the vast majority of the observed biofilms were formed by *E. coli* (Figures 26).

Overall, the results of the present study show that faecal indicator bacterium can survive in the presence of high cell densities of the autochthonous microflora in water biofilms, suggesting that these biofilms may act as a reservoir for bacterial pathogens in polluted rivers.

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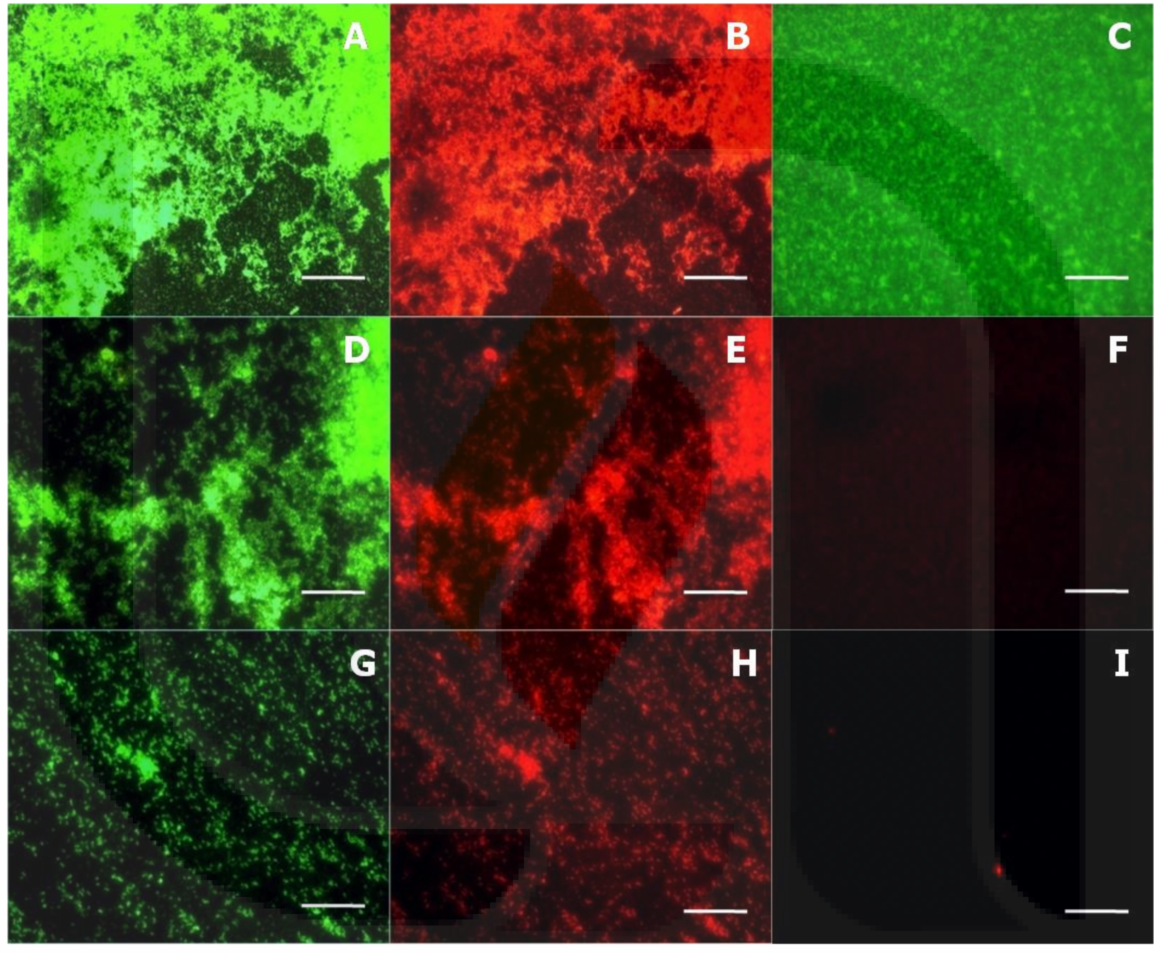

Figure 23. Images of bacterial cell observed by using fluorescence microscope in experiments performed with *E. coli* ATCC 25922 as positive control (A, B, D, E, G, and H) and *Actinobacillus pleuropneumoniae* environmental isolated as negative control (C, F, and I). *E. coli* detected was hybridized with ''Colinsitu'' probe labeled with the CY3 dye (B, E, H, F, and I). Samples were treated with Film Tracer FM 1-43 green biofilm cell station (Molecular Probes, USA) (A, D, G, and C). The fluorescent signals were acquired separately using three filter sets with Cy3, and fluorescein. Scale bar: 20 µm.

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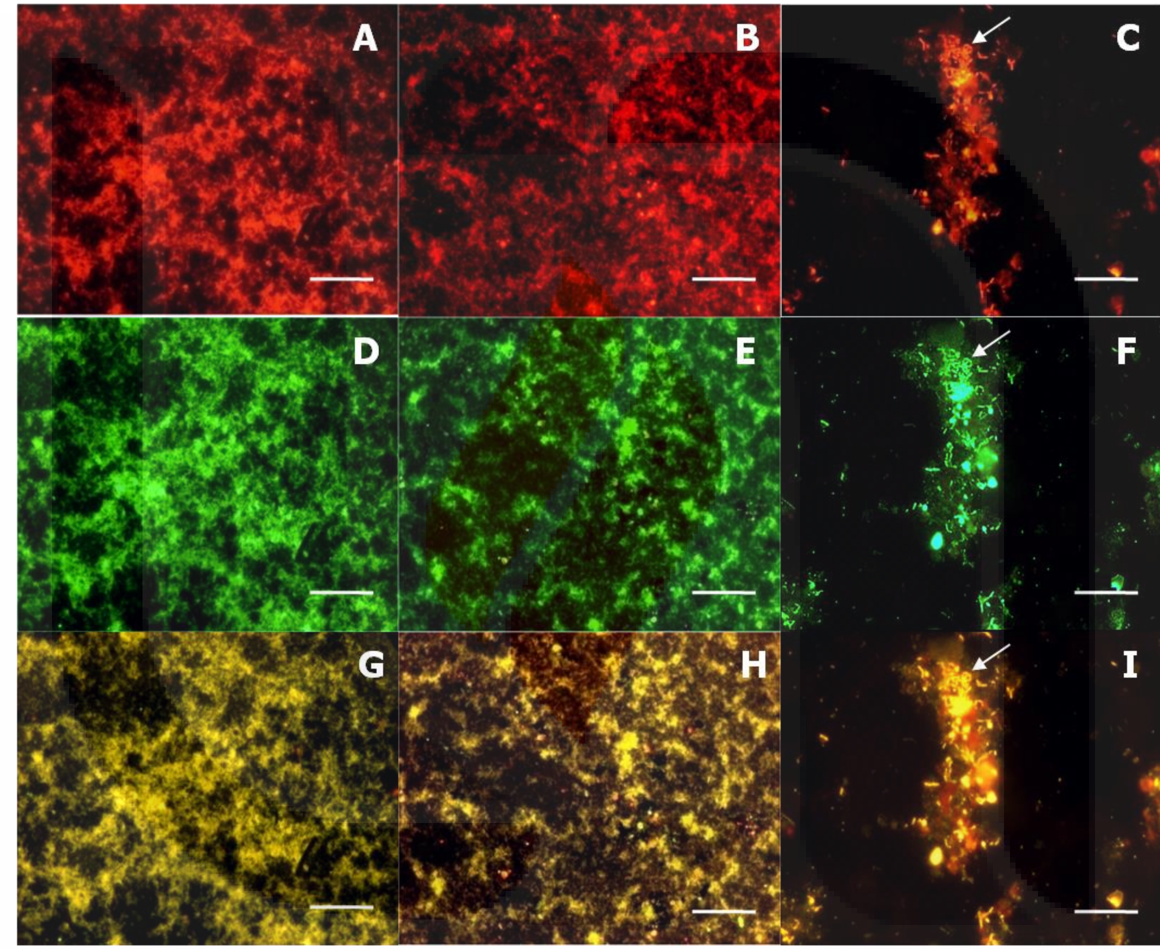

Figure 24. Images of biofilms producing *E. coli* in San Pedro River's water observed by fluorescence microscope. *E. coli* detected was hybridized with ''Colinsitu'' probe labeled with the CY3 dye (A, B and C). Samples were treated with Film Tracer FM 1-43 green biofilm cell station (Molecular Probes, USA) (D, E and F) in order to see the total amount of bacteria cells in the biofilm. Merge of two labels of the cells into the biofilm (G, H and I). Overall, biofilm samples were formed by *E. coli*. Organic debris masks the probe signal in river water (C, F and I). Arrows indicate the presence of *E. coli*. The fluorescent signals were acquired separately using three filter sets with Cy3, and fluorescein. Scale bar: 20 µm.

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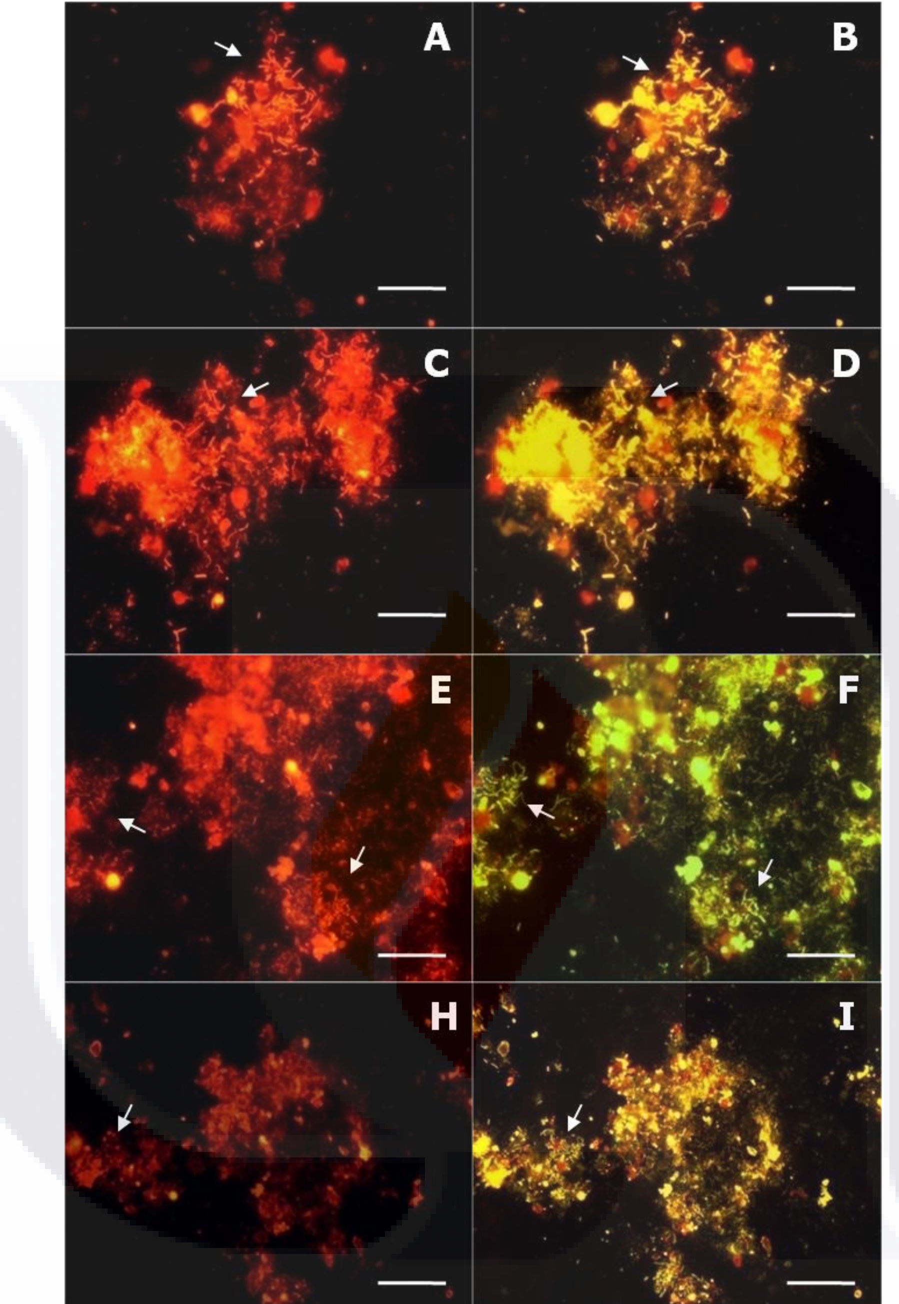

Figure 25. Images of biofilms in San Pedro River's water observed by fluorescence microscope. *E. coli* detected was hybridized with ''Colinsitu'' probe labeled with the CY3 dye (A, C, E, and H). Samples also were treated with Film Tracer FM 1-43 green biofilm cell station (Molecular Probes, USA) in order to see the total amount of bacteria cells in the biofilm. Merge of two labels of the cells into the biofilm (B, D, F and I). Overall, biofilm samples were formed by *E. coli*. Organic debris masks the probe signal in river water in almost all the samples. Arrows indicate the presence of *E. coli*. The fluorescent signals were acquired separately using three filter sets with Cy3, and fluorescein. Scale bar: 20 µm.

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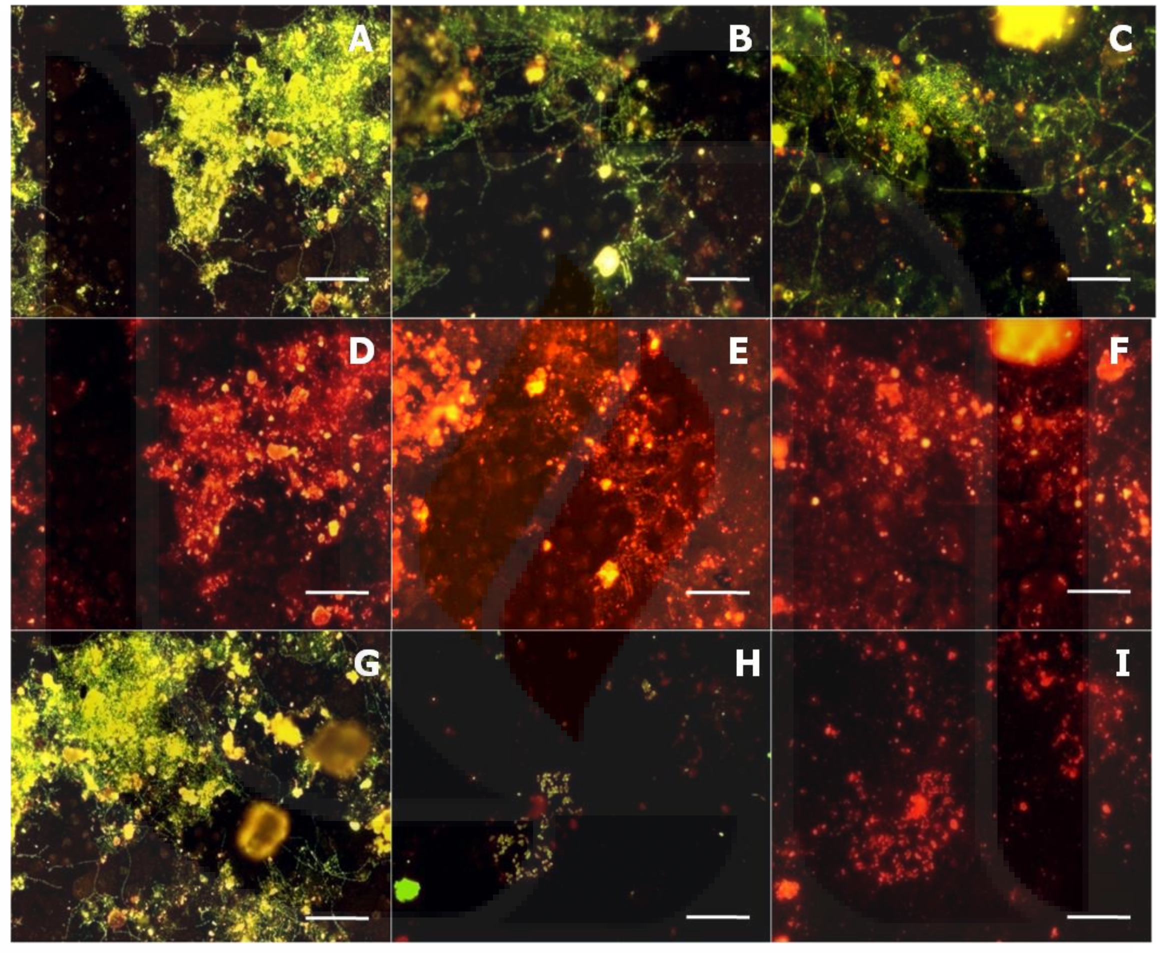

Figure 26. Images of biofilms producing bacteria in San Pedro River's water observed by fluorescence microscope. *E. coli* detected was hybridized with ''Colinsitu'' probe labeled with the CY3 dye. Samples also were treated with Film Tracer FM 1-43 green biofilm cell station (Molecular Probes, USA) in order to see the total amount of bacteria cells in the biofilm. Organic debris masks the probe signal in river water in almost all the samples. The samples were positive for biofilm formation (A, B, C, G and H). Must of biofilm forming bacteria are not *E. coli* (D, E, and F). The fluorescent signals were acquired separately using three filter sets with Cy3, and fluorescein. Scale bar: 20 µm.

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6.10.2 ADHERENCE TO BOROSILICATE TUBES.

Biofilm production was investigated by the tube adherence test proposed by Christensen *et al*., (1982). A positive result was defined as the presence of a layer of stained material adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid air interface was not considered to be positive. Overall, 80 percent of the strains tested were considered biofilm producers. Biofilm forming bacteria were further categorized as high producers (17.5%), moderate producers (20%) and weak producers (42.5%). Twenty percent of the strains were categorized as non biofilm forming. Tube test showed correlation with detection of biofilm forming strains by microtiter assay as well as Congo Red assay. The tube adherence test can be indicated for the routine detection of biofilm production because of its easy application and low cost.

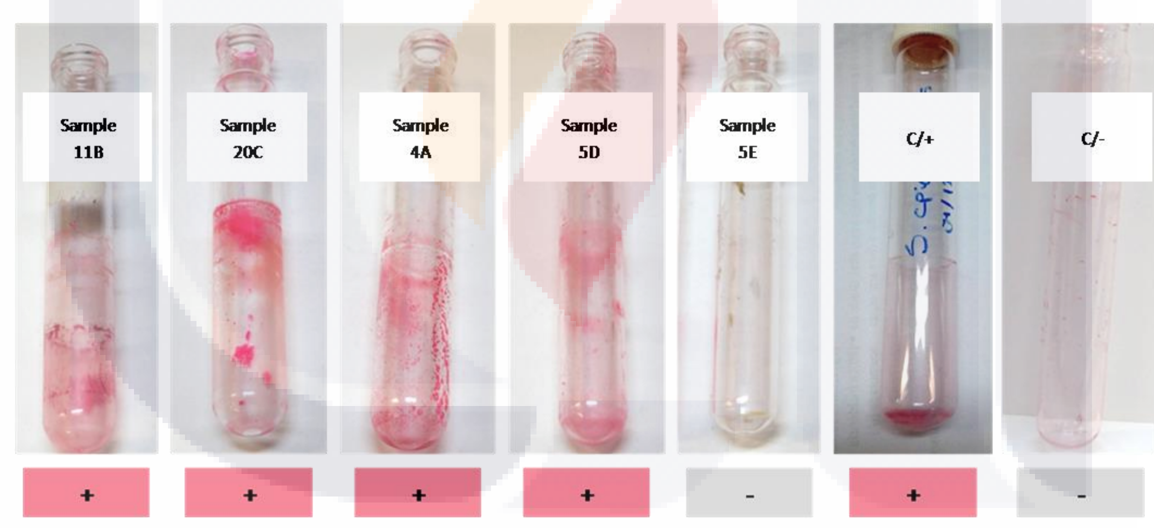

Figure 27. Tube adherence method. The biofilm formation was grown for 48h at 37°C in BHI broth. The cells adhered to glass tubes were stained with a 0.1% Safranin solution, washed with distilled water 3 times and dried. A positive result was defined as the presence of a layer of the stained material which adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid-air interface was considered as negative.

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6.10.3 BIOFILM FORMATION BY MICROTITER ASSAY

The mean crystal violet assay results of strains of different pathotypes grown in M9 medium plus 0.4% glucose at 30°C were compared to determine whether there was any significant pathotype difference in biofilm-forming capacity (Figure 29). A one way ANOVA and a *post hoc* Dunnett´s multiple comparison test revealed that the strains belonged to EAEC pathotype were significantly more competent at forming biofilms than isolates from any other pathotype groupings (P <0.01; P <0.001). This result was expected since EAEC are enteroaggregative pathogenic strains that show typicaltypical aggregative adhesion pattern implicated in the formation of biofilm. Reisner *et al*. (2006) demonstrate how human isolates, including a variety of pathogenic strains as well as and periphytic *E. coli*, have incredibly diverse biofilm-forming ability. This is consistent with our results, which show a large range in the ability of *E. coli* isolates from river sources to form biofilms *in situ* as well as *in vitro* in both, borosilicate tubes and polystyrene.

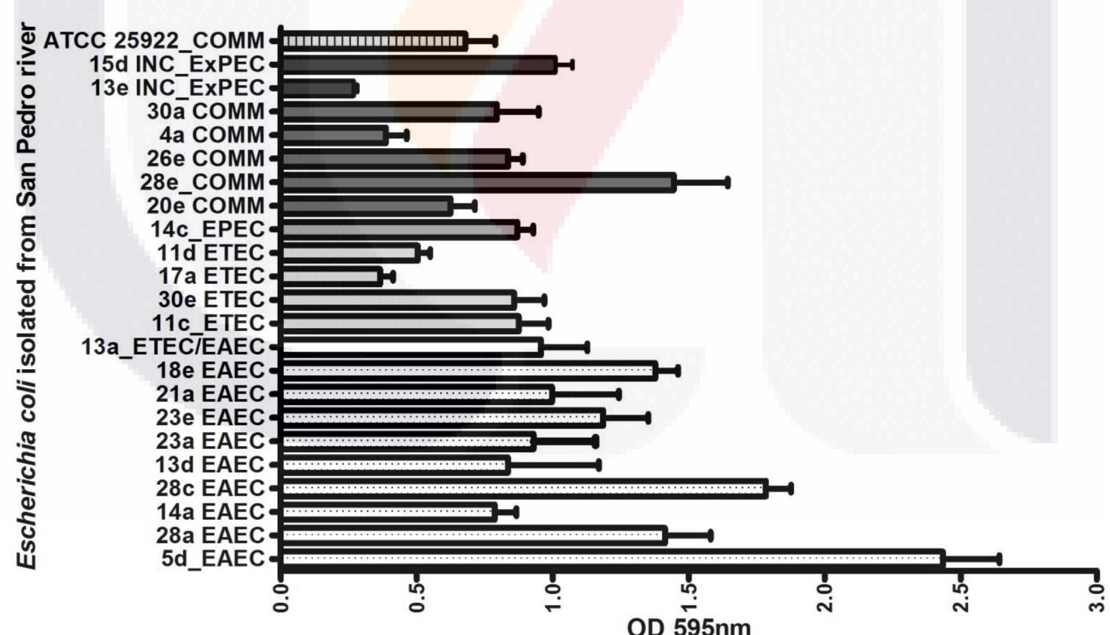

Figure 28. Biofilm formation by different *E. coli* pathotypes isolated from San Pedro River. Twenty-four hours biofilms of *E. coli* were performed in M9 + glucose 0.4%. Biofilms were measured as OD_{595nm} (OD, optical density) of solubilized crystal violet as describe by Puttamreddi & Minion, 2010. Data represent the mean ± SD of three replicates. These results are representative, confirmed by three independent experiments.

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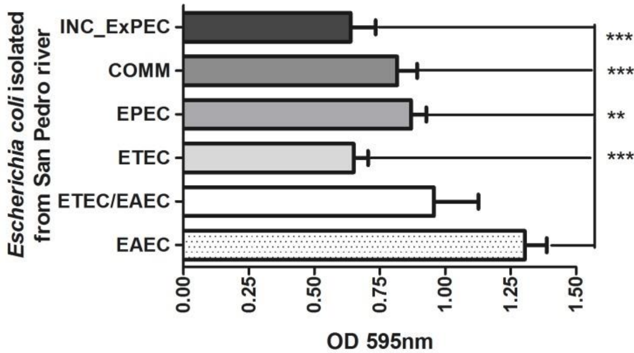

Figure 29. Biofilm formation by *E. coli* isolate from San Pedro River. The different pathotypes show difference among them. Significative differences were found among biofilm formation by EAEC strains and other pathotypes (* $0.05 \geq p \geq 0.01$; **, $0.01 \geq p \geq 0.001$; *** $0.001 \geq p$) .Twenty-four hour biofilms of *E. coli* were performed in M9 + glucose 0.4%. Biofilms were measured as OD_{595nm} of solubilized crystal violet as describe by Puttamreddi & Minion, 2010. Data represent the mean ± SD of three replicates.

6.10.4 SCANNING ELECTRON MICROSCOPY.

In order to learn more information on the potential of the strains isolated from San Pedro River to be pathogenic and phenotypically multi-resistant, we assayed by scanning electron microscopy the characteristics in five different isolates.

In the study, isolates as well as the strain *E. coli* ATCC 25922 seems to be biofilm formation strains. *E. coli* ATCC 25922 displayed the best biofilm formation among strains tested. We notice that bacterial aggregation in this isolates are mediated by putative pilus and expression of curli fibers. Curli is a proteinaceous component of the extracellular matrix produced by many *Enterobacteriaceae* species which is known as thin aggregative fimbriae (Pereira *et al.* 2010; Barnhart and Chapman, 2006), and has been identified as major determinant of cell-cell interactions and adherence to abiotic surfaces and to sustain biofilm formation in *E. coli* strains (Pereira *et al.,* 2010; Zogaj *et al.,* 2003) in according with our results.

We also noticed the presence of pilus, which suggest that strains isolates from natural stream water may be able to form biofilms, since has been shown that appendages such as pili are initial connectors in early stages of the biofilm formation allowing planktonic bacteria capable of engaging in biofilm formation by allowing cell-to-cell contact and interaction with surfaces (May and Okabe, 2008; Ghigo 2001).

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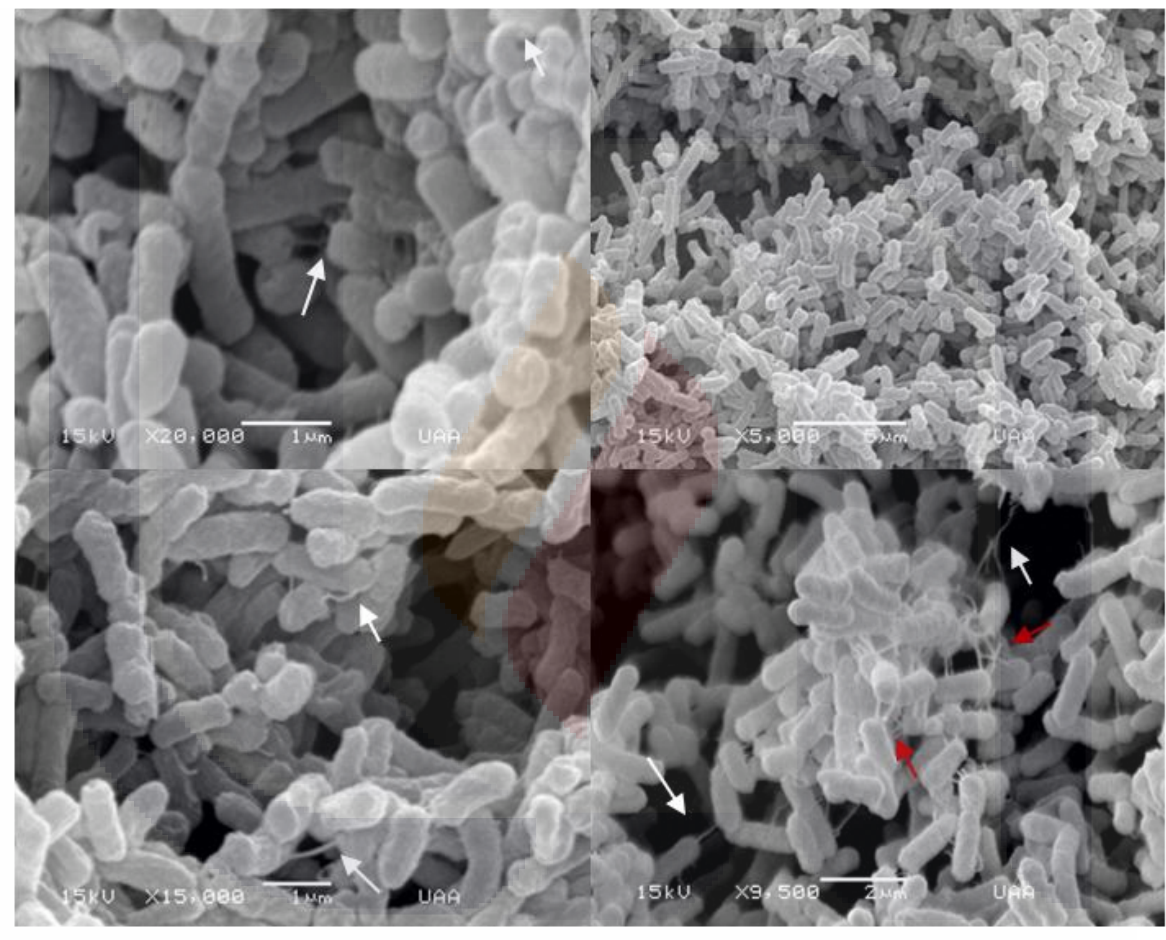

Figure 30. SEM micrograph showing *E. coli* (ID 14A) isolated from stream water. The strains 14A identified as ETEC by PCR and multidrug resistant strain including resistance to fluoroquinolone. The white arrows in the figure showed the use of structures as flexible pili mediating the formation of aggregates. Curli fibers were shown to mediate cell-cell adherence and interaction to abiotic surface fixating curli fibers (red arrows).

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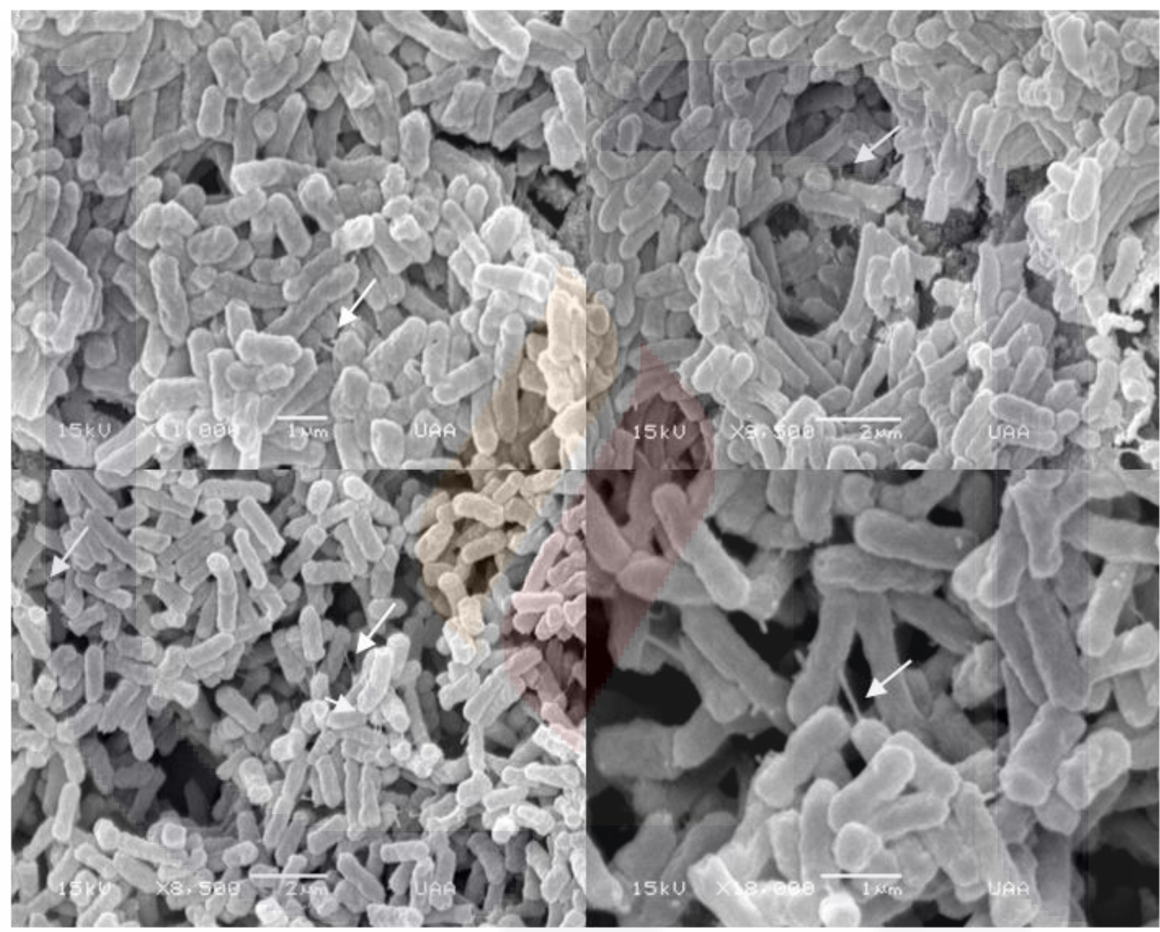

Figure 31. SEM micrograph showing *E. coli* isolated (ID 14C) from stream water. The white arrows show putative pilus used as structure to form biofilms. The individual bacteria cells of the strain have a rough surface.

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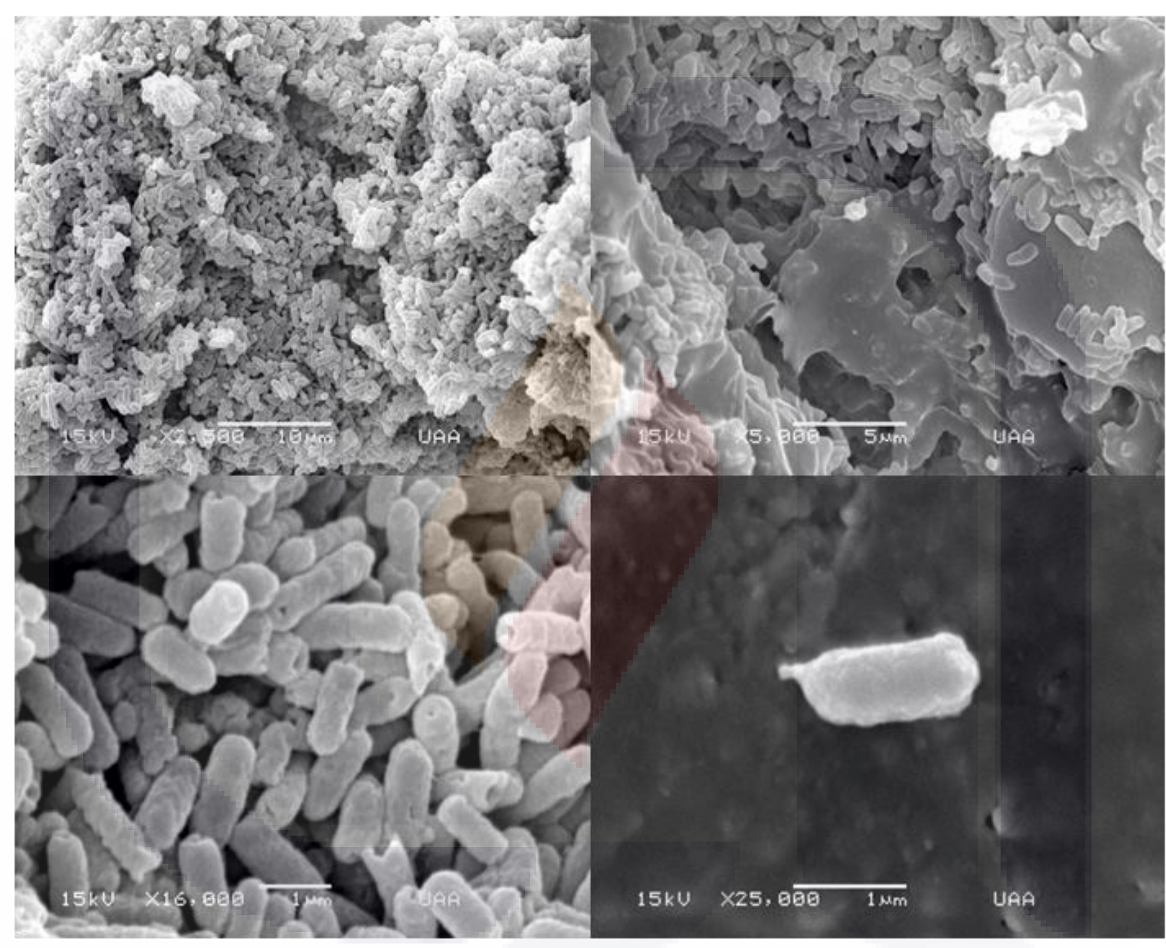

Figure 32. SEM micrograph showing *E. coli* (ID 4A) isolated from natural water stream. It is observed that the strains are embedded in a surrounding matrix.

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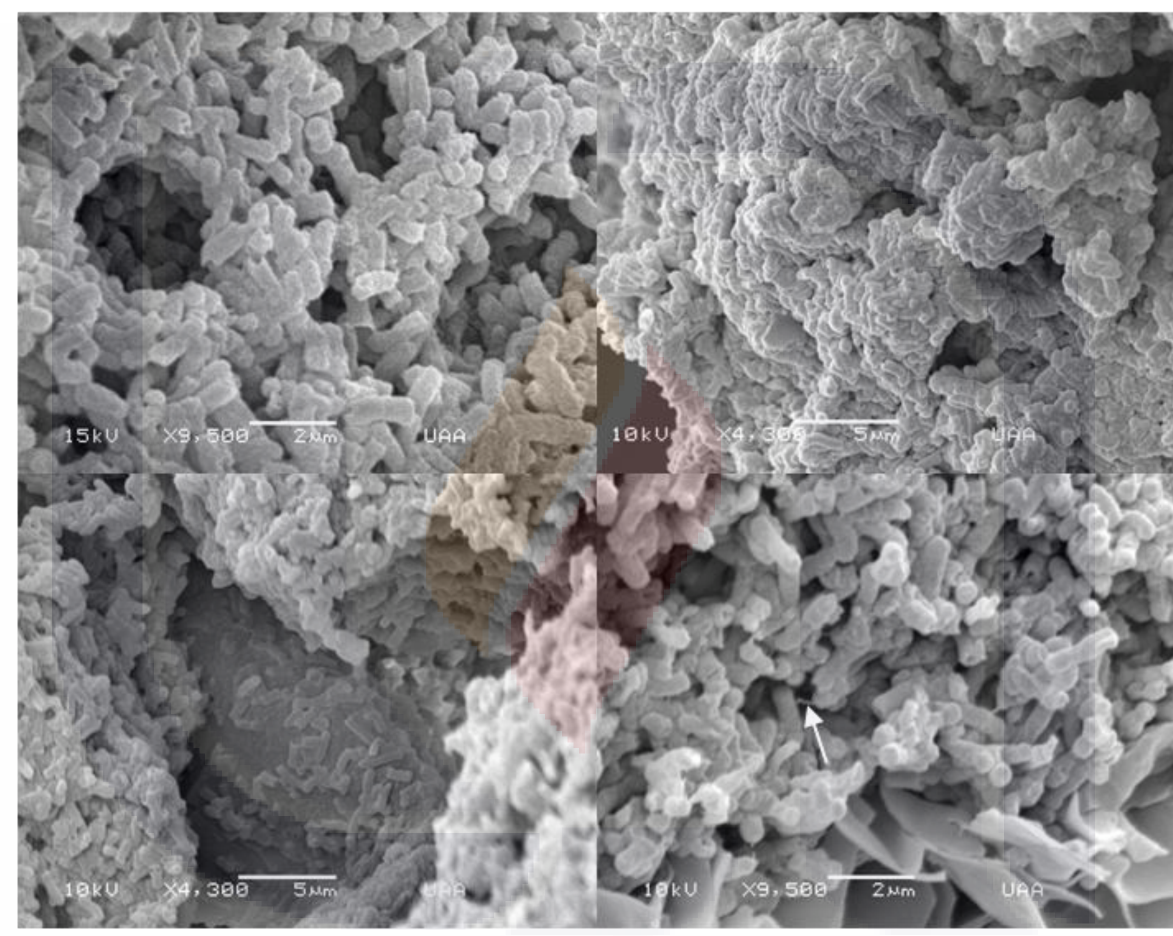

Figure 33. SEM micrograph showing *E. coli* (ID 5e) isolated from natural water stream. It is observed that the strains are embedded in a surrounding matrix. The white arrow indicate the pili structure.

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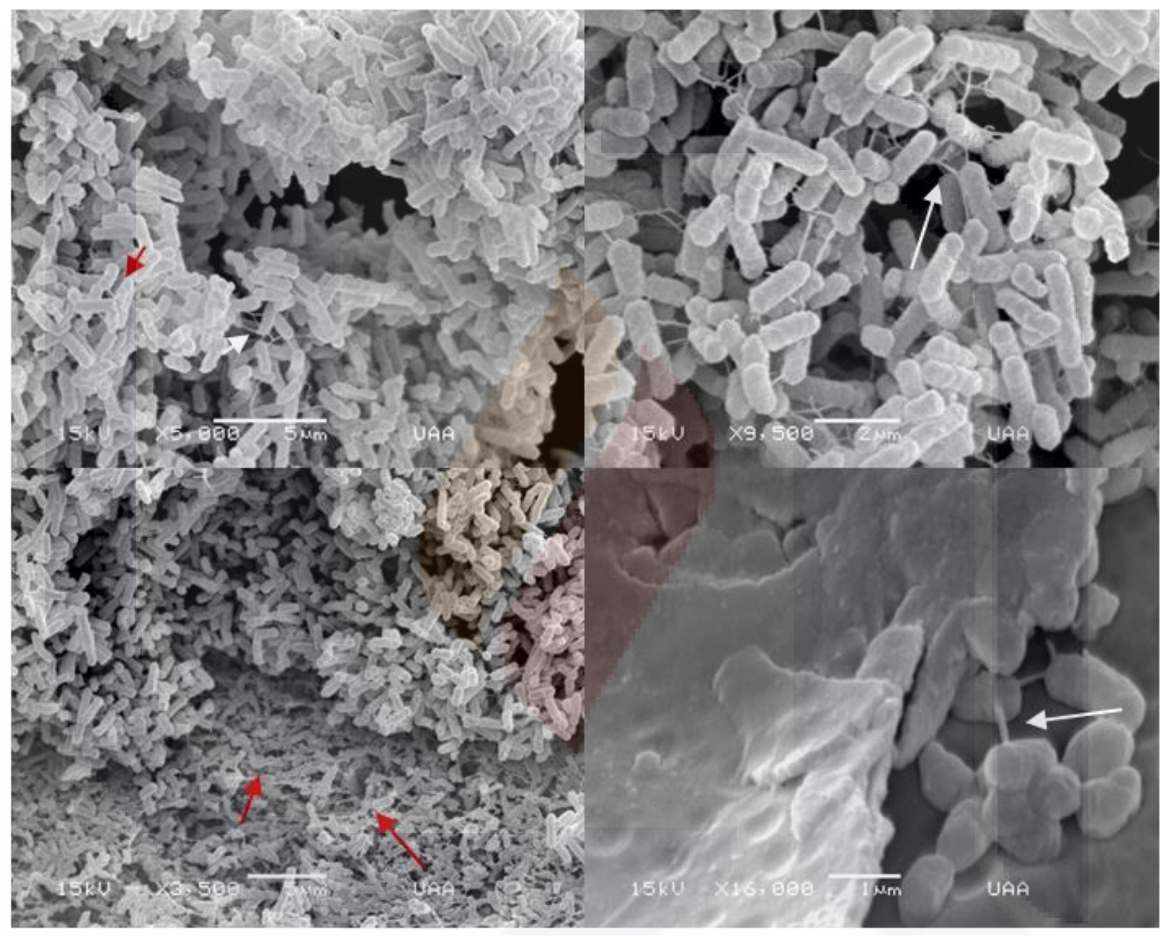

Figure 34. SEM micrograph showing *E. coli* (ATCC 25922). Most of the strains have a structure of smooth rods in comparison with environmental isolates with rough rods observed. This strain was strongest biofilm former. It is observed that the strains are embedded in a surrounding matrix. The white arrow Indicate the structures as pili. Curli fibers were shown to mediate cell-cell adherence and to interact with abiotic surface playing curli fibers (red arrows).

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6.10.5 CONGO RED ASSAY.

E. coli biofilm formation is controlled by a number of factors, including several surface proteins such as conjugative pili, type-1 fimbriae, and curli (Van Houdt and Michiels, 2005). Amyloid curli fibers are a mediating factor in the attachment stage of biofilm formation (Barnhart and Chapman, 2006) and have been shown to be a common determinant of adhesion in environmental isolates in a small sample population (Castonguay *et al*., 2006). Additionally, Carter *et al*. (2011) have demonstrated how curli-positive *E. coli* O157 variants grow better under nutrient-limited conditions than their curli-negative counterparts. If environmentally persistent *E. coli* isolates can be shown to have uniformly high curli production, it might indicate that the curli-mediated attachment phase largely determines whether a strain will be successful in colonizing the periphyton and persisting in the environment.

Since we saw putative pili and curli in the SEM assay, we investigated the production of cellulose and curli fimbriae by Congo red dye, which is has been correlated with biofilm formation and expression of multicellular behavior, dry and rough (rdar) morphotype of the cell (Da Re and Ghigo 2006; Romling *et al*., 2003; Solano *et al*., 2002; Zogaj *et al*., 2001).

Overall, most of the strains tested (52.4%) were Congo Red positive with rdar morphotype. Congo Red assay demonstrated that the production of curli and cellulose varies among isolates. Even when several congo red positive strains were biofilm positive (42.9%, Table 11) the production was not correlate with biofilm (p = 0.4502) indicating that curli and cellulose are involved in biofilm formation of *E. coli* in river water but others determinants such as adhesion, quorum sensing or metabolism are likely involved in biofilm formation of *E. coli* in river water besides of curli production.

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Figure 35. Different phenotypes observed on cellulose production and biofilm formation in *E. coli* strains isolated from San Pedro River (CR- binding phenotypes). Two microliters of a culture was spotted onto CR-LB plates and incubated for 48 h at 30°C (CR).

Table 11. *Escherichia coli curli* expression and biofilm formation in M9 medium plus glucose.

	Percentage of isolates (%)	
	[a]Biofilm positive	Biofilm negative
[b]Curli positive	42.9 (9)	9.5 (2)
Curli negative	33.3 (7)	14.3 (3)

[a]Isolates were grown in M9 medium supplemented with 0.4% glucose ad 30°C for 48 h and subjected to a crystal violet assay. [b]Isolates were grown on LB agar (without NaCl) supplemented with 0.02% Congo Red and 0.002% of Brilliant Blue for 48h. Colonies that appeared red were designated curli positive.

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7. DISCUSSION

In many parts of the world, health problems and diseases have often been caused by discharging untreated or inadequately treated wastewater. Agricultural and urban runoffs may be major sources of pollution of water bodies and major sources of bacteria affecting the quality of stream water as well as drinking water since bacterial pathogens can flow down through the soil to the subsoil and then to groundwater.

Wastewater used for irrigation could carry microbial pathogens, that constitutes a risk for the transfer of infections to humans or animals if they are exposed to pathogens in the wastewater that also could carry chemical component discharged from sewage and industries that contribute to oxygen demand and lead to a destabilized aquatic ecosystem (Salem *et al*., 2011; Morrison *et al*., 2001).

Surface waters, including dams, rivers and streams, constitute an important source of drinking water, domestic, agricultural, recreational and other purposes. However, they are vulnerable to pollution and are frequently contaminated with faecal matter (Kistemann *et al*., 2002; Effler *et al*., 2001). They are considered an ideal reservoir for antibiotic resistance dissemination, since antimicrobials and antimicrobial resistant bacteria are often directly released in the aquatic environment (Lupo *et al*., 2012).

In developing countries, the contribution of sewage and wastewater as point sources, as well as animal farming without adequate outlet control, represent extra loads of contamination for aquatic systems. Most of the water from these activities is subsequently used for irrigation, without any treatment which could represent potential risks of contamination (Mazari-Hiriart *et al*., 2008). In the State of Aguascalientes in Mexico, this is of great importance because river water is used for irrigation and in rural communities as a source of drinking water for livestock.

In this study, we evaluated stream water from San Pedro’s River, the major riverbed and main pluvial collector of Aguascalientes State, Mexico; relevant to automotive sector, agricultural area, livestock industry, irrigation and recreational use.

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Currently, the River is being contaminated by the influx of wastewater from several industries and sectors, highlighting agricultural activities and livestock industry. We hypothesize that this environment could harbor high levels of microorganisms and this selective pressure encourages the presence of pathogenic bacteria and the emerging of antimicrobial agents' resistant bacteria that could create potential risk to public health and environment.

Our results showed that the water quality of the San Pedro River found seriously contaminated by organic matter, nutrients, organic pollutants and fecal coliforms. The contamination at specific sites probably is associated to low flow-rate, and consequently low dilution capacity of this river (Dragun *et al*., 2011, Guzmán-Colis *et al*., 2011).

The physicochemical values and microbiological counts found in the San Pedro River are consistent with an important pollution originating from municipal and livestock wastewater (Table 5). Furthermore, these conditions provide a favorable environment for microbial growth (pH 7.4-7.7, Amxaka *et al*., 2004), specially to coliforms growth (temperature 22.6^{o}C -25.8^{o}C, Skraber *et al*., 2004). In general, DO concentrations (0.38-2.1) were at least four times lower than the desirable value (5.0 mg/L), far below the levels required to sustain aquatic life. Conductivity provides information on the concentration of dissolved salts in water. Overall, the conductivity values were below the desired level for good quality water 2000µS/cm (Guzmán-Colis *et al*., 2011).

Considering the desirable levels of quality of agricultural water, all sites exceeded the criteria for COD, BOD, P, N, anilines and MBAS, excepting the sampling location near to effluent of the wastewater treatment plant indicating its efficiently. Nevertheless, when the stream water meet with an untreated discharge, levels of COD and BOD return to high levels. Given that the water from the San Pedro River is used for irrigation, the water may constitute a source of bacterial contamination that could infect humans or animal through direct contact, aerosol or consumption of vegetables.

Major factors affecting the microbiological quality of surface water are discharges from sewage works and runoff from informal settlements. High total and faecal coliform counts in water are usually manifested in the form of diarrhea, fever and other secondary complications (Fatoki *et al*., 2001). Our results showed that the

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concentration of coliforms in San Pedro River and its major creeks was exceeding by more than one order of magnitude the WHO tolerance limits in fifty percent of the sampling locations (Figure 12). These concentrations and wide variations are similar to findings elsewhere (Chigor *et al*., 2013; Chigor *et al*. 2012; Dragun *et al*., 2011; Lata *et al*., 2009; Mazari-Hiriart *et al*., 2008; Schets *et al*., 2008).

The elevated coliform counts along the river coincide mostly with point sources of fecal contamination, agricultural industry, municipal wastewater, chemical industry, and dairy products, which discharge to the river and streams. Moreover, most of these sites are situated near known human point sources, recreational locations, or agricultural operations.

Comparing the values of total and fecal coliforms (FC) values (FC=3Log_{10}MPN /100mL) with those obtained previously by Carrazco-Rosales (2008, unpublished data), and Guzmán-Colis (2011, FC =9 Log_{10}MPN/100 mL and FC= 6.5 Log_{10}MPN/100 mL, respectively) obtained at the same River, the values in this study showed significant differences with lowers values of microbial densities. These are probably explained due to the levels of COD, BOD and nitrogen which also are lower in the present study and good practices have been implemented. Nevertheless, the levels biological and chemical water quality parameters can indicate serious anthropogenic impact characterized by high bacteria counts.

One of the objectives of our study was to obtain field microbiological data describing the fate of fecal bacteria at the scale of the whole watershed in order to help management of microbiological water quality of the River and give and estimated of the sanitary risk associated to these bacteria. To be able to do these it was necessary to estimate the total level of contamination of *E. coli* in the aquatic system. Established methods to detect and measure *E. coli* or fecal bacteria have different limitations such long response delay (24 to 48 h) and they do not take into account the viable but not culturable bacteria (VBNC) which nevertheless could represent a health threat since pathogenic VBNC bacteria could maintain their virulence even when they loose their ability to grow on culture media (Garcia-Armisen and Servais 2004; Kell *et al*., 1998; Pommepuy *et al*., 1996; Grimes and Colwell, 1986; Colwell *et al*., 1985). For this reason we tested and alternative method to detect *E. coli* by a fluorescent *in situ* hybridization

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with a specific probe coupled with a viability test. The conversion to the VBNC state is supposed to be a response to adverse environmental conditions such as lack of nutrients, unfavorable water temperature, the presence of disinfectants or toxic metal ions such as copper (Dwidjosiswojo *et al*., 2011).

In this work, a combination of the DVC (direct viable count) and the FISH procedures was successfully tested to enumerate *E. coli* in stream water from river (Figure 15). This method was chosen because allows to identify the substrate responsive cells, called viable cells (potential capacity to grow and not a real activity in the environmental conditions), and hybridization with the “colinsitu probe” allows the identification of low number of *E. coli* among a large number of non-target bacteria. In this study, approximately the same numbers of *E. coli* by the MPN method and the FISH differing from Garcia-Armisen and Servais (2004), whom saw an enumeration of 2 to 100 times more with the FISH procedure than with MPN method which could be due to the time elapsed before the experiment was performed since they tested to 200 h after taking the sample which increased the count of VBNC cells (Garcia-Armisen and Servais 2004; Petit *et al*., 2000) in contrast to this study (720 h approximately). All the same, we were able to detect VBNC *E. coli* in most of the sample locations (Figure 14) suggesting the presence of others bacteria present in the River in a VBNC state even in contaminated water and the large underestimation of the abundance of *E. coli* in stream water. Various environmental factors were related to the VBNC state in San Pedro River such as pH and conductivity. From a sanitary point of view, this means that the number of viable fecal bacteria is systematically underestimated by traditional culture-based methods and particularly in strongly contaminated water. This should raise the question on the real significance of the routine microbiological water quality analysis by classical methods and the deduced estimation of the sanitary risk.

Among phylogenetic groups, isolates of phylogroup A were the most abundant. This *E. coli* phylotype is regarded as nonpathogenic or commensal (Clermont *et al*., 2012; Dröge *et al*., 1999). However, here, almost of the isolates carried virulence genes associated to InPEC and ExPEC strains in agreement to Anastasi *et al*., 2012. Previous works suggest that strains belonging to the B1 phylogenetic group are adapted to surviving and multiplying in the environment outside host enteric conditions (Walk *et*

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al., 2007; Anderson *et al*., 2005; McLellan 2004; Gordon *et al*., 2002). Skurnik *et al*., (2008) found that the strains belonging to phylogenetic group A and to a lesser extent group B1 may have the genetic background to emerge as intestinal pathogens. Hamelin *et al*., (2007) observed that most commensal *E. coli* isolates are found in phylogenetic groups A and B1, nevertheless, obligatory pathogens responsible for acute and severe diarrhea also belonged to these phylogroups Additionally, Mokracka *et al*., 2011 showed that integron positive strains mainly belonged to phylogenetic groups D and B1 and have the most virulence factors score and were related to quinolone resistance in *E. coli* strains isolates from water sources. These results suggest that some environmental *E. coli* strains belonging to phylogenetic groups A and B1 should not be regarded merely as commensals, because they may possess virulence determinants associated with intestinal and/or extraintestinal strains.

Contrary to this finding, Mokracka *et al*. (2011) found the most abundant phylogroup among integron bearing *E. coli* strains was D, followed by A, B1, and B2. Nevertheless, we identified underassigned phylogroups for 10 percent of the isolates since we were used the newer reviewed method of Clermont *et al*. (2012). Thus, further assays such as sequence typing method are needed it to characterize these strains.

Based in Clermont *et al*. (2012) phylogroups C, F and E, are new phylogroups designed in the last years. Phylogroup C has been associated to commensal or InPEC strains; phylogenetic group E has been associated to EHEC strains and phylogenetic group F is closely associated to groups D and B2 since it possesses the *chuA* gene. In our study, members of the phylogroups D, F and B2 were identified. These groups are supposed to encompass most pathogenic *E. coli* strains and in our research they comprehend 12% of strains. It has been suggested that the protein coded by *chuA* is involved in human host colonization and thus can have impact on pathogenicity (Hoffmann *et al*., 2001). In our study, the frequency of *chuA* (14%) is lower than that reported by Hoffmann *et al*. (2001) and Mokracka *et al*. (2011) for strains isolated from environmental samples (59% and 30%, respectively) and from strains isolated from human sources (70%, Hoffmann *et al*., 2001).

In Mexico, antibiotics are medical drugs with high rate of consumption and their consumption is associated with a high rate of misuse (Dreser *et al*., 2008). Misuse is

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caused by unwarranted prescription, inappropriate choice of treatment, self-prescription, lack of adherence by consumers, as well as lax regulation on the use of antibiotics (Amabile-Cuevas, 2010; Zaidi *et al*., 2003). The misuse and overuse of antibiotics is particularly important because it may contribute to selection and increase occurrence of antimicrobial resistant bacteria.

The occurrence and spread of multi-drug resistant bacteria is a worldwide public health concern. The emergence of bacterial resistance to antibiotics is common in areas where antibiotics are heavily used, and antibiotic-resistant bacteria also increasingly occur in aquatic environments (Zhang *et al*., 2009). In contaminated water such as river water as well as wastewater treatment plants, dissemination of mobile antimicrobial resistance determinants often occurs as bacteria previously exposed to antibiotics mix with native strains (Leonard *et al*., 2014 ; Schlüter *et al*., 2007).

In our study, at least two thirds of all *E. coli* isolates were resistant to beta-lactams such as ampicillin and carbenicillin, and at least one third of the isolates were resistant to trimethoprim-sulfamethoxazole and chloramphenicol (Figure 16). The presence of antibiotic resistant *E. coli* was also observed in other studies from human and animal fecal sources, wastewater treatment plant and surface water (Mokracka *et al*., 2011; Ibekwe *et al*., 2011; Sayah *et al*., 2005). Similar resistance levels were found in *E. coli* isolated from children and adults in Latin America and the 53.2% and 57.7% isolates were resistant to ampicillin and trimethoprim-sulfamethoxazol, respectively (Estrada-Garcia *et al*., 2009; Estrada-Garcia *et al*., 2005). Thus, suggesting that human wastewater can be linked with the presence of bacterial antimicrobial resistance in the River. While this study did not determine the specific source of *E. coli* isolates, it is likely that the isolates from sampling locations nearby agricultural land runoff, industrial sewage and slaughterhouse discharges are potential source for *E. coli* pathotypes (Figure 21).

Moreover, 30.6% of *E. coli* isolates were considered multi-drug resistant. Similar trends were reported by Moges *et al*., in wastewater from non-hospital environment where others factors such as indiscriminate use of antibiotics in human medicine, animal husbandry and agriculture and wastewater from pharmaceutical plants may disrupt the microbial balance in favor of resistant bacteria. In this study, industrial sewage including

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textile dyes industries and slaughterhouse sewages was the main contributor in the selection of antibiotic-resistant bacteria in sewage (Figure 20). Most multi-drug resistant bacteria presented resistance to ampicillin, carbenicillin and trimethoprim-sulfamethoxazol at the same time, suggesting the presence of resistance genes and mobile elements as integrons. In the multi-resistant isolates, resistance to fluoroquinolones such as pefloxacin and levofloxacin was significatively associated with resistance to trimethoprim-sulfamethoxazol and ampicillin (Table 6). This association is likely due to the presence of *qnr* genes and beta-lactams resistance genes in the multi-resistant isolates (Wang *et al*., 2001).

Although we observed a high prevalence of antibiotic resistance in all samples, the isolates from river sources were generally more susceptible to antibiotics than levels reported for clinical isolates (Moges *et al*., 2014; Zhang *et al*., 2009b; Falagas *et al*., 2007), which is consistent with other previous reports (Mogas *et al*., 2014; Romeu Alvarez *et al*., 2012; Ibekwe *et al*., 2011). Since *E. coli* has a marked ability to develop antibiotic resistance, together with the fact that antibiotics were heavily used in clinical environments, it was not surprising that clinical isolates showed higher level of antibiotic resistance.

Most *E. coli* identified as of pathogenic or potentially pathogenic were classified as intestinal pathogens (61%, 91/150). EAEC was the most prevalent intestinal pathogenic *E. coli* (44% of the isolates), and this is consistent with previous studies conducted in Mexico (Estrada-Garcia *et al*., 2005), since in clinical settings, EAEC and ETEC are the most prevalent pathotypes in Mexico (Estrada-Garcia *et al*., 2009; Estrada-Garcia *et al*., 2005; Estrada-Garcia *et al*., 2004). Virulence genes for ExPEC pathotypes were also commonly detected (Table 8) which is consisted with previous reports (Colomer-Lluch *et al*., 2013; Anastasi *et al*., 2012; Anastasi *et al*., 2010; Hamilton *et al*., 2010; Hamelin *et al*., 2007; Hamelin *et al*., 2006; Muhldorfer *et al*., 1994) and towhich also demonstrated that certain clonal groups of *E. coli* with uropathogenic properties can persist throughout one or more treatment stages of WWTP and enhanced ability to survive in surrounding environmental waters (Anastasi *et al*., 2012; Anastasi *et al*., 2010) and even could reproduce and persist in secondary environments rather than being obligate intestinal flora in tropical (Anastasi *et al*., 2012;

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Solo-Gabriele *et al*., 2000) and temperate climates (Jjemba *et al*., 2010; Ishii *et al*., 2006; Gordon *et al*., 2002; McLellan 2004). Furthermore, in our study, several pathogenic strains as well as commensal were also positive for the amplification of other virulence genes belonging to other *E. coli* pathotypes (Table 9) including ExPEC virulence genes. One possible explanation for this could be that environmental isolates may acquire virulence genes when they are in the environment, thus combinations of VG provide evidence of genetic exchange between related bacteria (Anastasi *et al*., 2012; Hamelin *et al*., 2007; Dröge *et al*., 1999).

Contrary to other studies (Maal-Bared *et al*., 2013) EHEC and EIEC were no detected. In this study, *E. coli* strains were isolated from water based on a high incubation temperature (37°C-44.5°C) and selection for β-glucuronidase activity. However, as Hamelin *et al.,* (2006) mentionated, many studies have shown that isolates belonging to the O157:H7 serotypes do not grow at these temperatures. Thus, it is quite possible that the proportion of pathogenic *E. coli* in our water samples could have been higher if our culturing methods had not excluded detection of O157:H7 and possibly other pathogenic *E. coli* strains as enteroinvasive *E. coli*.

The occurrence of isolates not classified as pathotypes yet possessing a subset of virulence genes could be explained by the high genome plasticity of *E. coli* (Table 9). This plasticity exists due to the dynamic genetic exchange of virulence genes through plasmids, pathogenicity islands, and other mobile genetic elements. These genetic factors contribute to the rapid evolution of *E. coli* strains and to the formation of unusual virulence gene combinations that could potentially lead to the evolution of new pathotypes (Hamelin *et al*., 2007).

Furthermore, several intestinal pathogens with multiple resistances to antimicrobials were isolated from the River (Figure 18, Table 7). The high prevalence of strains resistant to antimicrobial agents could have two different origins, one directly from the human and animal treated with antibiotics, and the other one from the horizontal genetic transference between different bacterial species. Thus, the occurrence of pathogenic *E. coli* with multiple antimicrobial resistances in the San Pedro River represents a great concern due to possible transfer of resistant genes and may increase the probability of infections with a higher cost of treatment.

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Anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes and antimicrobial resistant bacteria usually relevant in clinical environments (Tacão *et al*., 2012). Our results indicated that high contaminated sample locations are related to the presence of resistant and multi-drug resistant bacteria (Figure 18). Furthermore, samples from industrial and slaughterhouses had important presence antibiotic resistance bacteria (Figure 17). The highest number of multi-resistant isolates was found in samples from wastewater discharges from human sewage, industrial sector, and farms. This suggests the importance of wastewater discharges in the dissemination of antimicrobial resistance strains.

Several studies indicated that among clinical *E. coli* strains, the production of virulence factors is negatively associated with resistance to some antibiotics, including fluoroquinolones (Kawamuro-Sato *et al*., 2010; Johnson *et al*., 2003). However, such phenomenon is not observed among animal or river water isolates (Koczura *et al*., 2012; Chen *et al*., 2011). It has been suggested that ecological factors may determine relationship between bacterial virulence and antibiotic resistance (Johnson *et al*., 2003). Our results indicate that higher levels of antimicrobial resistance are found in pathogenic than in commensal *E. coli* (Figure 19, Table 7). Thus, the occurrence of pathogenic *E. coli* with multiple antimicrobial resistances in the San Pedro River represents a great concern due to possible transfer of resistant genes and may increase the probability of infections with a higher cost of treatment.

Another interesting finding was the higher percentage of *E. coli* pathotypes carrying antimicrobial resistance genes (Table 10). This may reflect the influence of human antibiotic use, again suggesting the importance of municipal wastewater as a potential source of antimicrobial-resistant *E. coli* pathotypes. In our study, most of the isolates resistant to levofloxacin and pefloxacin had a multi-resistant phenotype and some were potentially pathogenic *E. coli* (Table 10). Similar results were found in a Mexican study on the prevalence of fluoroquinolone resistance among *E. coli* isolates from urinary tract infection (Llanes *et al*., 2012; Amabile-Cuevas *et al*., 2010; Zaidi *et al*., 2003) as well as from an environmental study (Amabile-Cuevas *et al*., 2010). Fluoroquinolone (FQ) is a family of widely used synthetic antimicrobial agents with a broad antibacterial spectrum that is used as a front line drug for urinary tract and

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intestinal infections. However, increase in the prevalence of FQ resistant bacteria has been a great concern worldwide in the last years. Several mechanisms have been described for FQ resistance. In *E. coli,* the resistance is primarily associated with the accumulation of mutations in the quinolone-resistance determining regions (QRDRs) of *gyrA* and *parC*, which encode topoisomerase II (DNA gyrase) and topoisomerase IV respectively (Hooper, 2001; Hopkins *et al.*, 2005). These mutations can lead to conformational changes in the enzymes and thus preventing quinolones from binding to the DNA-substrate complex (Tran *et al.*, 2005a; b). In addition, several other mechanisms can contribute to FQ resistance, including plasmid-mediated quinolone resistance (PMQR) determinants (Martinez-Martinez *et al.*, 1998), such as the Qnr protein (QnrA, QnrB, QnrC, QnrD, and QnrS), AAC(6′)-Ib-cr, the variant of the aminoglycoside-modifying enzyme (Robicsek *et al.*, 2006a), and the efflux pumps QepA (Yamane *et al.*, 2007), and OqxAB (Hansen *et al.*, 2004; Jacoby, 2005). In addition, we showed that the triple mutation profile (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*) was the most prevalent. The point mutations Ser-83 → Leu and Asp-87 → Asn found in *gyrA* and Ser-80 → Ile in *parC*, have been observed in other studies (Sun *et al*., 2012; Nambodiri *et al*., 2011). Previous studies have shown that *E. coli* with a single mutation (Ser-83 → Leu) in the *gyrA* subunit are resistant to nalidixic acid, a first generation quinolone (Sun *et al*., 2012; Vila *et al*., 1994). In addition, most fluoroquinolone resistance isolates carried horizontally acquired quinolone resistance genes and these were found primarily in combination with QRDR mutations. Qnr proteins may supplement resistance to quinolones due to altered quinolone target enzyme, efflux pump activation, or deficiencies in outer-membrane porins (Poirel *et al*., 2012; Jeong *et al*., 2008; Martinez-Martinez *et al*., 2003; Martinez-Martinez *et al*., 1998). The presence of Qnr determinants facilitates the selection of low-level of resistance to quinolones encoded on the chromosome-encoded and the selection of higher-level resistance mutation (Jacoby, 2005). Several strains that harbored *qnrS* also carried bla_{TEM} gene. It was reported that, *qnrS* genes are associated with transposons containing TEM-1 type -lactamases (Dalhoff *et al*., 2012; Hernandez *et al*., 2011). Tetracycline and streptomycin resistance genes were also detected along with fluoroquinolone resistance genes. In our study we were able to see the presence of beta-

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lactamases-encoding genes at the same time as *qnr* which explain the phenotype resistance to ampicillin, carbenicillin and quinolones. While the majority of isolates are resistant phenotype chloramphenicol, only one isolate presented the gene *cmlAI* encoding for efflux pump. Several isolates presented the *sulII* gene encode for sulfonamide-resistant dihydropteroate synthases while just one was positive for *sulI* which is according to other authors (Xi *et al*., 2009)

In this study, more that 40% of our isolates tested carried the resistant genes *tetA* or/and *tetB*. Since tetracycline resistance genes are located on the mobile genetic elements, they are transmissible between bacteria. The presence of tetracycline resistance genes is important since they are located on mobile genetic elements, and can be transmissible between bacteria (Ibekwe *et al.,* 2011a). Therefore, most fecal bacteria from human or agricultural sources released into the environment may carry antibiotic resistance genes (Ibekwe *et al*., 2011b; Allen *et al*., 2010).

In agreement with Hamelin *et al.,* all multi-resistant *E. coli* isolates (isolates carrying more than three antimicrobial resistance genes) and the majority of *E. coli* isolates with three resistance genes contained a class 1 integron, which are very efficient genetic mechanism for the diffusion of antimicrobial resistance. These genetic determinants contribute to the rapid evolution of *E. coli* strains and to the creation of new pathogenic variants since they are frequently subject to rearrangement, excision, and horizontal transfer (Hamelin *et al.* 2006, 2007). Furthermore, since microbial density is usually high, andconforms to diversity of microorganisms (commensal bacteria, environmental microorganisms, clinically pathogens, and biofilms from activated sludge), it may facilitate the antibiotic resistant dissemination by HGT (LaPara *et al*., 2012; Schluter *et al*., 2007). Elevated nutrient levels can also improve the efficiency of horizontal gene transfer between species by the bacterial processes of transformation (Thomas and Nielsen 2005) and conjugation (Arana *et al*., 2001, 1997). High fecal coliform densities, as seen in this study, increase the likelihood of humans and animals coming in contact with pathogenic bacteria.

On the other hand, routine monitoring of microbiological surface water quality is usually limited to testing of the water phase. In this study we tested the presence of *E. coli* as biofilms in stream water. Our results revealed the presence of *E. coli* as biofilms

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in half of the sampling locations through the River water. These results are in agreement with previous reports in river water (Maal-Bared *et al*., 2013; Balzer *et al*., 2010) and drinking water systems (Keevil, 2002; Rogers *et al*., 1994) and suggest that faecal indicator bacteria and thus possibly bacterial pathogens of faecal origin can accumulate in River biofilms and are expected to survive longer periods of time than their planktonic phase since usually *E. coli* and other organisms including bacterial pathogens of faecal origin (e.g., *Campylobacter spp*.) and opportunistic bacteria of environmental origin (e.g., *Legionella spp*., *Pseudomonas aeruginosa*) can attach to preexisting biofilms, where they become integrated and survive for days to weeks or even longer, depending on the biology and ecology of the organism and the environmental constitutions (Wingender and Flemming 2011).

Moreover, the results are consistent with the inorganic and organic matters which were abundant in almost all the sample locations. We did not detect biofilm formation by *E. coli* isolated in sampling locations nearby “open space” (lower contaminated sample locations) probably due to the limiting factors for biofilm growth that is usually the nutrient depletion since biofilms are related to the occurrence of contaminated water and malodours (Wingender and Flemming, 2004). In addition, there are indications that at least a part of the biofilm populations of pathogenic bacteria persists in a VBNC state and remains unnoticed by the methods appointed to their detections (Wingender and Flemming 2011). However, this biofilm-associated non-culturable state and the potential of resuscitation of these bacteria have not yet been characterized in detail. Additionally, we have demonstrated a continuing, stable *E. coli* population in stream water suggesting the persistence of *E. coli* in the environment long after their original input and their adaptation to the aquatic environment.

The presence of *E. coli* as biofilms in polluted surface waters may be reservoirs for faecal indicator bacteria and provide a possible habitat for hygienically relevant microbes in which they can persist and even multiply thus possibly bacterial pathogens of faecal origin and contribute to the deterioration of water quality by releasing faecally derived pathogens. From a public health perspective, this phenomenon is of great importance since contamination of water with coliforms from biofilms can interfere with their function to indicate faecal or other undesirable exogenous (Wingender and

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Flemming 2011; Wingender and Flemming 2004) contaminations and mask true failures in water treatment and maintenance of the network (Wingender and Flemming 2011).

The results in this study showed that commensal and pathogenic *E. coli* isolates from river sources are able to form biofilm *in vitro*. In generally no significative differences were observed between pathogenic and commensal strains, excepting by EAEC which showed a remarkable variation among the capacities to form biofilms *in vitro*. This is consistant with previous works (Moreira *et al*., 2012; and Reisner *et al*., 2006). Reisner *et al*., (2006) and demonstrates that the ability to form mature biofilms is not universal among human *E. coli* isolates (pathogenic ones). Instead, human isolates tend to exhibit a wide range of biofilm-forming competence and were dependence on growth medium composition. On the other hand, Moreira *et al*., (2012) compared the biofilm forming ability of *E. coli* isolated from diverse sources. Moreira *et al*., (2012) found that periphytic strains isolates from freshwater had the best biofilm forming ability (OD_{600} 0.906 ± 0.230) compared with human (OD_{600} 0.389 ± 0.455) and bovine (OD_{600} 0.120 ± 0.209) strains. In our case, commensal strains had a lower capacity to form (OD_{595} 0.8851 ± 0.2950) compared with pathogenic strains and specifically to EAEC (OD_{595} 1.364 ± 0.5112) that displayed higher biofilm-forming capacity. EPEC strains showed and OD_{595} 0.8697 ± 0.1753 which is consisting with other studies (Cendra *et al*., 2012; Easton *et al*., 2011). Furthermore, since EAEC and ETEC were the main pathogens detected in through San Pedro River and these strains possess also the ability to form biofilms. This could represent a high risk to public health.

Previous studies have been shown that expression of F-like conjugative pili, aggregative adherence fimbria, curli and cellulose strongly promote biofilm formation in *E. coli* K-12 (Reisner *et al*., 2006), STEC (De Re *et al*., 2006; Cookson *et al*., 2002) and EAEC strains (Pereira *et al*., 2010). In our study, SEM micrographics showed that bacterial aggregation in the environmental isolates could be mediated by putative pilus and the putative expression of curli fibers, which are consisted with our results of the biofilm-forming capacity of *E. coli* strains in both, *in situ* and *in vitro* assays.

Congo Red assay demonstrated that the production of curli and cellulose varies among isolates. Consisting with our SEM results, one third of the strains tested by Congo red were able to produce curli and cellulose, nevertheless no correlation was

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found between biofilm production and curli production, indicating that even when curli and cellulose are involved in biofilm formation of *E. coli* in river water others determinants such as adhesion, quorum sensing or metabolism are likely to also be involved in biofilm formation of *E. coli* in water.

Our results revealed the presence of pathogenic *E. coli* in the river with present mobile elements as integrons, and multi-drug resistance characteristics, including fluoroquinolone resistance, an antibiotic highly used in humans and animals worldwide, mostly found in locations of the river that have been impacted by industrial sewage. This situation highlights the risk of multi-drug resistance pathogens dissemination (Dalhoff 2012; Sun *et al*., 2012; Allou *et al*., 2009). Furthermore, our study was conducted in a populated area, a setting that is often observed in developing countries and that must be taken into account (Mazari-Hiriart *et al*., 2008). This poses a potential risk for human infections.

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CONCLUSION

The results showed that the poor water quality and lacking of adequate wastewater treatment result in negative impact in San Pedro River; which serves as reservoir of high density of microorganism such as coliforms and enteropathogenic bacteria. Besides the high level of culturable bacteria, we also found evidence of the presence of viable but not culturable *E. coli* in water sources as well as biofilm-forming *E. coli* directly from river water, suggesting that these biofilms may act as a reservoir for bacterial pathogens in polluted rivers and non-culturability bacteria may also provide an additional risk from public health since these bacteria conserve their virulence.

Most of the isolates presented multi-drug resistant phenotype and integron genes which increase the potential of dissemination of antimicrobial resistance genes between the river, humans and animals. Furthermore, the discharge of slaughterhouses, industrial sewage and urban runoff effluent may contribute to the dissemination of antibiotic resistance and pathogenic bacteria in the aquatic environment. The high prevalence of antibiotic-resistant bacteria has two origins: a) directly by downloading resistant bacteria mainly from industrial discharges traces and b) through chromosomal mutations and the acquisition of resistance genes by horizontal transfer. Resistance to fluroquinolone of second and third generation is present in the river and occurs through multiple mechanisms.

To our knowledge, this is the first study that detects several pathogenic *Escherichia coli* in Mexico in river water as well as antimicrobial resistance profile and their mechanisms. Further studies are needed to determine the risk factors that the river represent for the population.

This study highlighted that the San Pedro River has an important bacterial contamination with antimicrobial resistant *E. coli* including diarrheagenic and extra-intestinal pathogenic strains as well as commensal *E. coli* which carried virulence genes and antimicrobial resistance genes. The results show that the prevalence of pathogenic *E. coli* and antibiotic resistance *E. coli* are present in the San Pedro River stream water

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such as fluoroquinolone resistant and these are a potential threat to human and animal health.

Thus, some efforts have to be done to reduce the possibility of antimicrobial resistance genes transfer entering into and spread in the environments, such a prudent use of antibiotics in human and veterinary infections, as well as effective wastewater treatment processes are needed to improve removal efficiency of pathogenic and multi-drug resistant *E. coli*. It is also essential to control clandestine discharges into the river and enforce to satisfy the official norms and regulations. Water quality assessment must integrate detection of antibiotics in water as a routine quality parameter and new guidelines for treatment water must be made in order to include the management of antibiotic bacteria and resistance genes in wastewater, as well as the detection and removal of these pollutants.

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GLOSSARY

Adhesion: A stable interaction of a cell with respect to a surface. Living cells actively excrete chemicals from their surface to anchor themselves to a substratum. This is referred to as adhesion or attachment.

Antimicrobial resistance (AMR): is resistance of a microorganism to an antimicrobial medicine to which it was originally sensitive. Resistant organisms (they include bacteria, fungi, viruses and some parasites) are able to withstand attack by antimicrobial medicines, such as antibiotics, antifungals, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist increasing risk of spread to others.

Biochemical oxygen demand (BOD): measure of the quantity of oxygen used by microorganisms in the oxidation of organic matter. If organic matter is present in the dilution water, it may increase its oxygen demand.

Biofilm: Microbial biofilms are populations of microorganisms that are concentrated at an interface (usually solid–liquid) and typically surrounded by an extracellular polymeric substance (EPS) matrix.Aggregates of cells not attached to a surface are sometimes termed 'flocs'and have many of the same characteristics as biofilms.

Chemical oxygen demand (COD): measure of water and wastewater quality. This test is based on the fact that a strong oxidizing agent, under acidic conditions, can fully oxidize almost any organic compound to carbon dioxide. The COD is the amount of oxygen consumed to chemically oxidize organic water contaminants to inorganic end products

Coliform organisms: Organisms capable of aerobic growth either 35 ± 1 ° C or 37 ± 1 ° C in a liquid medium with lactosed acid and gas production within a period of 48 h.

Curli: proteinaceous extracellular fibers that are involved in surface and cell-cell contacts that promote community behavior and host colonization. Curli are the major proteinaceous component of a complex extra-cellular matrix produced by many *Enterobacteriaceae.* Curli fibers are involved in adhesion to surfaces, cell aggregation, and biofilm formation. Curli also mediate host cell adhesion and invasion, and they are

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potent inducers of the host inflammatory response. Structurally and biochemically, curli belong to a growing class of fibers known as amyloids.

Detachment: The loss of single cells or aggregates of cells from the biofilm, usually into an overlying flow of fluid. Detachment can be an active process (dispersal), a passively induced mechanical process (for example, through fluid shear) or a chemical process (by adding agents that 'dissolve' the EPS matrix).

Fecal coliforms (thermotolerant): Coliform organisms described as having the same fermentative properties fecal coliforms with growth at 44 ± 0.5 ° C.

Hemolytic-uremic syndrome: Hemolytic-uremic syndrome (HUS) is a disorder that usually occurs when an infection in the digestive system produces toxic substances that destroy red blood cells, causing kidney injury.

Hemorrhagic colitis: Type of gastroenteritis in which certain strains of the bacterium *Escherichia coli* infect the large intestine and produce a toxin (Shiga toxin) that causes bloody diarrhea and other serious complications.

Intermediate (I): The "intermediate" category includes isolates with antimicrobial agent MICs (minimal inhibitory concentrations) that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (eg, quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (eg, β-lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

Maximum permissible limit: Value or assigned to a parameter, which should not be exceeded by the party responsible for providing treated wastewater range.

MBAS: is a colorimetric analysis test method that uses methylene blue for the quantificative measurement of anionic surfactants (such as a detergent or foaming

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agent) including tetrapropylene alkyl benzene sulfonato (ABS) surfactants and alkylate sulfonate (LAS) compounds in a sample water. An anionic surfactant detected by the color reaction is called a methylene blue active substance (MBAS).

Mesophilic bacteria: Bacterial species involved in biodegradation (i.e., digestion and decomposition of organic matter), which are more active in temperatures ranging from approximately 15°–40°C, are termed mesophilic bacteria. Mesophilic bacteria are involved in food contamination and degradation, such as in bread, grains, dairies, and meats. Bacterial infections in humans are mostly caused by mesophilic bacteria that find their optimum growth temperature around 37°C (98.6°F), the normal human body temperature.

Pathotypes: group of strains of a single species that cause a common disease using a common set of virulence factors.

Periphyton: An assemblage of organisms attached to and living on submerged solid surfaces in natural environments such as rivers.

Planktonic cells: Planktonic (or suspended) cell cultures are those grown primarily as single cells in suspension, either in a chemostat or a shake flask.

Reclaimed water: former wastewater (sewage) that is treated to remove solids and certain impurities, and used in sustainable landscaping irrigation or to recharge groundwater aquifers. Is the end product of wastewater reclamation that meets water quality requirements for biodegradable materials, suspended matter and pathogens.

Resistant (R): The "resistant" category implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules, and/or that demonstrate MICs or zone diameters that fall in the range where specific microbial resistance mechanisms (eg, β-lactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

Resistome: is a proposed expression by Gerard D. Wright comprises all of the antibiotic resistance genes. It includes resistance elements found in both pathogenic bacteria and antibiotic-producing commensal bacteria and their precusors.

Susceptible (S): The "susceptible" category implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

Type 1 fimbriae: are adhesion organelles expressed by many Gram-negative bacteria. They mediate attachment to both biotic and abioticsurfaces and are involved in the early stages of biofilm formation. Thye facilitate adherence to mucosal surfaces and inflammatory cells in vitro, are a crucial factor for the virulence of uropathogenic *Escherichia coli* during the first steps of infection by mediating adhesion to epithelial cells. They are also required for the consequent colonization of the tissues and for invasion of the uroepithelium promoting the formation of intracellular bacterial communities.

Type IV pilus: An elongated structure extending from the surface of Gram-negative cells that is independent of flagella and which can retract and pull the cell forward.

Viable bacteria: bacteria metabolically or physiologically active.

Viable but Nonculturable Bacteria (VBNC): metabolically active bacterial cell that crossed a threshold and, for known or unknown reasons, became unable to multiply in or on a medium normally supporting its growth.

Virulence: ability to organisms that enable them to enter, replicate, and persist in a host.

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SUPPLEMENTARY DATA

SUPPLEMENTARY DATA	**A**	**PARAMETERS OF REFERENCE**
SUPPLEMENTARY DATA	**B**	**RAW DATA**
SUPPLEMENTARY DATA	**C**	**PCRS FOR ANALYZED GENES**
SUPPLEMENTARY DATA	**D**	**SEQUENCING ANALYSIS**
SUPPLEMENTARY DATA	**E**	**PUBLICATIONS**
SUPPLEMENTARY DATA	**F**	**PRESENTED WORKS**

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SUPPLEMENTARY DATA A

Table 1:S Parameters that include each of the subindexes for water quality and desirable level values of parameters in the water for use in agricultural irrigation (Guzman-Colis *et al*., 2011).

Subindex	Parameters	Desirable level
Field	pH	6.5-8-5
	DO	5.5-8.0 mg/L
	Conductivity	2000µS/cm
Organic matter	BOD	15 mg/L
	COD	20 mg/L
Nutrients	Phosphorus	0.1 mg/L
	Nitrogen	1 mg/L
Organic toxics	MBAS	0.5 mg/L
	Phenols	0.010 mg/L
	Anilines	0.002 mg/L
Fecal contamination	Total coliforms	1000 MPN/100mL
	Fecal coliforms	100 MPN/100mL

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Table 2:S MPN indicator and 95% reliable limit to results positives and negatives combinations.

Number of positive reactions.			Indicator (MPN/100mL)	Reliable limit of 95%	
3 tubes with 10 mL	**3 tubes with1 mL**	**3 tubes with 0.1 mL**		**Lowest**	**Highest**
0	0	0	<3		
0	0	1	3	<0.5	9
0	1	0	3	<0.5	3
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	1	460	71	2400
3	3	2	1100	150	4800
3	3	3	>=2400		

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Table 3:S. Zone of diameter interpretation criteria.

Antimicrobial	Code	Concentration (µg)	Resistentance	Intermediate	Sensitives
Amikacin	AK	30	≤14	15-16	≥17
Ampicillin	AM	10	≤13	14-16	≥17
Levofloxacin	LEV	5	≤13	14-16	≥17
Cephalothin	CF	30	≤14	15-16	≥18
Cefotaxime	CTX	30	≤14	15-22	≥23
Ceftriaxone	CRC	30	≤13	14-20	≥21
Cloramphenicol	CL	30	≤12	13-17	≥18
Gentamicin	GE	10	≤12	13-14	≥15
Netilmicin	NET	30	≤12	13-14	≥15
Nitrofurantoin	NF	300	≤14	15-16	≥17
Pefloxacin	PEF	5	≤14	15-22	≥23
Trimetroprim-Sulfametoxazol	SXT	25	≤10	11-15	≥16

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Table 4:S. Oligonucleotides used in this study.

Oligonucleotide name	Target gene	Oligonucleotide 5' → 3'	Amplification product (bp)	Reference
		E.coli marker		
uidA-forward	*uidA*	ATGTGCTGTGCCTGAACC	450	This study.
uidA-reverse		ATTGTTTGCCTCCCTGCTG		
		Virulence genes for intestinal pathogenic *E. coli*		
VTcom-u	*stx1/stx2*	GAGCGAAATAATTTATATGTG	518	(Toma et al., 2003)
VTcom-d		TGATGATGGCAATTCAGTAT		
East-upper	*astA*	ATGCCATCAACACAGTATAT	110	(Vila et al., 2000)
East-lower		GCGAGTGACGGCTTTGTAGT		
AafAf	*aafA*	AAATTAATTCCGGCATGG	918	(Huang et al., 2007)
AafAr		ATGTATTTTTAGAGGTTGAC		
aggRks1	*aggR*	GTATACACAAAAGAAGGAAGC	254	(Aranda et al., 2007)
aggRksa2		ACAGAATCGTCAGCATCAGC		
AL65	*st*	TTAATAGCACCCGGTACAAGCAGG	147	(Toma et al., 2003)
Al125		CCTGACTCTTCAAAAGAGAAAATTAC		
LTL	*lt*	TCTCTATGTGCATACGGAGC	322	(Toma et al., 2003)
LTR		CCATACTGATTGCCGCAAT		
IpaIII	*ipaH*	GTTCCTTGACCGCCTTTCCGATACCGTC	619	(Toma et al., 2003)
IpaIV		GCCGGTCAGCCACCCTCTGAGAGTAC		
BFP1	*bfpA*	AATGGTGCTTGCGCTTGCTGC	326	(Aranda et al., 2007)
BFP2		GCCGCTTTATCCAACCTGGTA		
eae1	*eae*	CTGAACGGCGATTACGCGAA	644	(Aranda et al., 2007)
eae3		CGAGACGATACGATCCAG		
		Virulence genes for extra-intestinal pathogenic *E. coli*		
papC-F	*papC*	GACGGCTGTACTGCAGGGTGTGGCG	350	(Blanco et al., 1997)
papC-R		ATATCCTTTCTGCAGGGATGCAATA		
SfaSf	*sfaS*	GTGGATACGACGATTACTGTG	240	(Johnson and Stell, 2000)
SfaSr		CCGCCAGCATTCCCTGTATTC		
Afaf	*afa/dra*	GGCAGAGGGCCGGCAACAGGC	592	(Johnson and Stell, 2000)
Afar		CCCGTAACGCGCCAGCATCTC		
FyuAf	*fyuA*	TGATTAACCCCGCGACGGGAA	880	(Johnson and Stell, 2000)
FyuAr		CGCAGTAGGCACGATGTTGTA		
KpsMIIf	*kpsMT II*	GCGCATTTGCTGATACTGTTG	272	(Johnson and Stell, 2000)
KpsMIIr		CATCCAGACGATAAGCATGAGCA		
		Quinolone resistance genes		
gyrA11753	*gyrA*	GTATAACGCATTGCCGC	251	(Wang et al., 2001)
gyrA12004		TGCCAGATGTCCGAGAT		
EC-PAR-A	*parC*	CTGAATGCCAGCGCCAAATT	189	(Deguchi et al., 1997)
EC-PAR-B		GCGAACGATTTCGGATCGTC		
qnrA-up	*qnrA*	TCAGCAAGAGGATTTCTCA	605	(Maynard et al., 2004)
qnrA-down		GGCAGCACTATTACTCCCA		
qnrB-up	*qnrB*	GATCGTGAAAGCCAGAAAGG	469	(Robicsek et al., 2006)
qnrB-down		ACGATGCCTGGTAGTTGTCC		
qnrS-up	*qnrS*	ACGACATTCGTCAACTGCAA	417	(Robicsek et al., 2006)
qnrS-down		TAAATTGGCACCCTGTAGGC		
aac-up	*acc-(6')-lb*	TTGCGATGCTCTATGAGTGGCTA	482	(Park et al., 2006)
aac-down		CTCGAATGCCTGGCGTGTTT		

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Table 4:S continuation. Oligonucleotides used in this study.

Oligonucleotide name	Target gene	Oligonucleotide 5' → 3'	Amplification product (bp)	Reference
		Phylogenetic groups		
chuA.1b	*chuA*	ATGGTACCGGACGAACCAAC	288	(Clermont *et al*., 2012; Clermont *et al*., 2000)
chuA.2		TGCCGCCAGTACCAAAGACA		
yjaA.1b	*yjaA*	CAAACGTGAAGTGTCAGGAG	211	(Clermont *et al*., 2012)
yjaA.2b		AATGCGTTCCTCAACCTGTG		
TspE4C2.1b	TspE4.C2	CACTATTCGTAAGGTCATCC	152	(Clermont *et al*., 2012)
TspE4C2.2b		AGTTTATCGCTGCGGGTCGC		
AceK.f	*arpA*	AACGCTATTCGCCAGCTTGC	400	(Clermont *et al*., 2012)
ArpA1.r		TCTCCCCATACCGTACGCTA		
ArpAgpE.f	*arpA*	GATTCCATCTTGTCAAAATATGCC	301	(Clermont *et al*., 2012)
ArpAgpE.r		GAAAAGAAAAAGAATTCCCAAGAG		
trpAgpC.1	*trpA*	AGTTTTATGCCCAGTGCGAG	219	(Clermont *et al*., 2012)
trpAgpC.2		TCTGCGCCGGTCACGCCC		

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Table 5:S. PCR control strains used in this study.

Control strain	Positive gene (s)
ETEC H10407	*elt / est*
EHEC EDL933	*sxt1* and *sxt2*
EPEC 2349/69	*eae* and *bfpA*
EAEC O42	*aggR* and *aaf genes*
Shigella flexneri	*ipaH*
J53pMG252	*qnrA*
J53pMG298	*qnrB*
J53pMG306	*qnrS*
Salmonella SA20042859	*aac(6')-Ib*
H10407	Phylogroup A
E22	Phylogroup B1
ECOR 70	Phylogroup C
EDL933	Phylogroup E
ECOR 48 / O42	Phylogroup D
ECOR 36	Phylogroup F
CFT073	Phylogroup B2

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Table 6:S. Virulence genes and function used in this study.

Virulence gene	Function		Associated pathotypes
eae	Intimin (attaching and effacing lesions)	Locus of enterocyte effacement (LEE)	EPEC, EHEC
bfpA	Major structural subunit of bundle-forming pilus (BFP)	Adhesins	EPEC
st	Heat-stable enterotoxin I (STI or STa) and variants	Toxin	ETEC
lt	Heat-labile enterotoxin	Toxin	ETEC
ipaH	Invasion plasmid antigen	Various functions	EIEC
stx1/stx2	Shiga-like toxin I, subunit A-shiga-like toxin II, subunit B, various variants	Toxin	EHEC
eastI	Enteroaggregative *E. coli* heat-stable enterotoxin 1, other name: *astA*	Toxin	EAEC
aafA	Major fimbrial subunit of aggregative adherence fimbria type II (AAF/II)	Adhesins	EAEC
aggR	Transcriptional activator for the AAFs of EAEC),	Adhesins	EAEC
fyuA	Pesticin/yersiniabactin receptor protein	Iron acquisition or transport systems	UPEC
kpsMTII	Involved in polysialic acid transport, group II (K1, K4, K5, K7, K12, K92...)	Capsular and somatinc antigen	ExPEC
papC	Outer membrane usher protein involved in P fimbrial assembly	Adhesins	UPEC
sfaS	Major fimbrial subunit of S fimbriae (SfaI)	Adhesins	ExPEC

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SUPPLEMENTARY DATA B

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Table 7:S. Values of physico-chemical parameters from San Pedro River per sample type location.

No.	Type of sample	Possible source of pollution	Temperature (ºC)	pH	Conductivity (µS/cm)	Dissolved oxygen (mg/L)
1	Urban runoff	Urban runoff	26,3	7,31	1082	0,03
2	Slaughterhouse	Slaughterhouse	26	8,08	4900	0,03
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	29	7,53	1118	9,73
14	Open space	Open space	28,5	7,6	1808	0,05
18	Slaughterhouse	Slaughterhouse	22,3	8,01	3660	0,06
19	Urban runoff	Human sewage + wastewater discharge	30	7,22	1860	0,62
4	Urban runoff	Urban runoff	23,7	7,45	1274	0,07
5	Farm	Agricultural + farm	25	8,18	6010	0,9
6	Farm	Agricultural + farm	22	8,17	6240	1,12
8	Urban runoff	Urban runoff	22	7,87	1176	4,81
9	Open space	Open space	23	8,17	1161	6,08
7	Open space	Open Space	25,5	7,3	1340	0,07
11	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	28,3	7,23	1782	0,06
12	Urban runoff	Urban runoff + agricultural + wastewater	22,5	7,8	1570	0,03
10	Slaughterhouse	Agricultural + slaughterhouse	21,4	7,72	1472	1,32
13	Agricultural	Urban runoff + agricultural	27,2	7,14	1947	0,09
15	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	26,5	7,28	1673	0,05
16	Farm	Agricultural + farm	24,3	7,05	1631	0,08
17	Farm	Agricultural + farm	24,4	7,01	1647	0,26
20	Open space	Open space	22,5	7,1	1345	0,08
21	Agricultural	Agricultural	19,9	7,9	1191	4,91
22	Open space	Open space	15	7,1	1659	1,08
24	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	27	7,54	1313	0,5
25	Urban runoff	Urban runoff + wastewater discharge	26	7,38	1467	0,16
23	Urban runoff	Urban runoff + agricultural + wastewater	13,3	6,66	800	0,63
26	Slaughterhouse	Slaughterhouse	21,1	7,9	2078	0,07
27	Urban runoff	Urban runoff	27,3	7,61	1354	0,28
28	Open space	Open space	23,7	7,54	1120	0,05
29	Industrial sewage	Industrial sewage	21,9	8,17	2081	0,25
30	Farm	Agricultural + farm + human sewage	22,3	7,24	2812	0,06

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Table 8:S. Values of parameters for organic matter and nutrients from San Pedro River per sample type location.

No.	Type of sample	Possible source of pollution	COD (mg/L)	BOD (mg/L)	P (mg/L)	N (mg/L)	SST (mg/L)
1	Urban runoff	Urban runoff	585	467	13,4	32,2	122,5
2	Slaughterhouse	Slaughterhouse	9400	4532	53	934,7	4135
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	40	0	1,5	2,6	31,3
4	Open space	Open space	920	123	42,5	12,9	171,4
5	Slaughterhouse	Slaughterhouse	2450	651	19,5	651,1	650
6	Urban runoff	Human sewage + wastewater discharge	2915	1739	22,1	100	1718,8
7	Urban runoffoo	Urban runoff	502	166	21,3	85	678,33
8	Farm	Agricultural + farm	3136	1007	48,7	875,9	622,2
9	Farm	Agricultural + farm	4688	1285	123,4	974	6988,1
10	Urban runoff	Urban runoff	434	89	12,6	143,7	344,5
11	Open space	Open space	352	125	13,4	160,2	126
12	Open space	Open Space	484	213	15,4	108,2	340
13	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	1826	890	15	56,2	353,3
14	Urban runoff	Urban runoff + agricultural + wastewater	1446	918	34	125,6	827,8
15	Slaughterhouse	Agricultural + slaughterhouse	594	164	12,5	48,8	126
16	Agricultural	Urban runoff + agricultural	3022	934	4,2	95,6	3450
17	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	6800	2854	245,2	193,7	13196,5
18	Farm	Agricultural + farm	472	223	6	41,3	325
19	Farm	Agricultural + farm	512	279	5,6	23,4	730
20	Open space	Open space	256	191	23,4	93	207
21	Agricultural	Agricultural	1045	865	21,5	66,83	350
22	Open space	Open space	876	568	24,3	30,8	125
23	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	932	426	54,1	19,8	230
24	Urban runoff	Urban runoff + wastewater discharge	820	365	33,9	12,9	209,1
25	Urban runoff	Urban runoff + agricultural + wastewater	494	100	10,5	37,6	410
26	Slaughterhouse	Slaughterhouse	712	98	22	44,6	140
27	Urban runoff	Urban runoff	1052	511	52,5	57,2	365,8
28	Open space	Open space	304	187	22,3	90	546,21
29	Industrial sewage	Industrial sewage	446	167	16,5	23,4	102,5
30	Farm	Agricultural + farm + human sewage	1285	445	7,5	482,5	260

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Table 9:S. Values of parameters for organic toxics from San Pedro River per sample type location.

No.	Type of sample	Possible source of pollution	Phenols (mg/L)	Anilines (mg/L)	MBAS (mgL)
1	Urban runoff	Urban runoff	1,58	30	41,7
2	Slaughterhouse	Slaughterhouse	0,1	19,5	39,4
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	0,49	11,2	33,4
4	Open space	Open space	0,52	0,29	57,4
5	Slaughterhouse	Slaughterhouse	0,43	80,9	12
6	Urban runoff	Human sewage + wastewater discharge	0,64	72,1	10
7	Urban runoff	Urban runoff	1,02	82,4	45,9
8	Farm	Agricultural + farm	0,39	58,5	18,4
9	Farm	Agricultural + farm	0,08	67,3	20,9
10	Urban runoff	Urban runoff	0,14	29,5	20,4
11	Open space	Open space	0,2	36,1	22,9
12	Open space	Open Space	0,23	33	13,9
13	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	2,8	76	12,9
14	Urban runoff	Urban runoff + agricultural + wastewater	0,51	169,7	20,4
15	Slaughterhouse	Agricultural + slaughterhouse	0,61	21	20,9
16	Agricultural	Urban runoff + agricultural	0,45	89,7	170
17	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	0,31	32,6	16,4
18	Farm	Agricultural + farm	0,49	114,1	20,9
19	Farm	Agricultural + farm	0,74	53,1	30,9
20	Open space	Open space	0,31	44,9	16,4
21	Agricultural	Agricultural	0,38	50,7	14,9
22	Open space	Open space	0,43	47,8	14
23	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	0,39	56,09	13,5
24	Urban runoff	Urban runoff + wastewater discharge	0,63	75,1	15
25	Urban runoff	Urban runoff + agricultural + wastewater	0,34	14,9	18,9
26	Slaughterhouse	Slaughterhouse	0,39	29,2	18,4
27	Urban runoff	Urban runoff	0,15	40,9	13,4
28	Open space	Open space	0,39	34,7	22,5
29	Industrial sewage	Industrial sewage	0,38		
30	Farm	Agricultural + farm + human sewage	0,34		

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Table 10:S. Values of parameters for microorganism and fecal contaminations from San Pedro River per sample type location.

No.	Type of sample	Possible source of pollution	Mesophilic (CFU/100mL)	Total coliforms (Log_{10}MPN/100mL)	Fecal coliforms (Log_{10}MPN/100mL)
1	Urban runoff	Urban runoff	5,9	3,7	3,7
2	Slaughterhouse	Slaughterhouse	6,1	3,7	3,7
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	4,1	1,0	0,5
4	Open space	Open space	6,5	4,0	3,7
5	Slaughterhouse	Slaughterhouse	5,7	1,7	1,7
6	Urban runoff	Human sewage + wastewater discharge	6,0	4,0	3,7
7	Urban runoffoo	Urban runoff	6,1	4,0	3,7
8	Farm	Agricultural + farm	5,8	2,6	1,1
9	Farm	Agricultural + farm	5,8	2,2	2,2
10	Urban runoff	Urban runoff	4,5	2,0	2,0
11	Open space	Open space	4,6	2,4	1,5
12	Open space	Open Space	5,1	3,7	3,7
13	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	5,8	3,2	3,2
14	Urban runoff	Urban runoff + agricultural + wastewater	5,0	1,4	1,4
15	Slaughterhouse	Agricultural + slaughterhouse	5,3	1,0	1,0
16	Agricultural	Urban runoff + agricultural	6,7	4,4	3,7
17	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	6,0	3,7	3,7
18	Farm	Agricultural + farm	5,5	2,0	2,0
19	Farm	Agricultural + farm	5,6	2,4	2,4
20	Open space	Open space	5,1	2,7	2,7
21	Agricultural	Agricultural	5,9	1,0	0,5
22	Open space	Open space	5,0	3,4	3,0
23	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	5,1	1,4	1,4
24	Urban runoff	Urban runoff + wastewater discharge	5,8	3,4	3,0
25	Urban runoff	Urban runoff + agricultural + wastewater	4,4	2,2	2,2
26	Slaughterhouse	Slaughterhouse	4,9	4,4	3,0
27	Urban runoff	Urban runoff	5,8	4,0	4,0
28	Open space	Open space	5,8	2,4	2,4
29	Industrial sewage	Industrial sewage	5,4	3,7	3,7
30	Farm	Agricultural + farm + human sewage	5,7	3,7	3,4

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Table 11:S. Number of viable culturable and viable but not culturable from San Pedro River per sample type location.

Sample location	Type of sample	Possible source of pollution	*E. coli* VC	*E. coli* VBNC
1	Urban runoff	Urban runoff	2,15	0,00
2	Slaughterhouse	Slaughterhouse	3,66	2,94
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	1,00	0,00
4	Open space	Open space	3,66	3,47
5	Slaughterhouse	Slaughterhouse	1,72	1,49
6	Urban runoff	Human sewage + wastewater discharge	3,32	3,19
7	Urban runoff	Urban runoff	1,46	0,00
8	Farm	Agricultural + farm	1,97	0,00
9	Farm	Agricultural + farm	2,15	3,19
10	Urban runoff	Urban runoff	1,97	1,04
11	Open space	Open space	1,88	1,17
12	Open space	Open Space	1,36	0,00
13	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	3,18	3,11
14	Urban runoff	Urban runoff + agricultural + wastewater	1,36	3,76
15	Slaughterhouse	Agricultural + slaughterhouse	1,00	0,35
16	Agricultural	Urban runoff + agricultural	3,50	1,97
17	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	3,66	3,23
18	Farm	Agricultural + farm	2,00	1,27
19	Farm	Agricultural + farm	2,38	1,38
20	Open space	Open space	2,66	3,81
21	Agricultural	Agricultural	1,04	0,35
22	Open space	Open space	3,04	2,94
23	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	1,30	0,00
24	Urban runoff	Urban runoff + wastewater discharge	2,20	2,18
25	Urban runoff	Urban runoff + agricultural + wastewater	2,18	1,88
26	Slaughterhouse	Slaughterhouse	2,38	2,88
27	Urban runoff	Urban runoff	3,18	2,99
28	Open space	Open space	2,38	1,88
29	Industrial sewage	Industrial sewage	2,90	1,38
30	Farm	Agricultural + farm + human sewage	3,40	2,89

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Table 12:S. Number of pathogenic, antimicrobial resistant and multi-drug resistant bacteria from San Pedro River per sample type location.

No.	Type of sample	Possible source of pollution	Pathogenic *E. coli* (CFU)	ARB *E. coli* (CFU)	MDR *E.coli* (CFU)
1	Urban runoff	Urban runoff	3	1	3
2	Slaughterhouse	Slaughterhouse	3	1	2
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	1	2	1
4	Open space	Open space	4	3	0
5	Slaughterhouse	Slaughterhouse	6	4	2
6	Urban runoff	Human sewage + wastewater discharge	2	1	3
7	Urban runoffoo	Urban runoff	2	2	0
8	Farm	Agricultural + farm	0	0	0
9	Farm	Agricultural + farm	2	2	1
10	Urban runoff	Urban runoff	3	4	0
11	Open space	Open space	1	3	0
12	Open space	Open Space	1	0	2
13	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	1	1	1
14	Urban runoff	Urban runoff + agricultural + wastewater	0	0	0
15	Slaughterhouse	Agricultural + slaughterhouse	0	0	1
16	Agricultural	Urban runoff + agricultural	3	2	1
17	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	4	3	1
18	Farm	Agricultural + farm	2	3	0
19	Farm	Agricultural + farm	1	0	3
20	Open space	Open space	3	0	1
21	Agricultural	Agricultural	1	0	0
22	Open space	Open space	3	0	5
23	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	2	2	0
24	Urban runoff	Urban runoff + wastewater discharge	2	4	0
25	Urban runoff	Urban runoff + agricultural + wastewater	2	1	3
26	Slaughterhouse	Slaughterhouse	1	1	0
27	Urban runoff	Urban runoff	2	2	0
28	Open space	Open space	0	0	3
29	Industrial sewage	Industrial sewage	4	4	0
30	Farm	Agricultural + farm + human sewage	0	0	0

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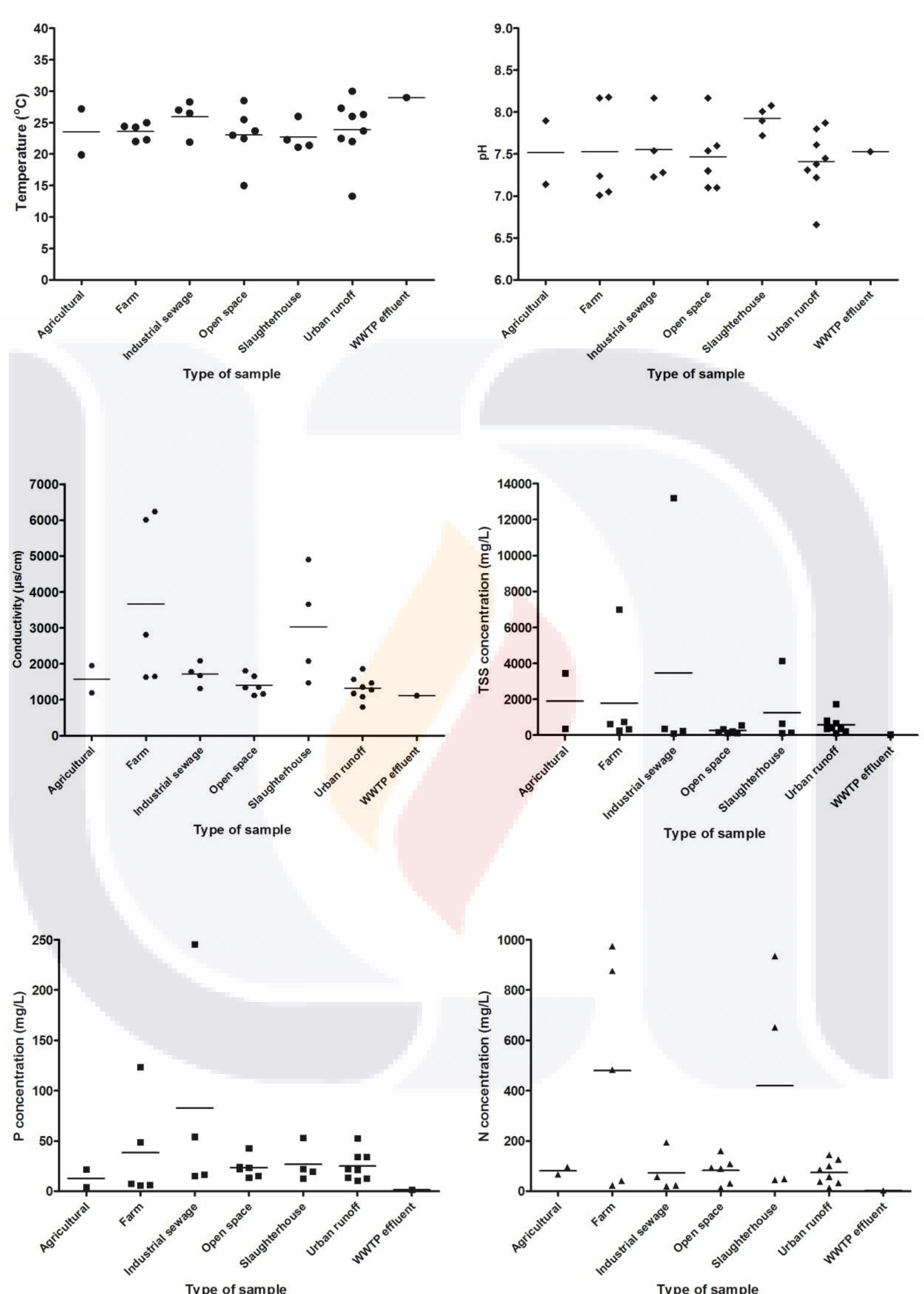

Figure 1:S. Spatial variation of physico-chemical parameters from San Pedro River per sample type location. AR, agriculturally impaired; F, farm; IS, industrial sewage; OS, open spaces; S, slaughterhouse; UR, urban runoff, WWTP-E, wastewater treatment plant effluent.

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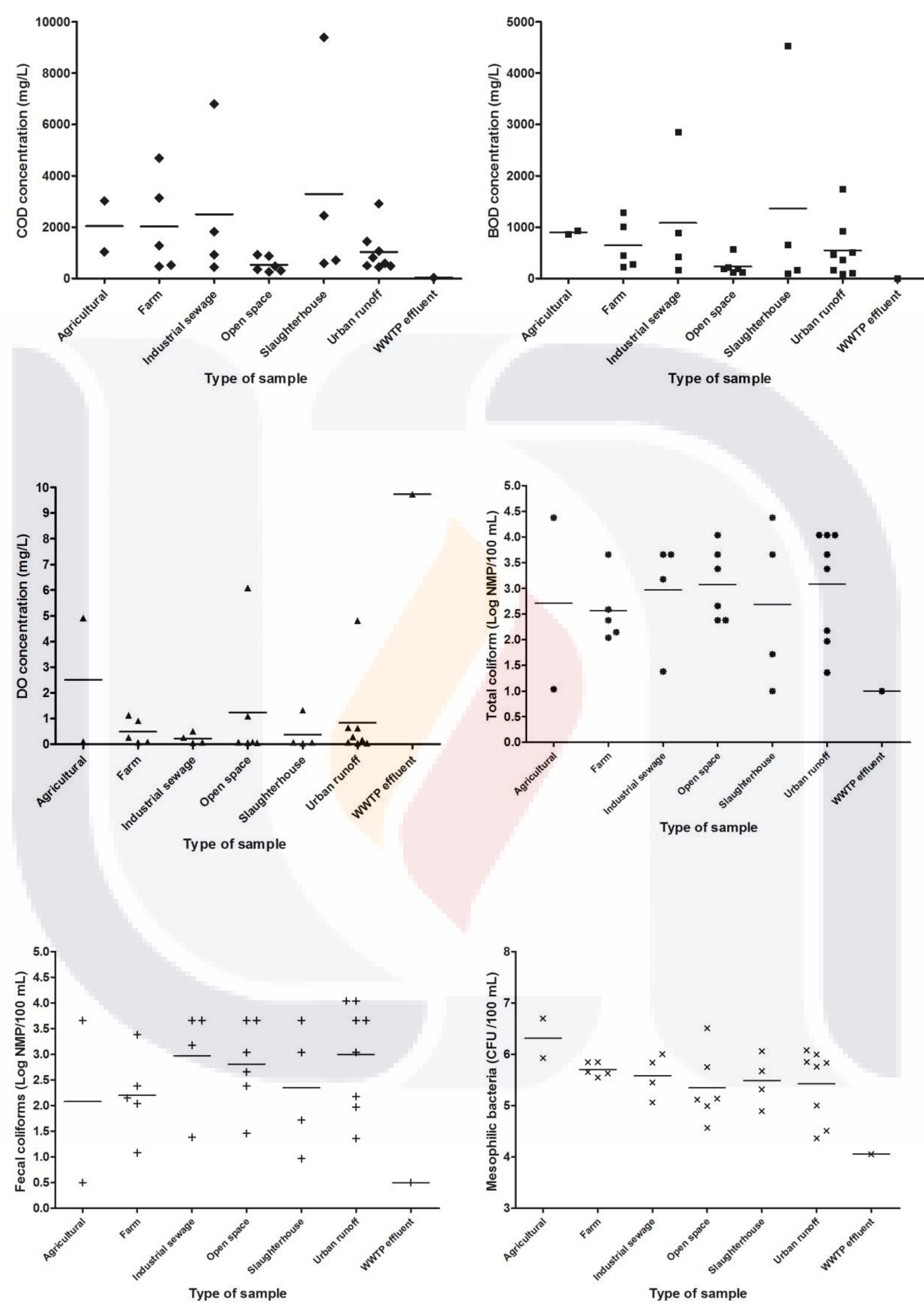

Figure 1:S, continuation. Spatial variation of physico-chemical parameters from San Pedro River per sample type location. AR, agriculturally impaired; F, farm; IS, industrial sewage; OS, open spaces; S, slaughterhouse; UR, urban runoff, WWTP-E, wastewater treatment plant effluent.

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Table 13:S. Antimicrobial resistant and multi-drug resistant patterns of *E. coli* isolates from San Pedro River per sample location.

Strain ID	Antimicrobial agent													Susceptibility				MDR pattern
	Am	**Cb**	**Cl**	**Pef**	**Lev**	**Sxt**	**G**	**Net**	**Ak**	**Cf**	**Ctx**	**Cro**	**Nf**	**S**	**I**	**R**	**MDR**	
1a	S	R	S	S	S	S	S	S	S	I	S	S	S	11	1	1	0	Cb
1b	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
1c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
1d	R	R	R	S	S	S	S	S	S	I	S	S	S	9	1	3	1	AmCbCl
1e	R	R	R	S	S	S	S	S	S	I	S	S	S	9	1	3	1	AmCbCl
2a	R	R	S	I	S	R	S	S	S	R	I	I	S	6	3	4	1	AmCbSxtCf
2b	R	R	S	I	S	R	S	S	S	R	S	S	S	8	1	4	1	AmCbSxtCf
2c	S	I	S	S	S	S	S	S	S	S	I	S	S	11	2	0	0	-
2d	S	S	S	I	S	S	S	S	S	S	S	S	S	12	1	0	0	-
2e	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
3a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
3b	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
3c	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
3d	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
3e	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
4a	I	S	I	R	S	S	S	S	S	R	S	S	R	8	2	3	1	PefCfNf
4b	S	S	S	S	S	S	S	S	S	R	S	S	I	12	1	1	0	Cf
4c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
4d	S	S	S	S	S	S	I	S	S	R	S	S	I	10	2	1	0	Cf
4e	I	S	S	S	S	R	S	S	S	R	S	S	R	9	1	3	1	CfNfSxt
5a	I	S	S	S	S	S	S	S	S	I	S	S	S	11	2	0	0	-
5b	R	R	R	S	S	R	S	S	S	I	S	S	S	8	1	4	1	AmCbClSxt
5c	S	S	R	S	S	S	S	S	S	I	S	S	S	12	1	1	0	Cl
5d	R	R	R	S	S	R	S	S	S	I	S	S	S	8	1	4	1	AmCbClSxt
5e	R	R	S	S	S	S	S	S	S	R	S	S	S	10	0	3	1	AmCbCf
6a	R	S	R	S	S	S	S	S	S	I	S	S	S	10	1	2	0	AmCl
6b	R	S	R	S	S	S	S	S	S	R	S	S	S	10	0	3	1	AmClCf
6c	R	S	R	S	S	S	S	S	S	S	S	S	S	11	0	2	0	AmCl
6d	R	S	R	S	S	S	S	S	S	I	S	S	S	10	1	2	0	AmCl
6e	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-

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Table 13:S, continuation. Antimicrobial resistant and multi-drug resistant patterns of *E. coli* isolates from San Pedro River per sample type location.

Strain ID	Antimicrobial agent													Susceptibility				MDR pattern
	Am	Cb	Cl	Pef	Lev	Sxt	G	Net	Ak	Cf	Ctx	Cro	Nf	S	I	R	MDR	
7a	R	R	S	I	S	R	S	S	S	R	I	I	S	6	3	4	1	AmCbSxtCf
7b	R	R	S	I	S	R	S	S	S	R	I	I	S	2	2	4	1	AmCbSxtCf
7c	R	R	R	I	S	R	S	S	R	R	I	I	S	4	3	6	1	AmCbClSxtAkCf
7d	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
7e	S	S	S	I	S	S	S	S	S	S	S	S	S	12	1	0	0	-
8a	R	R	R	S	S	R	S	S	S	R	S	S	R	7	0	6	1	AmCbClSxtCfNf
8b	R	R	R	S	S	R	S	S	S	R	S	S	I	7	1	5	1	AmCbClSxtCf
8c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
8d	R	R	R	S	S	R	S	S	S	R	S	S	I	7	1	5	1	AmCbClSxtCf
8e	R	R	R	S	S	R	S	S	S	R	S	S	R	8	0	5	1	AmCbClSxtCfNf
9a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
9b	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
9c	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
9d	S	I	S	S	S	R	S	S	S	S	S	S	S	11	1	1	0	Sxt
9e	S	S	S	S	S	R	S	S	S	S	S	S	S	12	0	1	0	Sxt
10a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
10b	S	S	S	I	S	S	S	S	S	S	S	S	S	12	1	0	0	-
10c	S	S	S	I	S	S	S	S	S	I	S	S	R	10	2	1	0	Nf
10d	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
10e	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
11a	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
11b	S	S	S	S	S	S	S	S	S	I	S	I	S	11	2	0	0	-
11c	R	R	R	R	R	R	S	S	S	I	S	S	S	6	1	6	1	AmCbClPefLevSxt
11d	R	R	R	R	S	R	S	S	S	S	S	I	S	7	1	5	1	AmCbClPefSxt
11e	S	S	S	I	S	S	S	S	S	I	S	S	S	11	2	0	0	-
11f	S	S	S	S	S	S	S	R	S	S	I	S	S	11	1	1	0	Net
12a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
12b	S	I	S	S	S	S	S	S	S	S	S	S	S	12	1	0	0	-
12c	S	I	S	S	S	S	S	S	S	S	S	S	S	12	1	0	0	-
12d	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
12e	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-

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Table 13:S, continuation. Antimicrobial resistant and multi-drug resistant patterns of *E. coli* isolates from San Pedro River per sample location.

Strain ID	Antimicrobial agent													Susceptibility				MDR pattern
	Am	**Cb**	**Cl**	**Pef**	**Lev**	**Sxt**	**G**	**Net**	**Ak**	**Cf**	**Ctx**	**Cro**	**Nf**	**S**	**I**	**R**	**MDR**	
13a	R	S	S	S	R	R	R	S	S	I	S	S	I	7	2	4	1	AmLevSxtG
13b	R	S	S	S	S	S	S	S	S	S	S	S	S	12	0	1	0	Am
13c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
13d	R	R	S	S	R	I	R	I	S	S	S	S	S	7	2	4	1	AmCbLevG
13e	R	R	S	S	R	I	S	S	S	S	S	S	S	9	1	3	1	AmCbLev
14a	R	S	S	R	R	R	S	S	S	R	R	R	I	5	1	7	1	AmPefLevSxtCfCtxCro
14b	R	R	S	S	S	R	S	S	S	R	S	S	S	9	0	4	1	AmCbCfSxt
14c	R	S	S	R	R	R	S	S	S	R	R	R	I	5	1	7	1	AmPefLevSxtCfCtxCro
14d	R	S	S	S	S	R	S	S	S	S	S	S	I	10	1	2	0	AmSxt
14e	S	S	S	S	S	S	S	S	S	S	S	S	I	12	1	0	0	-
15a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
15b	S	S	S	S	S	S	S	S	S	R	S	S	S	12	0	1	0	Cf
15c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
15d	S	S	S	I	S	S	S	S	S	S	S	S	S	12	1	0	0	-
15e	R	R	S	S	S	S	S	S	S	S	S	S	R	10	0	3	1	AmCbNf
16a	S	S	S	S	S	I	S	S	S	S	S	S	I	11	2	0	0	-
16b	I	S	S	S	S	S	S	R	S	S	S	S	S	11	1	1	0	Net
16c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
16d	S	S	S	S	S	S	S	S	S	R	S	S	S	12	0	1	0	Cf
16e	S	S	S	S	S	S	S	S	S	S	S	S	R	12	0	1	0	Nf
17a	R	S	R	R	S	R	S	S	S	S	S	S	S	9	0	4	1	AmClSxtPef
17b	R	S	R	S	S	R	S	S	R	R	S	S	S	8	0	5	1	AmClSxtAkCf
17c	S	S	S	I	S	S	S	S	S	I	S	S	S	11	2	0	0	-
17d	R	S	S	S	S	S	S	S	S	S	S	S	S	12	0	1	0	Am
17e	S	S	S	I	S	S	S	S	S	I	S	S	S	11	2	0	0	-
18a	R	S	R	R	S	R	S	S	S	S	S	S	S	9	0	4	1	AmClSxtPef
18b	R	S	S	S	S	S	S	S	S	I	S	S	S	11	1	1	0	Am
18c	I	S	S	S	S	S	S	S	S	I	S	S	S	11	2	0	0	-
18d	R	S	S	S	S	S	S	S	S	S	S	S	S	12	0	1	0	Am
18e	R	S	R	S	S	R	S	S	S	S	S	S	I	9	1	3	1	AmClSxt
19a	S	S	R	S	S	R	S	S	S	S	S	S	S	11	0	2	0	ClSxt
19b	S	S	S	S	S	S	S	S	S	R	S	S	S	12	0	1	0	Cf
19c	R	R	R	S	S	R	S	S	S	I	S	S	S	8	1	4	1	AmCbClSxt

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Table 13:S, continuation. Antimicrobial resistant and multi-drug resistant patterns of *E. coli* isolates from San Pedro River per sample location.

Strain ID	Antimicrobial agent													Susceptibility				MDR pattern
	Am	Cb	Cl	Pef	Lev	Sxt	G	Net	Ak	Cf	Ctx	Cro	Nf	S	I	R	MDR	
19e	R	R	R	R	S	R	R	S	S	I	S	S	R	5	1	7	1	AmCbClPefSxtGNf
20a	R	S	S	S	S	R	S	S	S	I	S	S	S	10	1	2	0	AmSxt
20b	R	S	S	S	S	I	S	S	S	I	S	S	S	10	2	1	0	Am
20c	R	S	S	S	S	R	S	S	S	S	S	S	S	11	0	2	0	AmSxt
20d	R	S	S	S	S	R	S	S	S	I	S	S	S	10	1	2	0	AmSxt
20e	S	S	I	R	S	S	S	S	S	S	I	S	S	10	2	1	0	Pef
21a	R	R	S	I	S	S	S	S	S	S	S	S	S	10	1	2	0	AmCb
21b	S	I	S	S	S	S	S	S	S	I	S	S	I	10	3	0	0	-
21c	R	R	S	S	S	S	S	S	S	S	S	S	I	10	1	2	0	AmCb
21d	R	S	S	S	S	S	S	S	S	S	S	S	S	12	0	1	0	Am
21e	R	R	S	I	S	S	S	S	S	R	S	S	I	3	1	1	1	AmCbCf
22a	S	S	I	S	S	S	S	S	S	S	S	S	S	12	1	0	0	-
22b	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
22c	S	S	I	S	S	S	S	S	S	I	S	S	S	11	2	0	0	-
22d	I	R	R	R	S	R	S	S	S	I	I	I	S	5	4	4	1	CbClPefSxt
22e	S	S	I	S	S	S	S	S	S	I	S	S	S	11	2	0	0	-
23a	R	R	S	I	S	R	S	S	S	S	S	S	R	8	1	4	1	AmCbSxtNf
23b	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
23c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
23d	S	S	S	S	S	S	S	S	S	S	S	I	S	12	1	0	0	-
23e	R	R	S	I	S	R	S	S	S	I	S	S	S	8	2	3	1	AmCbSxt
24a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
24b	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
24c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
24d	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
24e	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
25a	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
25b	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
25c	S	S	S	S	S	S	S	S	S	I	S	S	S	12	1	0	0	-
25d	S	S	S	S	S	S	S	S	S	I	S	I	S	11	2	0	0	-

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Table 13:S, continuation. Antimicrobial resistant and multi-drug resistant patterns of *E. coli* isolates from San Pedro River per sample location.

Strain ID	Antimicrobial agent													Susceptibility				MDR pattern
	Am	Cb	Cl	Pef	Lev	Sxt	G	Net	Ak	Cf	Ctx	Cro	Nf	S	I	R	MDR	
25e	S	S	S	S	S	S	S	S	S	I	I	S	S	11	2	0	0	-
26a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
26b	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
26c	S	S	I	I	S	I	S	S	S	S	S	S	R	9	3	1	0	Cl
26d	S	S	R	I	S	S	S	S	S	S	S	S	S	11	1	1	0	Cl
26e	S	S	R	I	S	I	S	S	S	I	S	S	S	9	3	1	0	Cl
27a	R	R	S	S	S	R	S	S	S	S	S	S	S	10	0	3	1	AmCbSxt
27b	R	R	S	S	S	R	S	S	S	I	S	S	S	9	1	3	1	AmCbSxt
27c	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
27d	R	R	S	S	S	R	S	S	S	S	S	S	S	10	0	3	1	AmCbSxt
27e	R	R	S	S	S	R	S	S	S	S	S	S	S	10	0	3	1	AmCbSxt
28a	R	R	R	R	S	R	S	S	S	R	I	S	S	6	1	6	1	AmCbClPefSxtCf
28b	R	R	R	S	S	R	S	S	S	I	S	S	S	8	1	4	1	AmCbClSxt
28c	R	R	R	R	S	R	S	S	S	I	S	S	S	7	1	5	1	AmCbClPefSxt
28d	S	S	S	I	S	S	S	S	S	S	S	S	S	12	1	0	0	-
28e	R	R	R	R	S	R	S	S	S	I	S	S	S	7	1	5	1	AmCbClPefSxt
29a	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
29b	R	R	R	S	S	R	S	S	S	R	S	S	S	8	0	5	1	AmCbClSxtCf
29c	S	I	S	I	S	S	S	S	S	S	S	S	S	11	2	0	0	-
29d	S	I	S	S	S	S	S	S	S	I	S	S	S	11	2	0	0	-
29e	R	R	R	S	S	R	S	S	S	S	S	R	S	8	0	5	1	AmCbClSxtCro
30a	R	S	S	I	S	S	S	S	S	I	S	S	S	11	1	1	0	Am
30b	S	S	R	S	S	S	S	S	S	S	S	S	S	12	0	1	0	Cl
30c	S	S	S	S	S	I	I	S	S	S	S	S	S	11	2	0	0	-
30d	S	S	S	S	S	S	S	S	S	S	S	S	S	13	0	0	0	-
30e	S	S	S	S	S	I	I	S	S	S	S	S	S	11	2	0	0	-

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Table 14:S. Association between antimicrobial resistance phenotype among EPEC strains from stream water.

Antibiotic		Association for the following antimicrobial susceptibility phenotype and virulence genes for EPEC.												
		Aminoglycoside		Phenicols	Quinolones		Sulfonamides	Beta-lactams			Cephalosporins			Nitrofunas
		Gentamicin	Netilmicin	Chloramphenicol	Pefloxacin	Levofloxacin	Solfamethoxazole-trimethorpirm	Ampicillin	Carbenicillin	Amikacin	Cephalotin	Cephatoxime	Ceftriaxone	Nitrofuration
Aminoglycosides	Gentamicin	-												
	Netilmicin	-	-											
Phenicols	Chloramphenicol	-	-	-										
Quinolones	Pefloxacin	-	-	-	-									
	Levofloxacin	-	-	-	-	-								
Sulfonamides	Solfamethoxazole-trimethorpirm	-	-	-	++	++	-							
Beta-lactams	Ampicillin	-	-	-	-	-	-	-						
	Carbenicillin	-	-	-	-	-	-	-	-					
	Amikacin	-	-	-	-	-	-	-	-	-				
Cephalosporins	Cephalotin	-	-	-	-	-	++	-	-	-	-			
	Cephatoxime	-	-	-	-	-	++	-	-	-	-	-		
	Ceftriaxone	-	-	-	-	-	++	-	-	-	-	-	-	
Nitrofunas	Nitrofuration	-	-	-	-	-	-	++	-	-	-	-	-	-

Only antimicrobial multi-resistance phenotype that exhibited an association with another gene at the $P < 0.05$ level are shown. The levels of significance of the association (as assessed by the chi-square exact test) were as follows: -, $P > 0.05$; +, $0.05 \geq P \geq 0.01$; ++, $0.01 \geq P \geq 0.001$; +++, $0.001 \geq P$.

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Table 15:S. Association between antimicrobial resistance phenotype among ETEC strains from stream water.

Association for the following antimicrobial susceptibility phenotype and virulence genes for ETEC.														
Antibiotic		Aminoglycoside		Phenicols	Quinolones		Sulfonamides	Beta-lactams			Cephalosporins			Nitrofunas
		Gentamicin	Netilmicin	Chloramphenicol	Pefloxacin	Levofloxacin	Solfamethoxazole-trimethorpirm	Ampicillin	Carbenicillin	Amikacin	Cephalotin	Cephatoxime	Ceftriaxone	Nitrofuration
Aminoglycosides	Gentamicin	-												
	Netilmicin	-	-											
Phenicols	Chloramphenicol	-	-	-										
Quinolones	Pefloxacin	-	-	+	-									
	Levofloxacin	-	-	+	-	-								
Sulfonamides	Solfamethoxazole-trimethorpirm	-	-	-	+	+	-							
Beta-lactams	Ampicillin	-	-	+	+	+	+	-						
	Carbenicillin	-	-	+	+	+	+	++	-					
	Amikacin	-	-	-	-	-	-	-	-	-				
Cephalosporins	Cephalotin	-	-	-	-	-	-	-	-	-	-			
	Cephatoxime	-	-	-	-	-	-	-	-	-	-	-		
	Ceftriaxone	-	-	-	-	-	-	-	-	-	-	-	-	
Nitrofunas	Nitrofuration	-	-	-	-	-	-	-	-	-	-	-	-	-

Only antimicrobial multi-resistance phenotype that exhibited an association with another gene at the P <0.05 level are shown. The levels of significance of the association (as assessed by the chi-square exact test) were as follows: -, $P > 0.05$; +, $0.05 \geq P \geq 0.01$; ++, $0.01 \geq P \geq 0.001$; +++, $0.001 \geq P$.

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Table 16:S. Association between antimicrobial resistance phenotype among EAEC strains from stream water.

Association for the following antimicrobial susceptibility phenotype and virulence genes for EAEC.														
Antibiotic		Aminoglycoside		Phenicols	Quinolones		Sulfonamides	Beta-lactams			Cephalosporins			Nitrofunas
		Gentamicin	Netilmicin	Chloramphenicol	Pefloxacin	Levofloxacin	Solfamethoxazole-trimethorpirm	Ampicillin	Carbenicillin	Amikacin	Cephalotin	Cephatoxime	Ceftriaxone	Nitrofuration
Aminoglycosides	Gentamicin	-												
	Netilmicin	+	-											
Phenicols	Chloramphenicol	-	-	-										
Quinolones	Pefloxacin	-	+	-	-									
	Levofloxacin	-	+	-	-	-								
Sulfonamides	Solfamethoxazole-trimethorpirm	-	-	-	+	+	-							
Beta-lactams	Ampicillin	-	-	+	-	-	-	-						
	Carbenicillin	-	-	++	-	-	-	-	-					
	Amikacin	-	-	-	-	-	-	-	-	-				
Cephalosporins	Cephalotin	-	-	-	-	-	-	-	-	-	-			
	Cephatoxime	-	+	-	-	-	-	-	-	-	+	-		
	Ceftriaxone	-	+	-	-	-	-	-	-	-	-	-	-	
Nitrofunas	Nitrofuration	+	+	-	-	-	-	-	-	-	-	-	+	-

Only antimicrobial multi-resistance phenotype that exhibited an association with another gene at the P <0.05 level are shown. The levels of significance of the association (as assessed by the chi-square exact test) were as follows: -, $P > 0.05$; +, $0.05 \geq P \geq 0.01$; ++, $0.01 \geq P \geq 0.001$; +++, $0.001 \geq P$.

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Table 17:S. Association between antimicrobial resistance phenotype among incomplete ExPEC strains from stream water.

Association for the following antimicrobial susceptibility phenotype and virulence genes for incomplete ExPEC.														
Antibiotic		Aminoglycoside		Phenicols	Quinolones		Sulfonamides	Beta-lactams			Cephalosporins			Nitrofunas
		Gentamicin	Netilmicin	Chloramphenicol	Pefloxacin	Levofloxacin	Solfamethoxazole-trimethorpirm	Ampicillin	Carbenicillin	Amikacin	Cephalotin	Cephatoxime	Ceftriaxone	Nitrofuration
Aminoglycosides	Gentamicin	-												
	Netilmicin	-	-											
Phenicols	Chloramphenicol	-	-	-										
Quinolones	Pefloxacin	-	-	-	-									
	Levofloxacin	-	-	-	-	-								
Sulfonamides	Solfamethoxazole-trimethorpirm	-	-	-	-	-	-							
Beta-lactams	Ampicillin	-	-	+	-	+++	++	-						
	Carbenicillin	-	-	-	-	-	-	+++	-					
	Amikacin	-	-	-	-	-	-	-	-	-				
Cephalosporins	Cephalotin	-	-	-	-	-	-	-	-	-	-			
	Cephatoxime	-	-	-	-	-	-	-	-	-	-	-		
	Ceftriaxone	-	-	-	-	-	-	-	-	-	-	-	-	
Nitrofunas	Nitrofuration	-	-	-	-	-	-	-	-	-	-	-	-	-

Only antimicrobial multi-resistance phenotype that exhibited an association with another gene at the $P < 0.05$ level are shown. The levels of significance of the association (as assessed by the chi-square exact test) were as follows: -, $P > 0.05$; +, $0.05 \geq P \geq 0.01$; ++, $0.01 \geq P \geq 0.001$; +++, $0.001 \geq P$.

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Table 18:S. Phylogenetic groups per *E. coli* isolate.

Sample	Target gene				Phylogroup
	arpA	***chuA***	***yjaA***	**TspE4.C2**	
1a	1	0	0	1	B1
1b	0	0	0	1	B1
1c	1	0	0	1	B1
1d	1	0	0	0	C
1e	1	0	0	0	C
2a	0	1	0	1	B2
2b	0	0	1	1	B1
2c	1	0	0	0	A
2d	0	1	1	1	B2
2e	0	0	0	1	U
3a	1	0	1	0	C
3b	1	0	1	0	C
3c	0	0	0	1	U
3d	0	0	0	1	U
3e	0	0	0	1	U
4a	0	1	0	0	F
4b	0	0	0	0	U
4c	0	0	0	0	U
4d	1	0	0	0	A
4e	0	0	0	0	U
5a	1	0	0	0	A
5b	1	0	0	0	A
5c	1	0	1	0	C
5d	1	0	0	0	A
5e	1	0	0	0	A
6a	1	0	0	0	A
6b	1	0	0	0	A
6c	1	0	0	0	A
6d	1	0	1	0	C
6e	1	1	1	0	D
7a	1	1	1	1	A
7b	0	1	1	1	B2
7c	1	1	1	1	U
7d	1	0	0	0	A
7e	1	0	0	0	A
8a	1	1	1	1	U
8b	0	0	1	1	U

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Table 18:S, continuation. Phylogenetic groups per *E. coli* isolate.

Sample	Target gene				Phylogroup
	arpA	***chuA***	***yjaA***	**TspE4.C2**	
8c	1	0	1	0	C
8d	0	0	0	1	U
8e	0	1	0	0	F
9a	1	0	0	0	A
9b	1	0	0	0	A
9c	1	0	0	0	A
9d	1	0	1	0	C
10a	1	1	1	0	D
10b	1	1	0	0	D
10c	1	1	0	0	D
10d	1	0	0	1	B1
10e	1	0	0	1	B1
11a	1	0	0	0	A
11b	1	0	0	0	A
11c	1	0	0	0	A
11d	1	0	1	0	C
11e	1	0	0	0	A
11f	1	0	0	1	B1
12a	1	0	0	0	A
12b	0	0	0	0	U
12c	1	0	1	0	C
12d	1	0	0	0	A
12e	1	0	0	0	A
13a	1	0	0	0	A
13b	1	0	0	0	A
13c	1	0	0	0	A
13d	1	0	1	0	C
13e	1	1	0	0	D
14a	1	0	1	0	C
14b	1	0	1	0	C
14c	1	0	1	0	C
14d	1	0	0	1	B1
14e	1	1	0	0	D
15a	1	0	0	0	A
15b	1	0	0	0	A
15c	1	0	0	0	A
15d	1	0	0	0	A

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Table 18:S, continuation. Phylogenetic groups per *E. coli* isolate.

Sample	Target gene				Phylogroup
	arpA	*chuA*	*yjaA*	TspE4.C2	
16a	0	1	0	0	F
16b	1	0	1	0	C
16c	1	0	1	0	C
16d	0	0	0	0	U
16e	0	0	0	0	U
17a	1	0	1	0	C
17b	1	0	0	0	B1
17c	1	0	0	0	A
17d	1	0	0	0	A
17e	1	1	0	0	D
18a	1	0	0	1	B1
18b	0	0	1	0	Clado I o II
18c	1	0	0	1	B1
18d	1	1	1	0	D
18e	1	0	0	0	A
19a	1	0	1	0	C
19b	1	0	1	0	C
19c	1	0	1	0	C
19d	1	0	0	0	A
19e	1	0	1	0	C
20a	1	0	0	0	A
20b	1	0	0	0	A
20c	1	0	1	0	C
20d	1	0	1	0	A
20e	1	0	0	0	A
21a	1	0	0	0	A
21b	1	0	0	0	A
21c	1	0	0	1	B1
21d	1	0	0	1	B1
21e	1	0	1	0	A
22a	1	0	0	0	A
22b	1	0	1	0	C
22c	1	0	0	0	A
22d	1	0	0	0	A
22e	1	0	0	0	A
23a	1	1	0	0	D
23b	1	0	0	0	A
23c	0	0	0	1	U

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Table 18:S, continuation. Phylogenetic groups per *E. coli* isolate.

Sample	Target gene				Phylogroup
	arpA	***chuA***	***yjaA***	**TspE4.C2**	
23d	1	0	1	0	C
23e	1	0	0	0	A
24a	1	0	0	1	B1
24b	1	0	0	1	B1
24c	1	0	0	1	B1
24d	1	0	0	1	B1
24e	1	0	0	1	B1
25a	1	0	0	0	A
25b	1	0	0	0	A
25c	1	0	0	1	B1
25d	1	0	0	1	B1
25e	1	0	0	1	B1
27a	0	0	1	1	U
27b	1	0	0	0	A
27c	1	0	0	0	A
27d	1	1	0	0	D
27e	1	1	0	0	D
28a	1	0	0	0	A
28b	1	0	1	0	C
28c	1	0	1	0	C
28d	1	0	0	0	A
28e	1	1	1	0	E
29a	1	0	0	0	A
29b	1	0	0	0	A
29c	1	0	0	0	A
29d	1	0	1	0	C
29e	1	0	0	0	A
30a	1	0	0	0	A
30b	1	0	1	0	C
30c	1	1	0	0	D
30d	1	0	1	0	C
30e	1	0	0	0	A

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Table 19:S. Virulence factors and pathotype per isolate strain.

Strain ID	Target gene													Pathotype
	eae	***bfpA***	***est***	***elt***	***ipaH***	***stx1/stx2***	***eastI***	***aafA***	***aggR***	***fyuA***	***kpsMTII***	***papC***	***sfaS***	
1a	0	1	0	0	0	0	0	0	0	0	0	0	0	COMM
1b	0	0	0	0	0	0	1	0	1	1	0	0	0	EAEC
1c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
1d	1	0	0	0	0	0	1	0	0	1	0	0	0	EPEC
1e	1	1	0	0	0	0	1	0	0	0	0	0	0	EPEC
2a	0	0	1	0	0	0	1	0	1	0	0	0	0	EAEC
2b	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
2c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
2d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
2e	0	0	1	0	0	0	0	0	0	0	0	0	0	ETEC
3a	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
3b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
3c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
3d	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
3e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
4a	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
4b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
4c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
4d	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
4e	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
5a	1	0	0	0	0	0	0	0	0	0	0	1	0	EPEC
5b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
5c	0	0	0	0	0	0	1	0	1	0	0	0	1	EAEC INC_ExPEC
5d	0	1	0	0	0	0	1	0	1	0	0	1	0	EAEC
5e	1	0	0	0	0	0	0	0	1	0	0	0	0	EPEC
6b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
6a	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
6c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
6d	1	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
6e	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
7a	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
7b	0	1	0	0	0	0	0	0	0	0	0	0	0	COMM
7c	0	1	0	0	0	0	0	0	0	0	0	0	0	COMM
7d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
7e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM

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Table 19:S, continuation. Virulence factors and pathotype per isolate strain.

Strain ID	Target gene													Pathotype
	eae	*bfpA*	*est*	*elt*	*ipaH*	*stx1/stx2*	*eastI*	*aafA*	*aggR*	*fyuA*	*kpsMTII*	*papC*	*sfaS*	
8d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
8e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
8a	0	0	0	0	0	0	0	0	1	0	0	0	0	EAEC
8b	0	0	0	0	0	0	0	1	1	0	0	0	0	EAEC
8c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
9a	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
9b	0	0	1	0	0	0	0	0	0	0	0	0	0	ETEC
9c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
9d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
9e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
10a	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
10b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
10c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
10d	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
10e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
11a	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
11b	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
11c	0	1	1	0	0	0	1	0	0	0	0	0	0	ETEC
11d	0	1	0	0	0	0	0	0	0	0	0	0	0	COMM
11e	0	0	1	1	0	0	1	0	1	1	0	0	0	EAEC ETEC
11f	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
12a	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
12b	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
12c	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
12d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
12e	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
13a	0	1	0	0	0	0	1	0	1	0	0	0	0	EAEC
13b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
13c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
13d	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
13e	0	0	0	0	0	0	0	0	0	0	1	0	0	INC_ExPEC
14a	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
14c	1	0	0	0	0	0	1	0	0	0	0	0	0	EPEC
14d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
14e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM

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Table 19:S, continuation. Virulence factors and pathotype per isolate strain.

Strain ID	Target gene													Pathotype
	eae	*bfpA*	*est*	*elt*	*ipaH*	*stx1/stx2*	*eastI*	*aafA*	*aggR*	*fyuA*	*kpsMTII*	*papC*	*sfaS*	
15a	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
15b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
15c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
15d	0	0	0	0	0	0	1	0	0	1	0	0	0	INC_ExPEC
15e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
16a	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
16b	0	0	0	0	0	0	0	0	0	0	0	1	0	INC_ExPEC
16c	1	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
16d	0	0	0	0	0	0	0	0	0	0	0	0	1	COMM
16e	0	0	0	0	0	0	1	0	0	0	0	0	1	INC_ExPEC
17a	0	0	1	0	0	0	1	0	1	1	1	0	0	ETEC
17b	0	1	0	0	0	0	0	0	0	0	0	0	0	COMM
17c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
17d	0	0	0	0	0	0	0	1	1	0	0	0	0	EAEC
17e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
18a	0	0	0	1	0	0	1	0	0	1	0	0	0	EAEC
18b	0	0	0	0	0	0	1	0	1	0	0	1	1	EAEC
18c	0	0	0	0	0	0	0	0	0	1	0	0	1	EPEC
18d	0	0	0	0	0	0	0	0	0	0	0	0	1	COMM
18e	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
19a	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
19b	1	1	0	0	0	0	1	0	0	0	0	0	0	EPEC
19c	0	0	1	0	0	0	1	0	0	0	0	0	0	ETEC
19d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
19e	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
20a	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
20b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
20c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
20d	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
20e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
21a	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
21b	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
21c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
21d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
21e	0	1	1	0	0	0	1	0	0	0	0	0	0	ETEC

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Table 19:S, continuation. Virulence factors and pathotype per isolate strain.

Strain ID	Target gene													Pathotype
	eae	***bfpA***	***est***	***elt***	***ipaH***	***stx1/stx2***	***eastI***	***aafA***	***aggR***	***fyuA***	***kpsMTII***	***papC***	***sfaS***	
22a	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
22b	1	1	0	0	0	0	0	0	0	0	0	0	0	EPEC
22c	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
22d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
22e	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
23a	1	1	1	0	0	0	1	0	1	0	0	0	0	EPEC
23b	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
23c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
23d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
23e	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
24a	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
24b	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
24c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
24d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
24e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
25a	1	1	0	0	0	0	1	0	0	0	0	0	0	EPEC
25b	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
25c	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
25d	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
25e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
26a	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
26b	0	0	0	0	0	0	0	1	1	0	0	0	0	EAEC
26c	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
26d	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
26e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
27a	1	1	1	0	0	0	1	0	0	0	0	0	0	ETEC
27b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
27c	0	1	0	0	0	0	1	0	0	0	0	0	0	EAEC
27d	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
27e	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
28a	0	0	0	0	0	0	1	0	1	0	0	0	1	EAEC/ INC_ExPEC
28b	0	0	0	0	0	0	1	0	1	0	0	0	0	EAEC
28c	0	1	0	0	0	0	0	0	0	0	0	0	0	COMM
28d	0	0	0	0	0	0	0	1	0	0	0	0	0	EAEC
28e	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM

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Table 19:S, continuation. Virulence factors and pathotype per isolate strain.

Strain ID	Target gene													Pathotype
	eae	***bfpA***	***est***	***elt***	***ipaH***	***stx1/stx2***	***eastI***	***aafA***	***aggR***	***fyuA***	***kpsMTII***	***papC***	***sfaS***	
29a	0	0	0	0	0	0	0	0	0	0	0	0	0	COMM
29b	0	0	0	0	0	0	0	0	1	1	0	0	0	EAEC
29c	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
29d	0	0	0	0	0	0	1	0	0	0	0	0	0	EAEC
29e	0	0	0	0	0	0	1	1	0	0	0	0	0	EAEC
30a	0	0	0	0	0	0	1	0	0	0	0	0	0	EAEC
30b	0	0	0	0	0	0	1	0	0	0	0	0	0	EAEC
30c	0	0	1	0	0	0	1	0	0	0	0	0	0	ETEC
30d	0	0	0	0	0	0	0	0	1	0	0	0	0	EAEC
30e	0	0	0	1	0	0	1	0	0	0	0	0	0	ETEC

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Table 20:S. Biofilm formation in stream water per sample location.

Sample location	Type of sample	Possible source of pollution	*E. coli* biofilm at stream water
1	Urban runoff	Urban runoff	Presence
2	Slaughterhouse	Slaughterhouse	Ausence
3	Effluent of wastewater treatment plant	Effluent from wastewater treatment plant	Presence
4	Open space	Open space	Presence
5	Slaughterhouse	Slaughterhouse	Ausence
6	Urban runoff	Human sewage + wastewater discharge	Presence
7	Urban runoff	Urban runoff	Ausence
8	Farm	Agricultural + farm	Presence
9	Farm	Agricultural + farm	Presence
10	Urban runoff	Urban runoff	Presence
11	Open space	Open space	Presence
12	Open space	Open Space	Presence
13	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage	Ausence
14	Urban runoff	Urban runoff + agricultural + wastewater	Presence
15	Slaughterhouse	Agricultural + slaughterhouse	Ausence
16	Agricultural	Urban runoff + agricultural	Presence
17	Industrial sewage	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	Ausence
18	Farm	Agricultural + farm	Ausence
19	Farm	Agricultural + farm	Presence
20	Open space	Open space	Ausence
21	Agricultural	Agricultural	Ausence
22	Open space	Open space	Ausence
23	Industrial sewage	Urban runoff + wastewater discharge + industrial sewage	Ausence
24	Urban runoff	Urban runoff + wastewater discharge	Presence
25	Urban runoff	Urban runoff + agricultural + wastewater	Presence
26	Slaughterhouse	Slaughterhouse	Presence
27	Urban runoff	Urban runoff	Ausence
28	Open space	Open space	Ausence
29	Industrial sewage	Industrial sewage	Ausence
30	Farm	Agricultural + farm + human sewage	Presence

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Table 21:S. Biofilm formation by microtiter assay in microplate in M9 medium plus 0.4% glucose per isolate.

Strain ID	OD 595nm									MD	SD
5d_EAEC	2,173	1,754	3,208	2,697	2,141	1,697	2,396	3,558	2,284	2,43	0,624
28a EAEC	1,468	1,398	1,663	1,42	0,908	0,682	1,021	2,319	1,838	1,41	0,501
14a EAEC	0,747	1,108	0,938	0,296	0,876	0,536	0,837	0,886	0,856	0,79	0,239
28c EAEC	2,32	1,772	1,99	1,725	1,713	1,535	1,334	1,85	1,82	1,78	0,276
13d EAEC	0,822	0,352	0,407	0,254	0,209	0,231	0,798	1,065	3,381	0,84	1,003
23a EAEC	1,09	0,526	0,487	0,637	0,682	0,501	0,808	0,961	2,671	0,93	0,686
23e EAEC	1,624	0,919	1,05	0,925	0,732	0,784	1,927	1,924	0,787	1,19	0,496
21a EAEC	0,365	0,33	0,313	0,288	1,777	2,177	1,722	1,055	0,947	1,00	0,737
18e EAEC	1,393	0,818	1,511	1,603	1,423	1,475	1,289	1,23	1,655	1,38	0,250
13a_ETEC/EAEC	0,754	0,268	1,876	1,359	0,734	0,431	1,117	0,717	1,356	0,96	0,511
11c_ETEC	0,694	0,777	0,645	0,499	0,922	1,444	0,772	0,726	1,395	0,87	0,329
30e ETEC	0,732	0,667	0,461	1,188	1,249	0,742	0,79	1,39	0,5	0,86	0,336
17a ETEC	0,268	0,273	0,238	0,364	0,46	0,304	0,353	0,705	0,323	0,37	0,143
11d ETEC	0,511	0,638	0,429	0,538	0,287	0,748	0,412	0,413	0,56	0,50	0,138
14c_EPEC	1,053	0,577	0,768	0,906	1,096	1,057	0,773	0,866	0,731	0,87	0,175
20e COMM	0,257	0,394	0,444	0,979	0,683	0,669	1,06	0,456	0,678	0,62	0,268
28e_COMM	1,325	1,258	2,456	0,833	0,849	2,088	0,96	1,196	2,037	1,44	0,598
26e COMM	0,941	0,985	0,793	0,957	0,96	0,924	0,763	0,701	0,5	0,84	0,162
4a COMM	0,163	0,237	0,138	0,453	0,312	0,339	0,425	0,89	0,521	0,39	0,229
30a COMM	0,732	0,734	0,788	0,301	0,364	0,214	1,257	1,498	1,262	0,79	0,461
13e INC_ExPEC	0,266	0,195	0,305	0,294	0,329	0,253	0,258	0,273	0,243	0,27	0,039
15d INC_ExPEC	1,317	1,034	1,037	0,86	0,93	1,153	0,862	1,185	0,708	1,01	0,190
ATCC 25922_COMM	0,312	0,744	0,531	0,863	0,377	0,62	0,72	0,516	1,423	0,68	0,330

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Table 22:S. Biofilm formation by microtiter assay in microplate in M9 medium plus 0.4% glucose, tube adherence method (BHI broth) and curli assay (congo red agar) per isolate.

Type of *E. coli*	No. Strain	Tube method	Microplate method	Curli positive
INC_EXPEC	13e	0	0	1
INC_EXPEC	15d	0	1	0
EPEC	14c	1	1	0
ETEC	11c	0	1	0
ETEC	11d	0	0	0
ETEC	17a	0	0	0
ETEC	30e	0	0	1
EAEC	5d	1	1	1
EAEC	13a	0	1	1
EAEC	13d	0	1	1
EAEC	14a	1	1	0
EAEC	18e	0	1	0
EAEC	21a	1	1	1
EAEC	23a	1	1	0
EAEC	23e	0	1	1
EAEC	28a	1	1	1
EAEC	28c	1	1	0
COMM	4a	0	0	0
COMM	20e	1	1	1
COMM	26e	0	0	0
COMM	28e	0	1	1
COMM	30a	1	1	1

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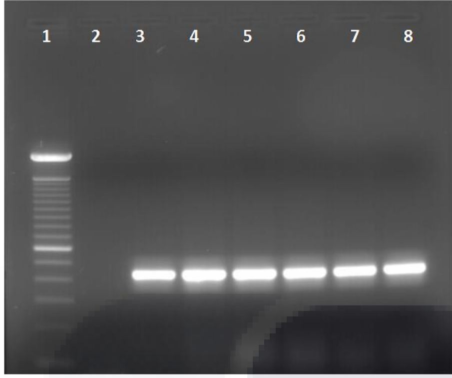

Figure 2:S. Detection of *uidA* gene (amplification at 450bp). Agarose gel electroctrophoresis (1%). Lane: 1, DNA ladder (100pb); lane 2: negative control (water); lane 3: positive control CFT073; lane 4-8: *E. coli*.

Figure 3:S. Agarose gel electroctrophoresis (1%) for multiplex PCR by *arpA* (400pb), *chuA* (288pb), *yjaA* (211 bp) genes and the TspE4.C2 fragment (152pb) amplifications. Lane: 1, DNA ladder (100pb); lane 2-17: *E. coli* isolates; lane 19: positive control CFT073; lane 20: negative control (water).

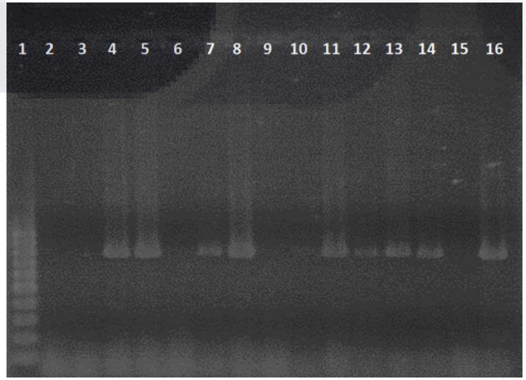

Figure 4:S. Agarose gel electroctrophoresis (1.5%) *fyuA* gene (ferric yersiniabactin) amplification (880pb). Lane: 1, DNA ladder (100pb); lane 2-14: *E. coli* isolates; lane 15: negative control (water); lane 16: positive control CFT073.

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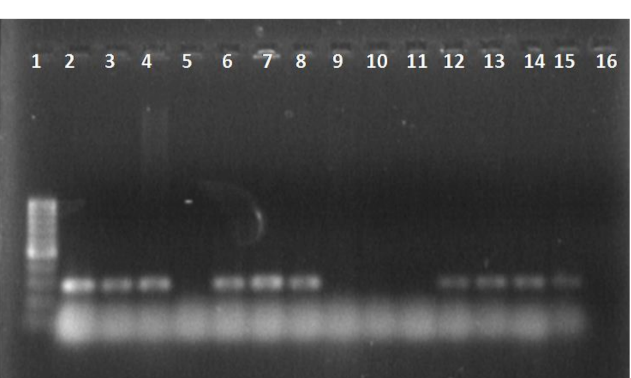

Figure 5:S. Agarose gel electroctrophoresis (1.5%) *kpsMII* gene (capsule synthesis) amplification (272pb). Lane: 1, DNA ladder (100pb); lane 2-14: *E. coli* isolates; lane 15: positive control CFT073; lane 16: negative control (water).

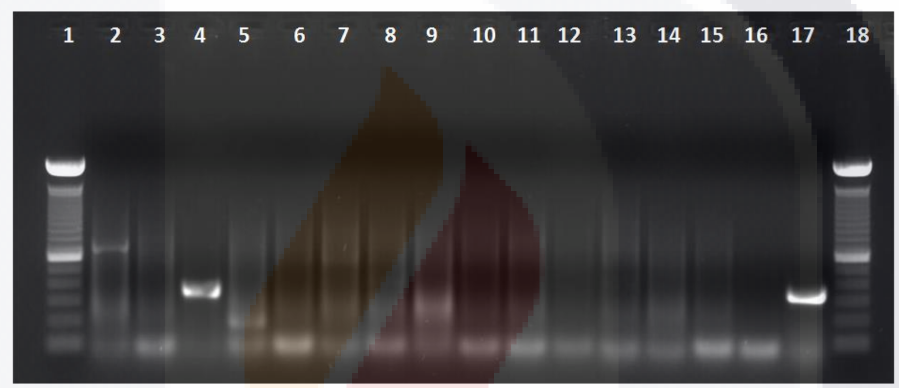

Figure 6:S. Agarose gel electroctrophoresis (1.5%) *papC* gene (outer membrane usher protein involved in P fimbrial assembly) amplification (350pb). Lane: 1, DNA ladder (100pb); lane 2-14: *E. coli* isolates; lane 15: positive control CFT073; lane 16: negative control (water); lane: 18, DNA ladder (100pb).

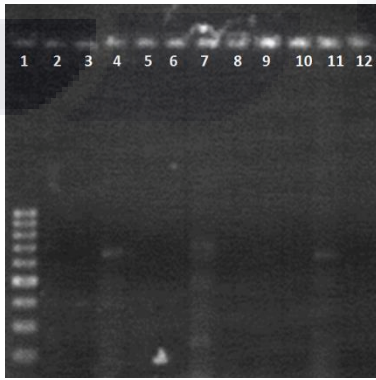

Figure 7:S. Agarose gel electroctrophoresis (1.5%) *afa/dra* gene (Dr family adhesins) amplification (592pb). Lane: 1, DNA ladder (100pb); lane 2-10: *E. coli* isolates; lane 11: positive control CFT073; lane 12: negative control (water).

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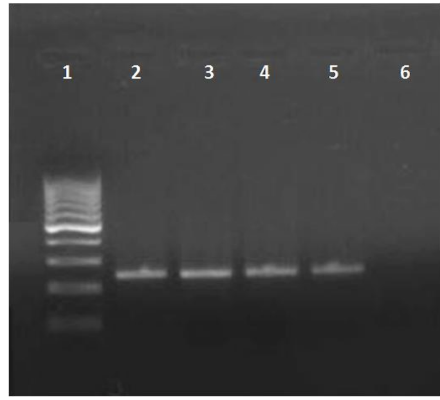

Figure 8:S. Agarose gel electroctrophoresis (1.5%) *sfaS* gene (S fimbriae) amplification (240pb). Lane: 1, DNA ladder (100pb); lane 2-4: *E. coli* isolates; lane 5: positive control CFT073; lane 6: negative control (water).

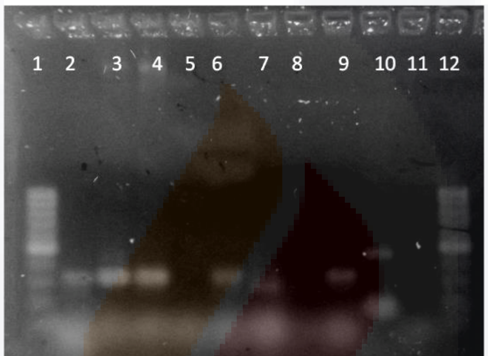

Figure 9:S. Agarose gel electrophoresis of products from PCR for detection *st* (147 pb) and *lt* (322pb) for ETEC strains. Lane 1, 100-bp DNA ladder; lane 2-8, *E. coli* isolates: lane 11, negative control (water); lane 9-10 positive controls (O42, H10407).

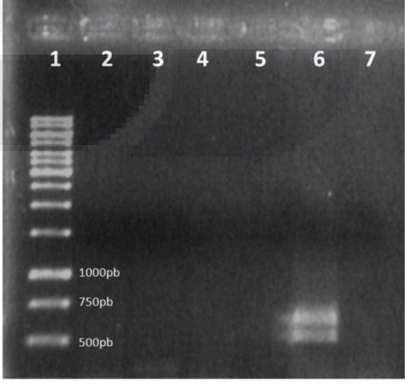

Figure 10:S. Agarose gel electrophoresis of products from PCR for detection *ipaH* (619 pb) and *stx1/stx2* (518pb) for EIEC and EHEC strains. Lane 1, 100-bp DNA ladder; lane 2-8, *E. coli* isolates: lane 11, negative control (water); lane 9-10 positive controls (*Salmonella* spp., and EDL933).

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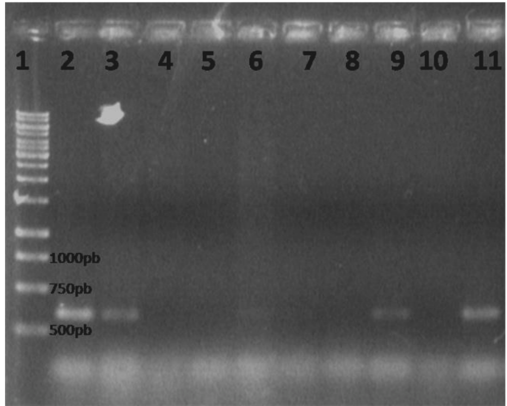

Figure 11:S. Agarose gel electrophoresis of products from PCR for detection *eae* (644pb) for EPEC strains. Lane 1, 1000-bp DNA ladder; lane 2-9, *E. coli* isolates; lane 10, negative control (water); lane 11 positive control (E2348/69).

Figure 12:S. Agarose gel electrophoresis of products from PCR for detection *bfp* (326pb) for EPEC strains. Lane 1, 1000-bp DNA ladder; lane 2-9, *E. coli* isolates; lane 10, positive control (E2348/69); lane 11, negative control (water).

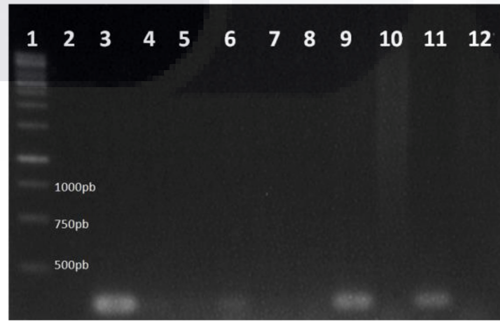

Figure 13:S. Agarose gel electrophoresis of products from PCR for detection *astA* (110 pb) for EAEC strains. Lane 1, 100-bp DNA ladder; lane 2-8, *E. coli* isolates: lane 11, negative control (water); lane 9-10 positive controls (O42).

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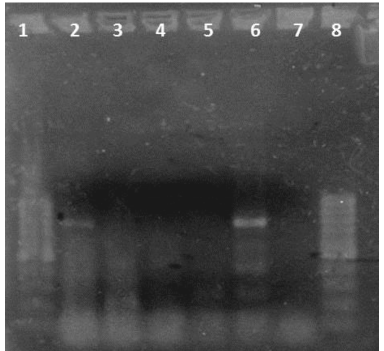

Figure 14:S. Agarose gel electrophoresis of products from PCR for detection *aafA* (110 pb) for EAEC strains. Lane 1, 100-bp DNA ladder; lane 2-5, *E. coli* isolates: lane 7, negative control (water); lane 6 positive controls (O42).

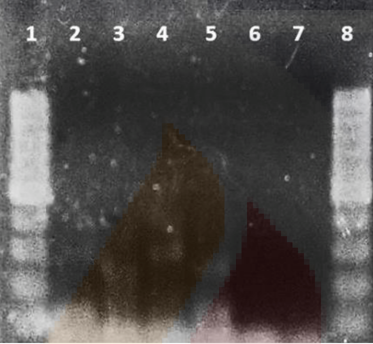

Figure 15:S. Agarose gel electrophoresis of products from PCR for detection *aggR* (254 pb) for EAEC strains. Lane 1, 100-bp DNA ladder; lane 4, *E. coli* isolates: lane 6-77, negative control (water); lane 5 positive controls (O42).

Figure 16:S. Antimicrobial susceptibility assay for *E. coli* isolates from stream water.

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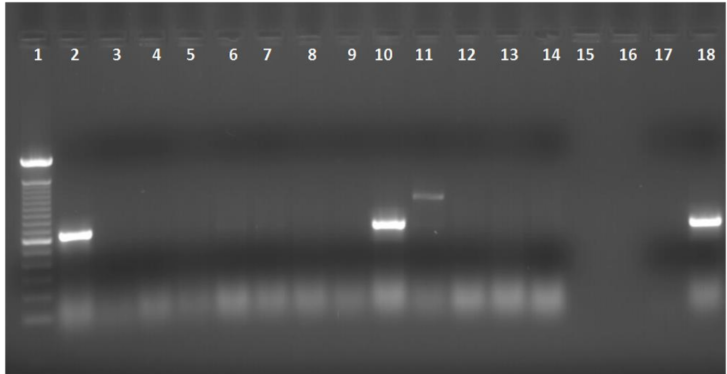

Figure 17:S. Agarose gel electroctrophoresis (1.5%) for detection of *qnrA* resistance genes (605pb). Lane: 1, DNA ladder; lane 2-16; *E. coli* isolates; lane 17: negative control (water); lane 18: positive control for *qnrA* Jacoby J53 pMG252.

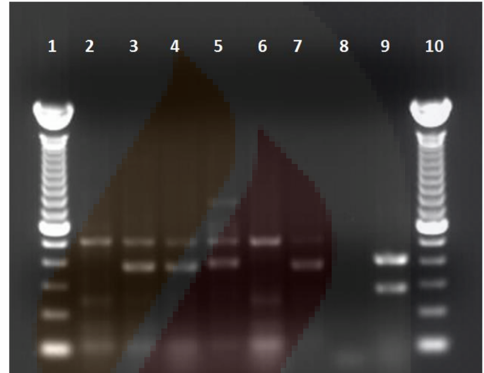

Figure 18:S. Agarose gel electrophoresis of products from PCR for detection of *qnrS* (417pb) genes. Lane 1: DNA ladder; lane 2-7, *E. coli* isolates; lane 8: negative control (water); lane 9 positive control: SA20070201.

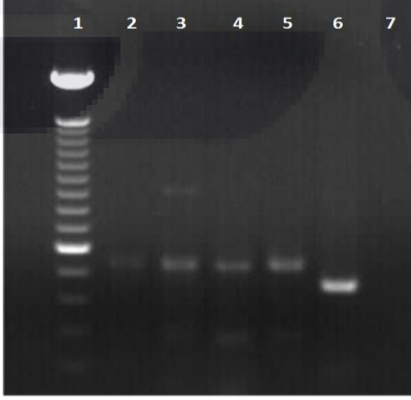

Figure 19:S. Agarose gel electrophoresis of products from PCR for detection of *qnrB* (469pb) genes. Lane 1: DNA ladder; lane 2-4, *E. coli* isolates; lane 5, J53pMG306; lane 6, positive control: SA20042589; lane 7: negative control (water).

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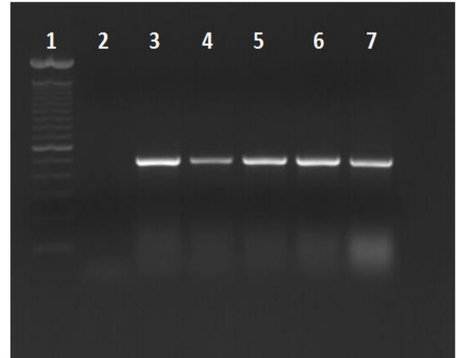

Figure 20:S. Agarose gel electrophoresis of products from PCR for detection of *gyrA* gene (475pb) for sequencing. Lane 1, 100-bp DNA ladder; lane 2, negative control (water); lane 3, positive control KL16 Wild Type; lane 4, positive control BN49; lane 5, positive control BN59, lane 6-7 *E. coli* isolates.

Figure 21:S. Agarose gel electrophoresis of products from PCR for detection *parC* (422 pb) for sequencing. Lane 1, 100-bp DNA ladder; lane 2, negative control (water); lane 3, CFT073; lane 4-7, *E. coli* isolates.

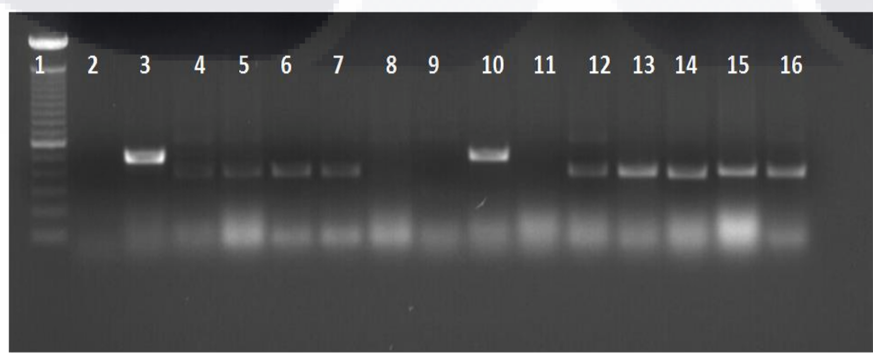

Figure 22:S. Agarose gel electrophoresis of products from PCR for detection *aac-(6')-lb* (482 pb) for sequencing. Lane 1, 100-bp DNA ladder; lane 2, negative control (water); lane 3, SA20042859; lane 4-16, *E. coli* isolates.

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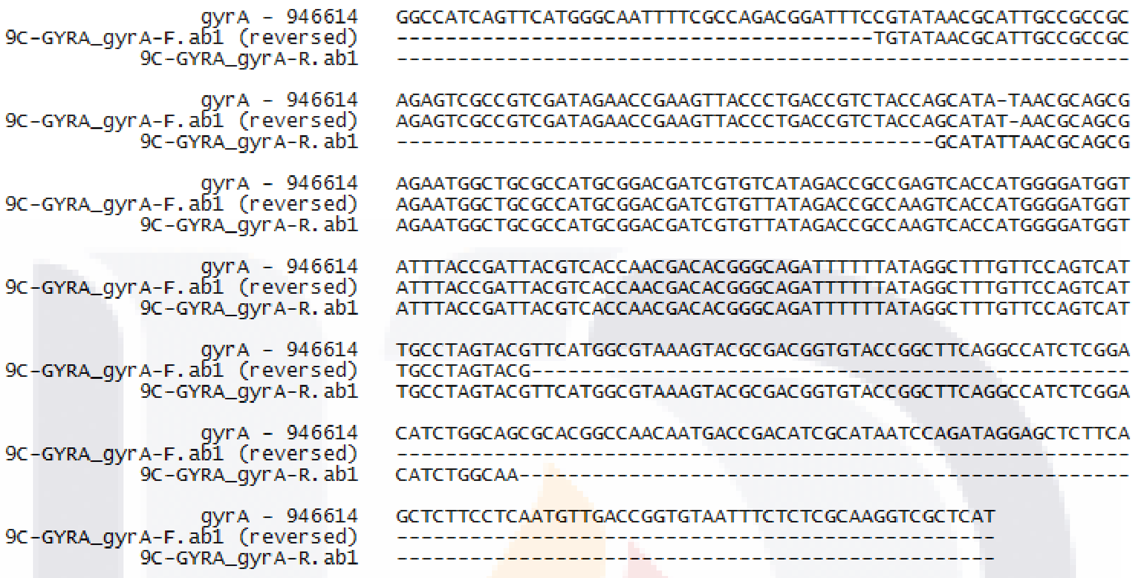

Figure 27:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 9C was change the number to 11C to give a geographical distribution of sample locations.

Figure 28:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 9C was change the number to 11C to give a geographical distribution of sample locations.

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Figure 29:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 9D was change the number to 11D to give a geographical distribution of sample locations.

Figure 30:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 9D was change the number to 11D to give a geographical distribution of sample locations.

Figure 31:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 12C was change the number to 14C to give a geographical distribution of sample locations.

Figure 32:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 12C was change the number to 14C to give a geographical distribution of sample locations.

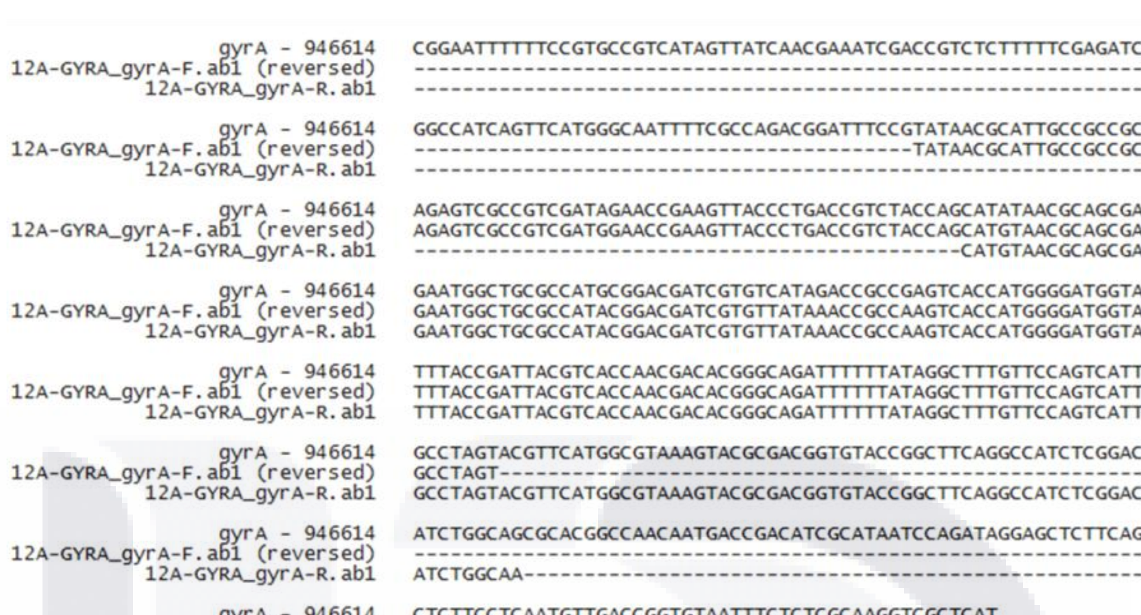

Figure 33:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 12A was change the number to 14A to give a geographical distribution of sample locations.

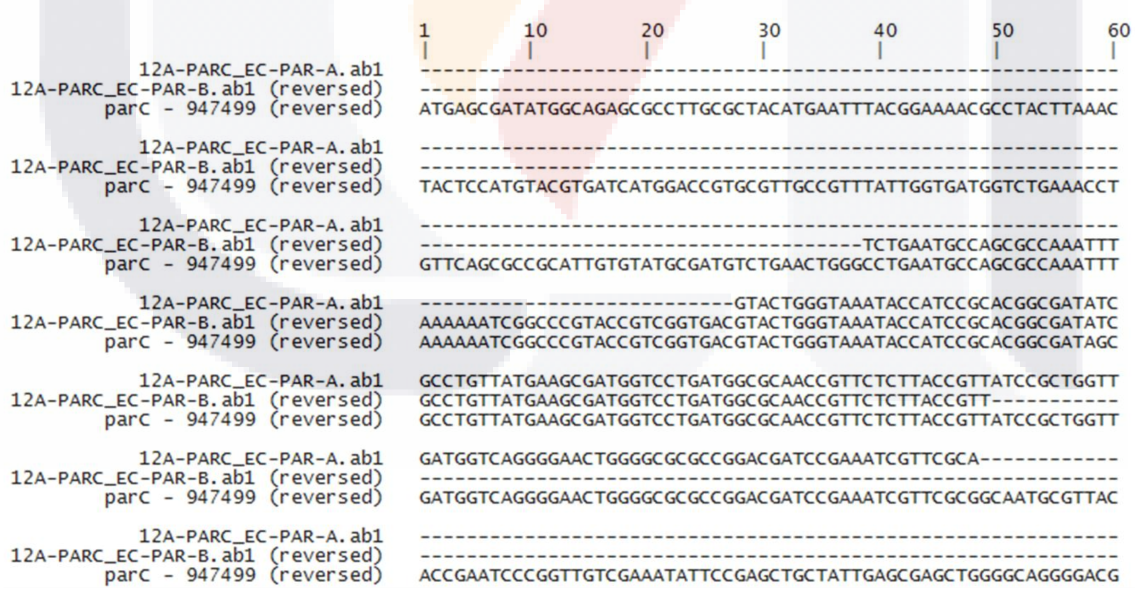

Figure 34:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 12A was change the number to 14A to give a geographical distribution of sample locations.

Figure 35:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 11D was change the number to 13D to give a geographical distribution of sample locations.

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Figure 36:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 11D was change the number to 13D to give a geographical distribution of sample locations.

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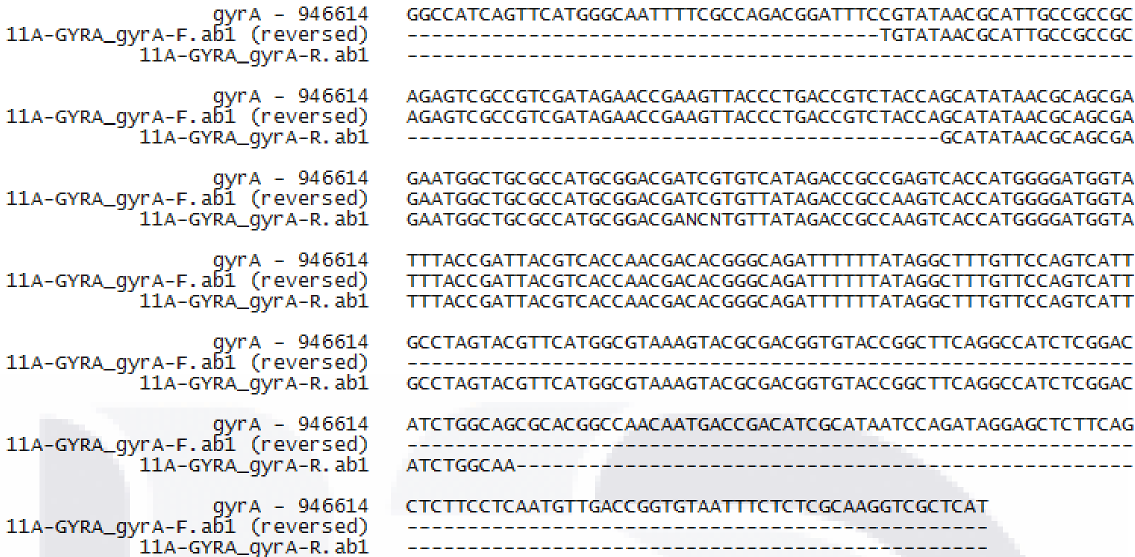

Figure 37:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 11D was change the number to 13D to give a geographical distribution of sample locations. Sample 11A was change the number to 13A to give a geographical distribution of sample locations.

Figure 38:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 11A was change the number to 13A to give a geographical distribution of sample locations.

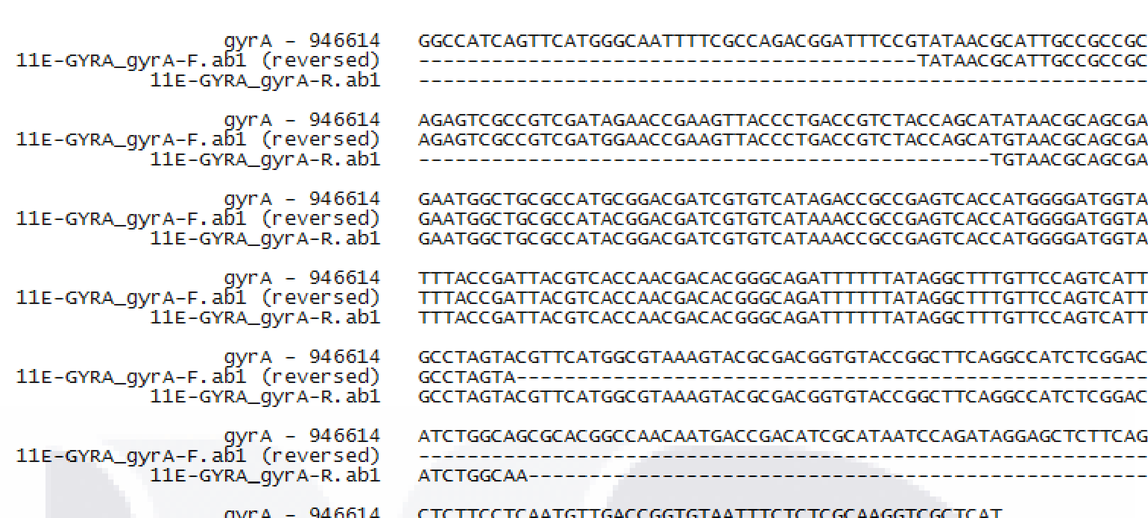

Figure 39:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 11E was change the number to 13E to give a geographical distribution of sample locations.

Figure 40:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 11E was change the number to 13E to give a geographical distribution of sample locations.

Figure 41:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance of sample 28A.

Figure 42:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance for sample 28A.

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Figure 43:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 14A was change the number to 4A to give a geographical distribution of sample locations.

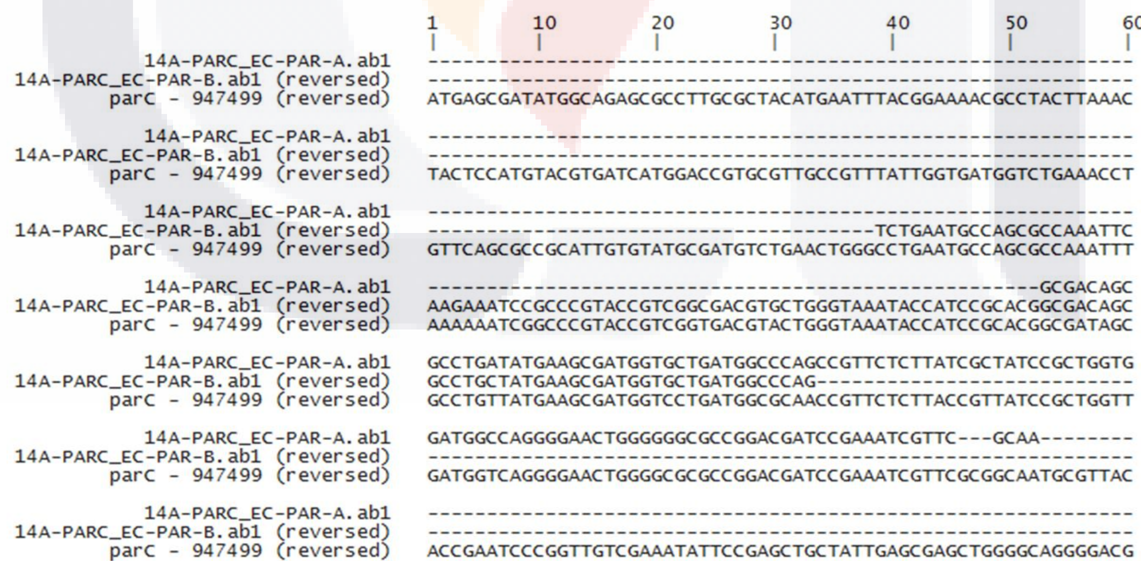

Figure 44:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 14A was change the number to 4A to give a geographical distribution of sample locations.

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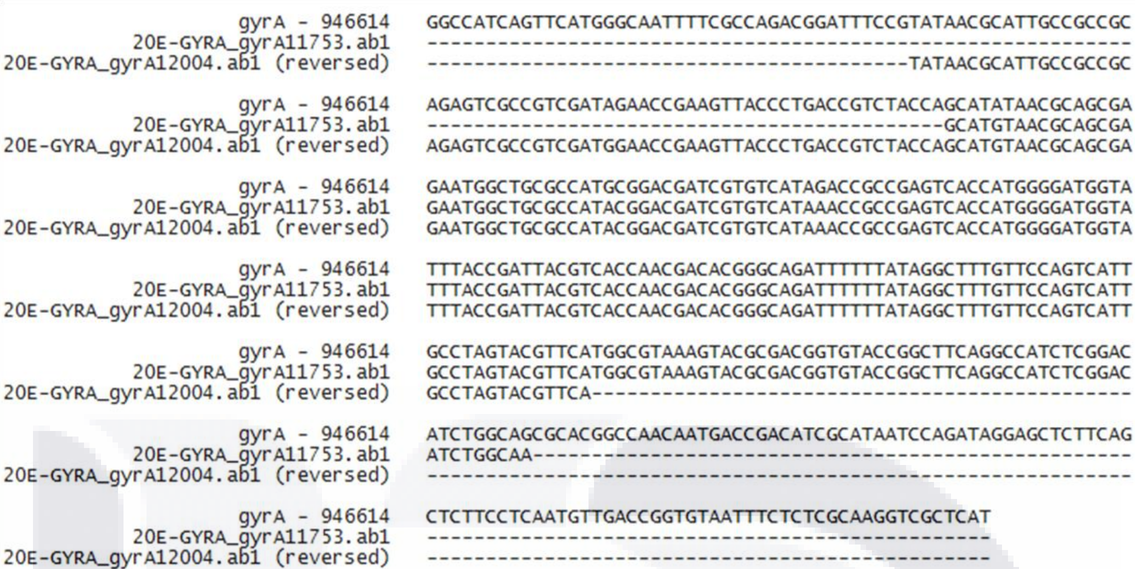

Figure 45:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance of sample 20E.

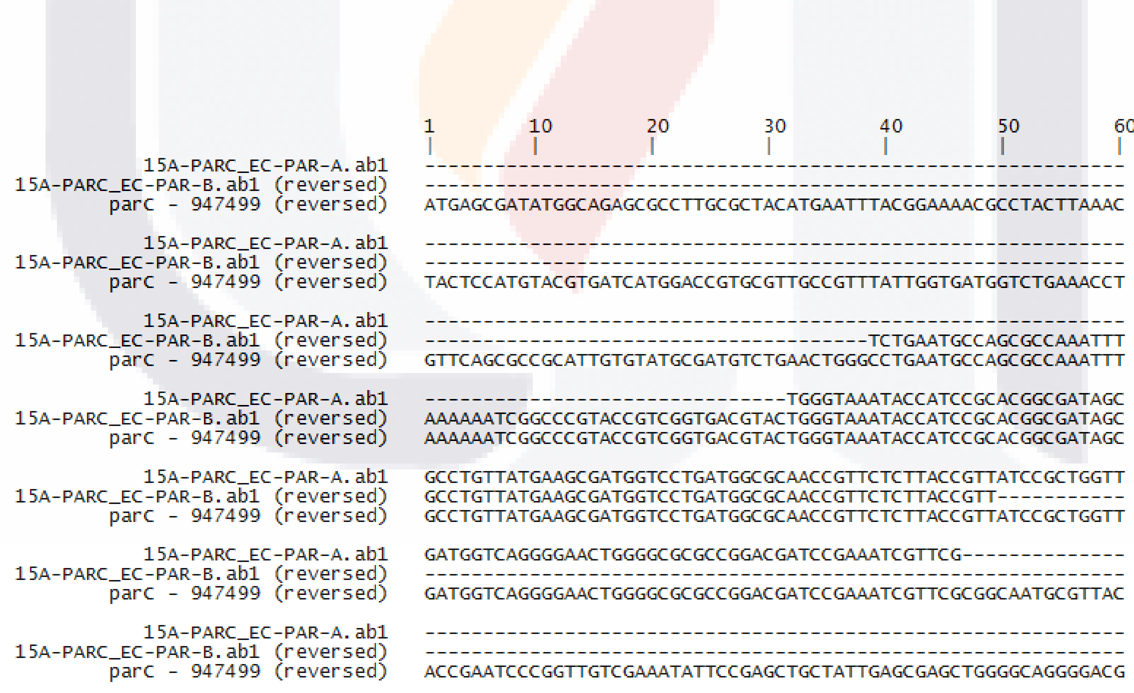

Figure 46:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 15A was change the number to 20E to give a geographical distribution of sample locations.

Figure 47:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 24A was change the number to 23A to give a geographical distribution of sample locations.

Figure 48:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 24A was change the number to 23A to give a geographical distribution of sample locations.

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Figure 49:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 24E was change the number to 23E to give a geographical distribution of sample locations.

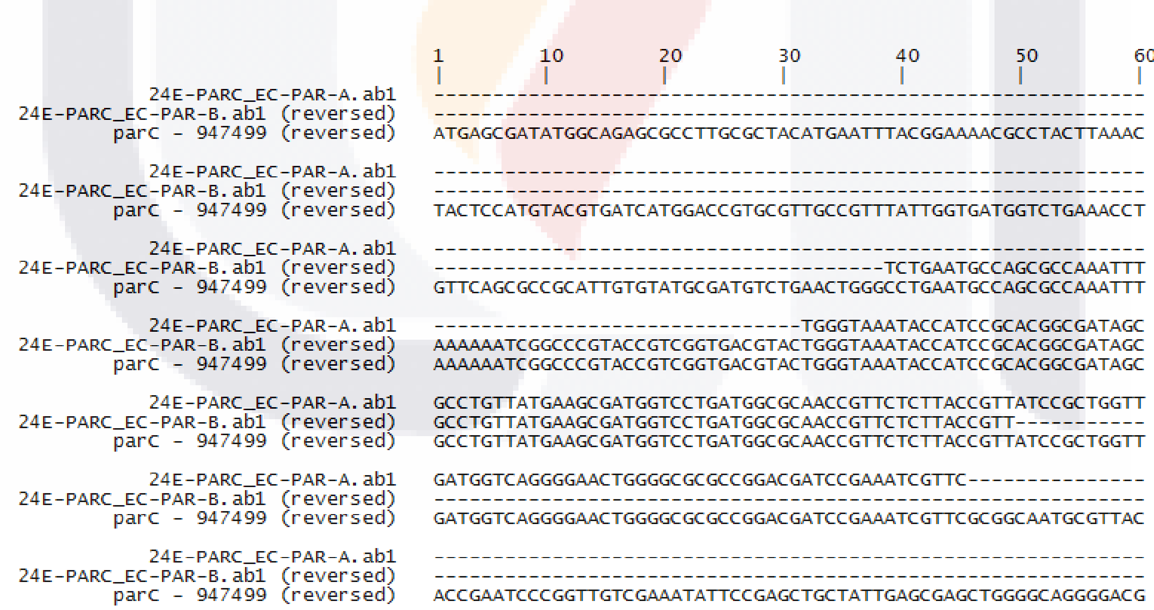

Figure 50:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 24E was change the number to 23E to give a geographical distribution of sample locations.

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Figure 51:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance of sample 30A.

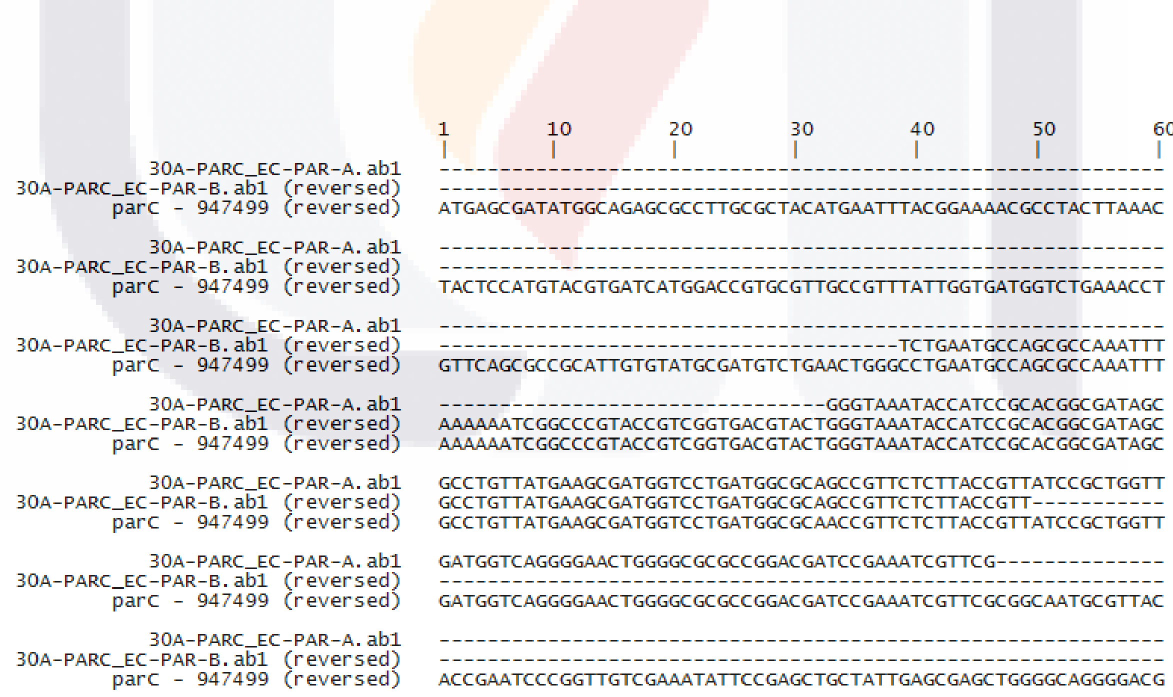

Figure 52:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance of sample 30A.

Figure 53:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance of sample 21A.

Figure 54:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance of sample 21A.

Figure 55:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 15A was change the number to 17A to give a geographical distribution of sample locations.

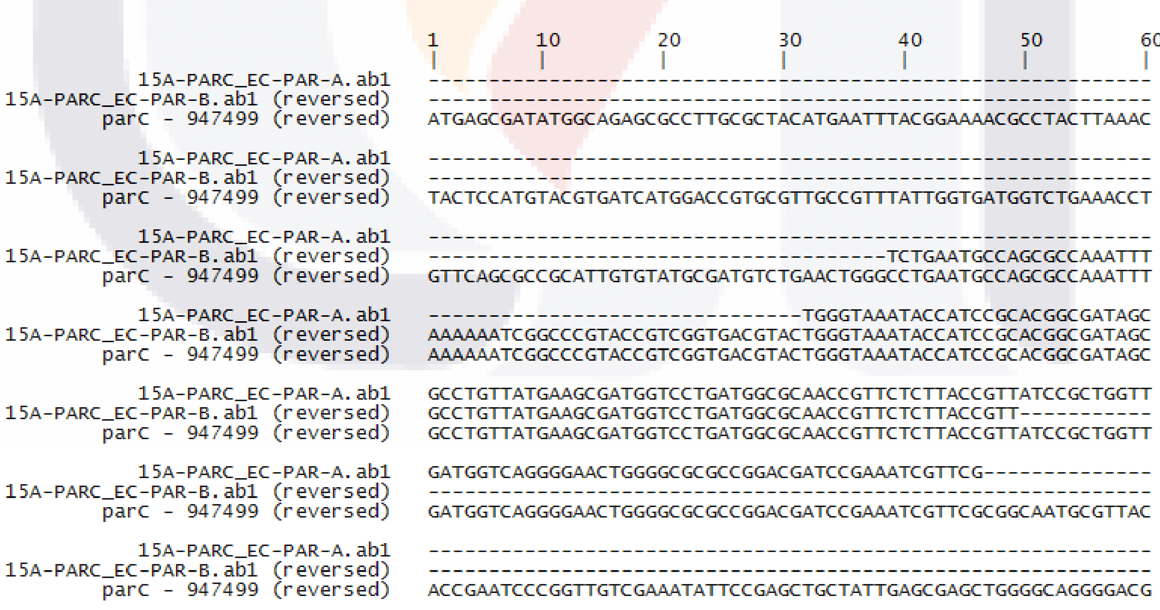

Figure 56:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 15A was change the number to 17A to give a geographical distribution of sample locations.

Figure 57:S. Aligment of sequencing products of *gyrA* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 28E was change the number to 28C to give a geographical distribution of sample locations.

Figure 58:S. Aligment of sequencing products of *parC* to chromosomal detect mutations in the quinolone-resistance determining regions (QRDRs) for fluoroquinolone resistance. Sample 28E was change the number to 28C to give a geographical distribution of sample locations.

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SUPPLEMENTARY DATA E

Some of the results presented in this work have been published or have been presented during scientific mettings:

1. Ramírez-Castillo, F.Y., Avelar-González, F.J., Garneau P., Marquez-Diaz, F., Guerrero-Barrera, A.L., Harel, J. (2013). Presence of multi-drug resistant pathogenic *Escherichia coli* in the San Pedro River located in the State of Aguascalientes, Mexico. *Front Microbiol*. 4:147.
2. Ramírez-Castillo, F.Y., Harel, J., Moreno-Flores, A.C., Loera-Muro, A., Guerrero-Barrera, A.L., Avelar-González, F.J. (2014). Antimicrobial resistance: the role of aquatic environments. *International Journal of Current Research and Academic Review*. 2 (7):231-246.
3. Loera-Muro, V.M., Loera-Muro, A.. Morfín-Mata, M., Jacques, M., Avelar-González, F.J., Ramírez-Castillo, F.Y., Ramírez-López, E.M., Guerrero-Barrera, A.L. (2014). Porcine respiratory pathogens in swine farms environment in Mexico. *Open Journal of Animal Sciences*, 4, 196-205.
4. Loera-Muro, A., Ramírez-Castillo, F.Y., Avelar-González, F.J., Guerrero-Barrera, A.L., (2012). Biopelículas multi-especie: asociarse para sobrevivir. *Investigación y ciencia de la Universidad Autónoma de Aguascalientes.* 54:53-60.
5. Díaz-Galindo, M.C., Loera-Muro A., Ramírez-Castillo F.Y., Olvera-Rarías, R., Guerrero-Barrera A.L. (2012). Matriz extracelular: ¿es el andamio de los tejidos?. *Investigación y ciencia de la Universidad Autónoma de Aguascalientes*. 56:49-56.

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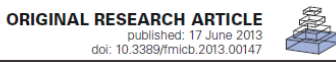

ORIGINAL RESEARCH ARTICLE
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Presence of multi-drug resistant pathogenic *Escherichia coli* in the San Pedro River located in the State of Aguascalientes, Mexico

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Contamination of surface waters in developing countries is a great concern. Treated and untreated wastewaters have been discharged into rivers and streams, leading to possible waterborne infection outbreaks and may represent a significant dissemination mechanism of antibiotic resistance genes. In this study, the water quality of San Pedro River, the main river and pluvial collector of the Aguascalientes State, Mexico was assessed. Thirty sample locations were tested throughout the River. The main physicochemical parameters of water were evaluated. Results showed high levels of fecal pollution as well as inorganic and organic matter abundant enough to support the heterotrophic growth of microorganisms. These results indicate poor water quality in samples from different locations. One hundred and fifty *Escherichia coli* were collected and screened by PCR for several virulence genes. Isolates were classified as either pathogenic ($n = 91$) or commensal ($n = 59$). The disc diffusion method was used to determine antimicrobial susceptibility to 13 antibiotics. Fifty-two percent of the isolates were resistant to at least one antimicrobial agent and 30.6% were multi-resistant. Eighteen *E. coli* strains were quinolone resistant of which 16 were multi-resistant. Plasmid-mediated quinolone resistance (PMQR) genes were detected in 12 isolates. Mutations at the Ser-83→Leu and/or Asp-87→Asn in the *gyrA* gene were detected as well as mutations at the Ser-80→Ile in *parC*. An *E. coli* microarray (Maxivirulence V 3.1) was used to characterize the virulence and antimicrobial resistance genes profiles of the fluoroquinolone-resistant isolates. Antimicrobial resistance genes such as bla_{TEM}, *sulI*, *sulII*, *dhfrIX*, *aph3* (*strA*), and *tet* (B) as well as integrons were found in fluoroquinolone (FQ) resistance *E. coli* strains. The presence of potential pathogenic *E. coli* and antibiotic resistance in San Pedro River such as FQ resistant *E. coli* could pose a potential threat to human and animal health.

Keywords: water quality, antibiotic resistance, fluoroquinolone, virulence factors, pathogenic *Escherichia coli*

INTRODUCTION

Escherichia coli is a commensal member of the intestinal flora of humans and warm-blooded animals. Several genotypes have acquired specific virulence factors and are capable of causing disease as gastrointestinal diseases, urinary tract infections (UTIs) and sepsis/meningitis. Intestinal pathogenic strains causing diarrhea include enteropathogenic *E. coli* (EPEC), enterohemorragic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC, Nataro and Kaper, 1998). The extraintestinal pathogenic *E. coli* (ExPEC) group is composed of uropathogenic *E. coli* (UPEC), which is the main causes of UTIs, meningitis-associated *E. coli* (MNEC), sepsis-associated *E. coli* (SEPEC) and the avian pathogenic *E. coli* (APEC), which is associated with respiratory infections, pericarditis, and septicaemia in poultry (Kaper et al., 2004).

The presence of *E. coli* in water is widely used as a microbiological indicator of fecal pollution and water quality (WHO, 2006). The presence of pathogenic *E. coli* in environmental water creates a potential risk for infections in humans and animals especially since water is used for irrigation, a source of drinking water, and for recreational purposes (Hamelin et al., 2006, 2007; Kümmerer, 2009a; Koczura et al., 2012). Furthermore, the presence of antimicrobial resistant bacteria in the environment is another great concern for public health.

The contamination of aquatic and soil environments by untreated sewage or manure coming from human or animals treated with antibiotics (Baquero et al., 2008; Kümmerer, 2009b;

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Lupo et al., 2012; Suzuki and Hoa, 2012), as well as the presence of heavy metal pollution (McArthur and Tuckfield, 2000) and quaternary ammonium compounds (QACs; Hegstad et al., 2010) might result in the presence of antimicrobial resistant bacteria. Furthermore, most antibiotics are not fully eliminated during the sewage treatment process. Thus, an aquatic environment such as rivers or streams could act as an antibiotic resistant genes reservoir and facilitate the dissemination of these genes (Kümmerer, 2004; Lupo et al., 2012). The emergence of antimicrobial resistance mechanisms, especially those associated with mobile genetic elements, may enhances the possibility that virulence factors genes and antibiotic resistance genes are spread simultaneously, inducing the emergence of new pathogens (Chen et al., 2011; Da Silva and Mendoça, 2012; Koczura et al., 2012).

Fluoroquinolone (FQ) is a family of widely used synthetic antimicrobial agents with a broad antibacterial spectrum that is used as a front line drug for urinary tract and intestinal infections. However, increase in the prevalence of FQ resistant bacteria has been a great concern worldwide in the last years. Several mechanisms have been described for FQ resistance. In *E. coli*, the resistance is primarily associated with the accumulation of mutations in the quinolone-resistance determining regions (QRDRs) of *gyrA* and *parC*, which encode topoisomerase II (DNA gyrase) and topoisomerase IV respectively (Hooper, 2001; Hopkins et al., 2005). These mutations can lead to conformational changes in the enzymes and thus preventing quinolones from binding to the DNA-substrate complex (Tran et al., 2005a,b). In addition, several other mechanisms can contribute to FQ resistance, including plasmid-mediated quinolone resistance (PMQR) determinants (Martinez-Martinez et al., 1998), such as the Qnr protein (QnrA, QnrB, QnrC, QnrD, and QnrS), the variant of the aminoglycoside-modifying enzyme, AAC(6′)-Ib-cr (Robicsek et al., 2006a), and the efflux pumps QepA (Yamane et al., 2007), and OqxAB (Hansen et al., 2004; Jacoby, 2005).

Pollution of surface waters may represent a pathway for the global dissemination of antibiotic resistance (Chen et al., 2011). Water contamination is a major environmental problem in Mexico. In the last decades, treated, and untreated wastewater have been released into natural streams leading to possible waterborne disease outbreaks resulting from the use of unsafe water for consumption, irrigation or recreational activities (Mazari-Hiriart et al., 2008; Chávez et al., 2011). Several studies on antibiotic resistant bacteria release and their occurrence in sewage and natural environments have been conducted (Sayah et al., 2005; Amábile-Cuevas et al., 2010; Chen et al., 2011; West et al., 2011; Czekalski et al., 2012; Sun et al., 2012; Tacão et al., 2012). However, the significance of rivers as environments providing irrigation water and recreational activities as well as the possibility of the potential spread of pathogenic and antibiotic resistant bacteria between the environment and humans and animals, marks them as highly relevant study subjects (Czekalski et al., 2012). In addition, anthropogenic activities might impact on water environment promoting the dissemination of antibiotic resistance (Pruden et al., 2006; Martinez, 2009; Tacão et al., 2012). Thus, it is of major importance to study how human activities can impact antimicrobial resistant bacteria and antimicrobial resistance genes in the environment in order to understand their spread and health implications (Tacão et al., 2012).

In this study, the water quality of San Pedro River of Aguascalientes State in Mexico was evaluated. Currently, the river is being contaminated by wastewater from urban municipal sewers, industrial activities, and livestock farms. The objectives of this study were to evaluate the quality of the water between sample locations in the river polluted by different human activities, to determine the presence of pathogenic *E. coli* and assess the antimicrobial resistance profiles for the *E. coli* isolates, focusing in FQs agents.

MATERIALS AND METHODS

WATER SAMPLING AND *E. coli* ISOLATION

Thirty sample locations were selected throughout San Pedro River and its main tributaries (**Figure 1**). All locations were sampled once and selected due to the presence of important discharges of treated or untreated wastewater into the river (**Table 1**). San Pedro River is the main watershed and pluvial collector of Aguascalientes State. The river belongs to the hydrological region of Lerma-Santiago-Pacifico which is one of the most important drainage basin of Mexico (77,000 km^2), draining 80% of the Aguascalientes State. Currently, the San Pedro River is being contaminated by the influx of wastewater from population centers, industrial activities, agricultural activities, and livestock which results in different levels of surface water quality. It is estimated that ~60% of the sewage water is discharged into the river without prior treatment (CONABIO, 2008). Nevertheless, the river is still used as important source for agricultural irrigation as well as recreational purposes in the cleanest zones (Gúzman-Colis et al., 2011).

Sampling was performed from June to November according to procedure described by the American Public Health Association (APHA, 1998) and the World Health Organization (WHO, 2006; **Table 1**). Water samples were collected in 180 mL sterile glass bottles in triplicates. The samples were stored at 4°C until analysis, which was done within 24 h of the sample collection. To isolate *E. coli*, serial dilutions of the sample were prepared in 0.85% NaCl, and were used to inoculate MacConkey agar and Eosin Methylene Blue medium agar. Plates were incubated overnight at 37°C. For further identification, possible *E. coli* were confirmed by testing for glucuronidase activity (growth and fluorescence in EC-MUG), citrate utilization, indole production, methyl red test and Voghes–Proskauer test. Isolates meeting the *E. coli* test profile were confirmed by detecting the *uidA* gene using *uidA* primers listed in **Table 2**. The *E. coli* isolates were stored at −80°C in tryptic soy broth and 20% (vol/vol) glycerol.

PHYSICOCHEMICAL PARAMETERS

All techniques were performed according to Standard Methods (APHA, 1998). Water temperature (Method 2550B), pH (Method 4500 − H + B), conductivity (2510B) and dissolved oxygen (4500 OG) were determined electrometrically *in situ*. The level of organic matter pollution was determined using the biological oxygen demand (BOD, 5210B) and chemical oxygen demand (COD, 5220D). Total nitrogen (4500–NorgB), total phosphorus (4500–PE) and total suspended solids (TSS) (2540B–F) were also determined.

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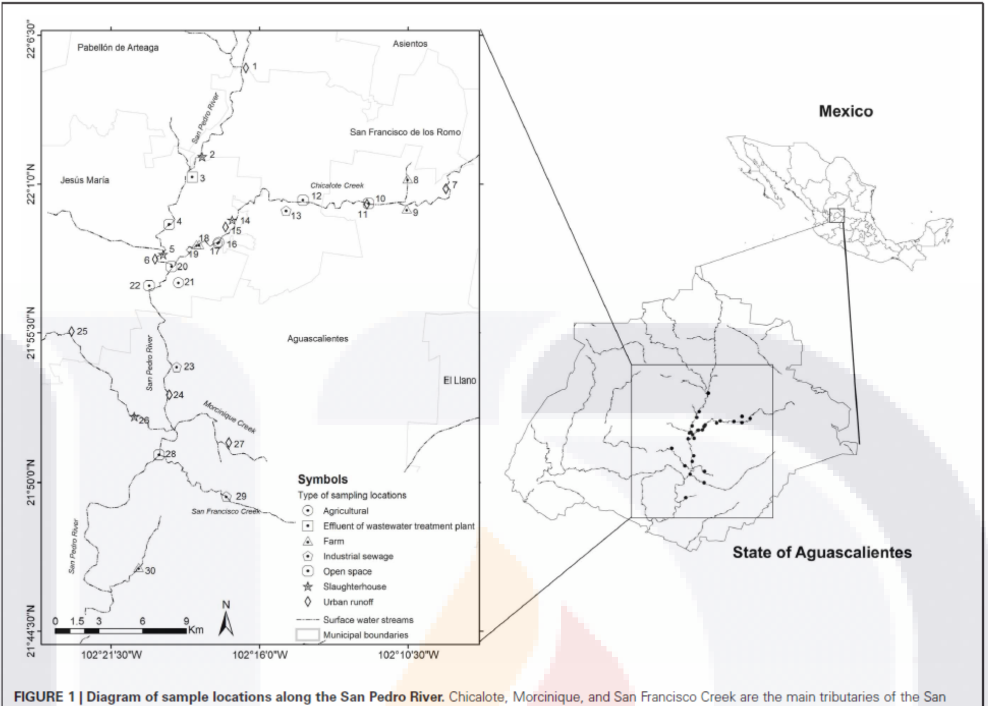

FIGURE 1 | Diagram of sample locations along the San Pedro River. Chicalote, Morcinique, and San Francisco Creek are the main tributaries of the San Pedro River.

MICROBIOLOGICAL ANALYSIS

The amount of total bacteria was estimated by testing for mesophilic microorganisms using the pour plates technique (9215B). Fecal contamination was determined by measuring total coliform (9221C) and fecal coliform (9221E) using the standard fermentation technique for the most probable number (MPN; APHA, 1998).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility test of 150 *E. coli* isolated from stream water was performed using a disc diffusion assay according to CLSI standards, 2010. Isolates that were resistant to three or more antimicrobial agents were defined to have a multiple drug resistant (MDR) phenotype. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, US), was included in each assay as a negative control. Antimicrobial agents were tested using a Bio-Rad (Hercules, CA, US) Sensi–Disc antimicrobial susceptibility test multidisc for Gram–negative bacteria with the following antimicrobial agents: amikacin-30, ampicillin-10, cephalothine-30, cephotaxim-30, ceftriaxone-30, chloramphenicol-30, gentamicin-10, netilmicin-30, nitrofurantoin-300, pefloxacin-5, carbenicillin-30, and trimethoprim-sulfamethoxazole-1.25/23.75 μg. The quinolone levofloxacin-5 μg was tested separately using a Bio-Rad (Mexico, DF, Mexico) Sensi-Disc.

DNA EXTRACTION

E. coli isolates were grown overnight in 5 mL of Luria–Bertani broth at 37°C without shaking. An aliquot (1 mL) of the overnight culture was transferred to 1.5 mL tubes and centrifuged at 15,500 × *g* for 2 min. The supernatant was removed, and the cell pellet was resuspended by vortexing in 200 μL of sterile water. The suspension was boiled for 15 min, centrifuged (15,500 × *g*, 2 min), and 150 μL of the supernatant was collected (Hamelin et al., 2006). The DNA quality and purity extracted was analyzed by spectrophotometer at 260 and 280 nm wavelengths. The sample with ratio ($\lambda_{260}/\lambda_{280}$) ≥ 1.5 was considered adequate to continue with identification by polymerase chain reaction (PCR).

PATHOTYPE AND VIRULENCE GENES DETERMINATION BY PCR

Detection of intestinal pathogenic *E. coli* (InPEC) and ExPEC virulence genes were performed by PCR with primers described in **Table 2**. InPEC isolates were defined according to criteria established by Aranda et al., 2007 and ExPEC isolates were defined by

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Table 1 | Location and main sources of pollution of the 30 sampling locations studied.

Sample no.	Sample locations	[a]Main source of pollution	[b]Type of sampling location
1	San Pedro River. Las Animas.	Urban runoff	UR
2	San Pedro River. FREASA	Slaughterhouse	S
3	San Pedro River. FREASA-PT.	Wastewater treatment plant effluent	WWTP-E
4	San Pedro River. San Antonio de los Horcones.	Open space	OS
5	San Pedro River. Rastro municipal de Jesus Maria.	Slaughterhouse	S
6	San Pedro River. Los Ramiez-Los Vasquez.	Human sewage + wastewater discharge	UR
7	Chicalote Creek. Jaltomate.	Urban runoff	UR
8	Chicalote Creek. El Becerro II.	Agricultural + farm	F
9	Chicalote Creek. El Becerro I.	Agricultural + farm	F
10	Chicalote Creek. Cañada Honda I.	Urban runoff	UR
11	Chicalote Creek. Cañada Honda II.	Open space	OS
12	Chicalote Creek. Loretito.	Open Space	OS
13	San Pedro-Chicalote River. Confluencia PIVA-Chicalote-Gomez Portugal.	Urban runoff + agricultural + wastewater + industrial sewage	IS
14	Chicalote Creek. Gomez Portugal - Area oeste.	Urban runoff + agricultural + wastewater	UR
15	Chicalote Creek. Gomez Portugal.	Agricultural + slaughterhouse	S
16	Chicalote Creek. Reserva Brandy.	Urban runoff + agricultural	AR
17	San Pedro-Chicalote River. Confluencia PIVA-Chicalote.	Urban runoff + agricultural + wastewater + industrial sewage + slaughterhouses	IS
18	Chicalote Creek. La Florida I.	Agricultural + farm	F
19	Chicalote Creek. La Florida II.	Agricultural + farm	F
20	San Pedro River-Chicalote Creek.	Open space	OS
21	San Pedro River. Paso Blanco.	Agricultural	AR
22	San Pedro River. Tepetate-San Miguelito.	Open space	OS
23	San Pedro River. Canal interceptor.	Urban runoff + wastewater discharge + industrial sewage	IS
24	San Pedro River. Puente Curtidores.	Urban runoff + wastewater discharge	UR
25	Morcinique Creek. Los Arquitos.	Urban runoff + agricultural + wastewater	UR
26	Morcinique. Los Negritos primera seccion.	Slaughterhouse	S
27	San Pedro River-Morcinique Creek. Aguascalientes Lopez Portillo.	Urban runoff	UR
28	San Pedro River-San Francisco Creek.	Open space	OS
29	San Pedro River-San Francisco Creek. Puente Bonaterra.	Industrial sewage	IS
30	San Pedro River. Fatima.	Agricultural + farm + human sewage	F

[a]*Main source of pollution of location was determined based on the presence of discharge nearby and the type of land activity.*
[b]*AR, agriculturally impaired; F, farm; IS, industrial sewage; OS, open spaces; S, slaughterhouse; UR, urban runoff; WWTP-E, wastewater treatment plant effluent.*

criteria described by Johnson and Stell, 2000. Positive controls are listed in **Table 3**.

CHROMOSOMAL-ENCODED AND ACQUIRED QUINOLONE RESISTANCE GENES

Eighteen quinolone resistant and intermediate resistant isolates were selected to investigate mutations in the QRDR of *gyrA* and *parC* genes, as well as the presence of the acquired genes *qnrA*, *qnrB*, *qnrS*, and *aac (6′)-Ib cr*. The oligonucleotides and PCR conditions used in this study are listed in **Table 2**. The quinolone resistance determining regions of *gyrA* and *parC* genes were amplified and sequenced as described by Namboodiri et al. (2011). Amplicons were sequenced on both strands and predicted peptide sequences were compared to the corresponding gene from the MG1655 genome using BLAST program in Geneious R6 software (v. 6.0., Biomatters Ltd., New Zealand). The strains J53pMG252, J53pMG298, and J53pMG306 were used as positive controls for *qnrA*, *qnrB*, and *qnrS*, respectively (Jacoby et al., 2003) and water was used as negative control. The *aac (6′)-Ib cr* genes was detected as described by Park et al. (2006) with some modifications. Primers were used as follows: aac-forward (5′-TTGCGATGCTCTATGAGTGGCTA-3′) and aac-reverse (5′-CTCGAATGCCTGCGCTGTTT-3′) to yield an amplicon of 482 bp. The PCR condition were 94°C for 4 min, 35 cycles of 94°C of 30 s, 58°C for 30 s, and 68°C for 45 s, and a final step of 68°C for 10 min. Positive controls are listed in **Table 3**. The *aac (6′)-Ib cr* variant was identified by sequencing the PCR products (Park et al., 2006).

DNA MICROARRAY ANALYSIS

Microarray hybridizations were performed using *E. coli* Maxivirulence version 3.1 microarray as previously described

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Table 2 | Oligonucleotides used in this study.

Oligonucleotide name	Target gene	Oligonucleotide 5′ → 3′	Amplification product (bp)	Reference
***E. coli* MARKER**				
uidA-forward	*uidA*	ATGTGCTGTGCCTGAACC	450	This study.
uidA-reverse		ATTGTTTGCCTCCCTGCTG		
VIRULENCE GENES FOR INTESTINAL PATHOGENIC *E. coli*				
VTcom-forward	*stx1/stx2*	GAGCGAAATAATTTATATGTG	518	Toma et al., 2003
VTcom-reverse		TGATGATGGCAATTCAGTAT		
East-forward	*eastI*	ATGCCATCAACACAGTATAT	110	Vila et al., 2000
East-reverse		GCGAGTGACGGCTTTGTAGT		
AafAf	*aafA*	AAATTAATTCCGGCATGG	518	Huang et al., 2007
AafAr		ATGTATTTTTAGAGGTTGAC		
aggRks1	*aggR*	GTATACACAAAAGAAGGAAGC	254	Aranda et al., 2007
aggRksa2		ACAGAATCGTCAGCATCAGC		
AL65	*est*	TTAATAGCACCCGGTACAAGCAGG	147	Toma et al., 2003
Al125		CCTGACTCTTCAAAAGAGAAAATTAC		
LTL	*elt*	TCTCTATGTGCATACGGAGC	322	Toma et al., 2003
LTR		CCATACTGATTGCCGCAAT		
IpaIII	*ipaH*	GTTCCTTGACCGCCTTTCCGATACCGTC	619	Toma et al., 2003
IpaIV		GCCGGTCAGCCACCCTCTGAGAGTAC		
BFP1	*bfpA*	AATGGTGCTTGCGCTTGCTGC	326	Aranda et al., 2007
BFP2		GCCGCTTTATCCAACCTGGTA		
eae1	*eae*	CTGAACGGCGATTACGCGAA	880	Aranda et al., 2007
eae3		CGAGACGATACGATCCAG		
VIRULENCE GENES FOR EXTRA-INTESTINAL PATHOGENIC *E. coli*				
papC-forward	*papC*	GACGGCTGTACTGCAGGGTGTGGCG	350	Blanco et al., 1997
papC-reverse		ATATCCTTTCTGCAGGGATGCAATA		
SfaSf	*sfaS*	GTGGATACGACGATTACTGTG	240	Johnson and Stell, 2000
SfaSr		CCGCCAGCATTCCCTGTATTC		
Afaf	*afa/dra*	GGCAGAGGGCCGGCAACAGGC	592	Johnson and Stell, 2000
Afar		CCCGTAACGCGCCAGCATCTC		
FyuAf	*fyuA*	TGATTAACCCCGCGACGGGAA	880	Johnson and Stell, 2000
FyuAr		CGCAGTAGGCACGATGTTGTA		
KpsMIIf	*kpsMT II*	GCGCATTTGCTGATACTGTTG	272	Johnson and Stell, 2000
KpsMIIr		CATCCAGACGATAAGCATGAGCA		
QUINOLONE RESISTANCE GENES				
gyrA11753	*gyrA*	GTATAACGCATTGCCGC	251	Wang et al., 2001
gyrA12004		TGCCAGATGTCCGAGAT		
EC-PAR-A	*parC*	CTGAATGCCAGCGCCAAATT	189	Deguchi et al., 1997
EC-PAR-B		GCGAACGATTTCGGATCGTC		
qnrA-forward	*qnrA*	TCAGCAAGAGGATTTCTCA	605	Maynard et al., 2004
qnrA-reverse		GGCAGCACTATTACTCCCA		
qnrB-forward	*qnrB*	GATCGTGAAAGCCAGAAAGG	469	Robicsek et al., 2006b
qnrB-reverse		ACGATGCCTGGTAGTTGTCC		
qnrS-forward	*qnrS*	ACGACATTCGTCAACTGCAA	417	Robicsek et al., 2006b
qnrS-reverse		TAAATTGGCACCCTGTAGGC		
aac-forward	*acc-(6′)-Ib*	TTGCGATGCTCTATGAGTGGCTA	482	Park et al., 2006
aac-reverse		CTCGAATGCCTGGCGTGTTT		

(Jakobsen et al., 2011). It allows the detection of 348 virulence genes and 98 antibiotic resistance genes and variants. DNA extraction and hybridizations were performed as described previously (Bruant et al., 2006). Each isolate was assigned to a specific *E. coli* pathotype according to its virulence gene profile and based on classification described previously (Bonnet et al., 2009; Jakobsen et al., 2011). *E. coli* isolates were also assigned to a phylogenetic group based on the presence of *chuA*, *TspE4.C2*, and *yjaA* as described previously (Clermont et al., 2000).

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Table 3 | PCR control strains used in this study.

Strain	Positive gene (s)
ETEC H10407	*elt* and *est*
EHEC EDL933	*sxt1* and *sxt2*
EPEC 2349/69	*eae* and *bfpA*
EAEC O42	*aggR* and *aaf genes*
Shigella flexneri	*ipaH*
J53pMG252	*qnrA*
J53pMG298	*qnrB*
J53pMG306	*qnrS*
Salmonella SA20042859	*aac(6')-Ib*

STATISTICAL ANALYSIS

Comparisons of associations between resistant phenotypes in *E. coli* isolated from stream were performed separately by using Pearson's chi-square exact test and Fisher's exact test (STATISTICA V. 10, StatSoft, US). Spearman's rank correlation (West et al., 2011) was used to examine the relationships among temperature, pH, conductivity, dissolved oxygen, COD, BOD, total phosphorus, nitrogen, total solid suspended, mesophilic bacteria and total and fecal coliform density across all sample locations.

RESULTS

PHYSICOCHEMICAL ANALYSES OF SAN PEDRO RIVER

The physicochemical properties of each sample locations of the San Pedro River (**Figure 1**) were characterized using parameters such as temperature, DO, pH, BOD, and COD. These parameters were included because they have a major influence on bacterial growth (Salem et al., 2011). Results of physicochemical parameters are shown in the **Figure 2**. The mean value for water temperature was 24.2°C, which is usually considered a favorable temperature for the growth of microorganisms as well as for a wide range of human and animal pathogens. The pH values of water samples varied between 6.7 and 8.2 units, with a mean value of 7.6 units. The pH values were within the permissible limits (6.0–9.0) established by the WHO for wastewater discharge into the sea or the environment. Furthermore, this pH range value is optimal for bacterial growth.

The dissolved oxygen concentrations (DO) for almost all the sample locations were lower than 1 mg/L suggesting high organic matter levels. These DO concentrations are near anoxygenic conditions, which allow the growth of a broad spectrum of both aerobic and anaerobic microorganisms. For conductivity, 96.6% of the samples were above the WHO guideline value of 1000 µS/cm for wastewater discharging into a stream. These conductivity levels imply high concentrations of dissolved inorganic matter suggesting that high concentrations of inorganic nutrients are available for microbial proliferation. Significant differences in conductivity were observed between location sites close to farms and slaughterhouses ($p < 0.001$) compared to other types of sample locations.

The indicators of organic matter, COD and BOD, were generally high along sample locations (**Figure 2**). This confirms the discharge of raw wastewater of urban runoff including municipal origin into the river. The mean values were 722 and 1723 mg/L for BOD and COD, respectively, representing a high organic load in the river. Sample locations with highest levels of BOD and COD were related to farms, urban runoff, industrial sewage and slaughterhouses. All water samples were above the maximum permissible limit of organic matter (BOD < 200 mg/L) set by Mexican Norms (NOM-001-ECOL-1996) for streams discharged into rivers used for agricultural irrigation. These results showed abundant carbon and energy sources to support the heterotrophic growth of microorganisms.

Total solid suspended (TSS) were correlated with concentration of COD, BOD, and mesophilic bacteria [TSS–COD, $r_s = 0.79$, $p = 0.05$; TSS–BOD, $r_s = 0.79$, $p = 0.05$; TSS–mesophilic bacteria density, $r_s = 0.89$, $p = 0.012$)]. The measured concentrations of COD and BOD showed a strong correlation ($r_s = 1$, $p > 0.001$). All the sample locations exceed the total phosphorus threshold of 0.1 mg/L as well as the nitrogen threshold (1 mg/L). Temperature and nitrogen concentration were negatively correlated ($r_s = -0.79$, $p = 0.048$). Total phosphorus concentration was strongly negatively correlated with dissolved oxygen concentration ($r_s = -0.96$, $p = 0.003$). Literature classifies wastewater TSS as follows: TSS less than 100 mg/L as weak, TSS greater than 100 mg/L but less than 220 mg/L as medium and TSS greater than 220 mg/L as strong wastewater. Results of this study classified the sample locations located near to wastewater treatment plant effluent as weakly contaminated wastewater, which reflects the efficiency of wastewater treatment. Less contaminated sample locations close to open space were classified as medium wastewater. In total, 27 sample locations were classified as polluted sites and only three as less polluted. Locations number three (wastewater treatment plant effluent), 15 (slaughterhouse) and 25 (urban runoff) were categorized as less polluted sites in base of their physico-chemical results (**Figure 1**).

BACTERIOLOGICAL ANALYSIS

The mesophilic microorganism counts were between 10^4 and 10^6 CFU and these measurements were consistent with the high levels of organic and inorganic nutrients found in the San Pedro River, and the favorable physicochemical conditions for microbial growth found in the river (**Figure 3**). Although agricultural, farm and industrial sewage site locations tended to have greater counts of mesophilic bacteria than open space, no significative differences were found ($p > 0.05$). Half of the samples exceeded the limit of 1000 MPN/100 mL (WHO, 2006). Some samples were as low as 1 MPN and others as high as 2.4×10^4 MNP/100 mL. Some samples presented low fecal coliforms counts as low as 0.5 MPN and others were as high as 1×10^4 MNP/100 mL. Statistically significant associations were found between the levels of total and fecal coliforms and water temperature ($p = 0.02$), and between coliforms and conductivity ($p = 0.03$), suggesting fecal bacteria proliferation due to appropriate conditions in the water environment. Total and fecal coliform were strongly correlated ($r_s = 0.86$, $p = 0.023$). Industrial sewage and urban runoff sites tended to have greater total and fecal coliform densities than the agricultural, farm locations and wastewater treatment plant effluent.

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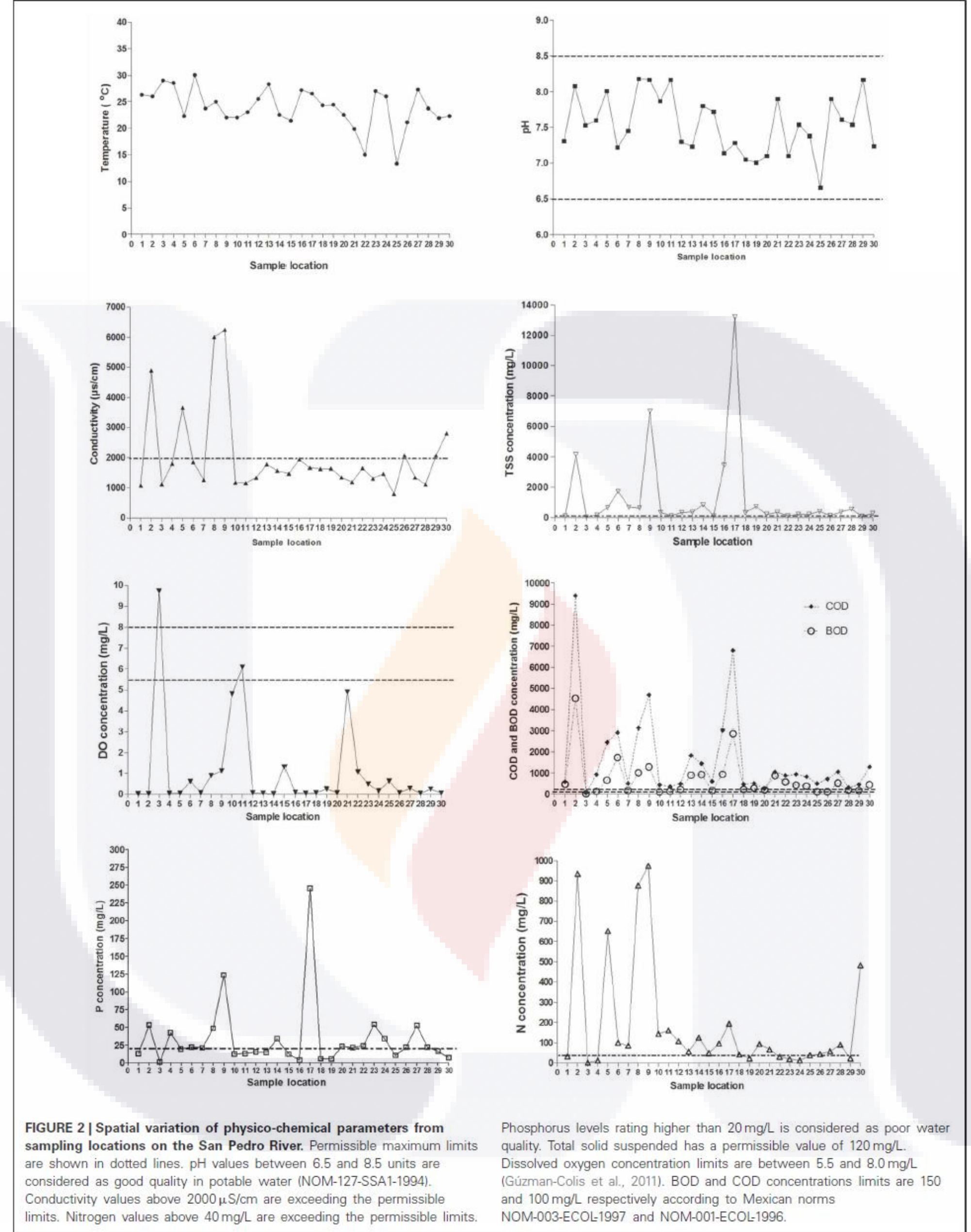

FIGURE 2 | Spatial variation of physico-chemical parameters from sampling locations on the San Pedro River. Permissible maximum limits are shown in dotted lines. pH values between 6.5 and 8.5 units are considered as good quality in potable water (NOM-127-SSA1-1994). Conductivity values above 2000 µS/cm are exceeding the permissible limits. Nitrogen values above 40 mg/L are exceeding the permissible limits. Phosphorus levels rating higher than 20 mg/L is considered as poor water quality. Total solid suspended has a permissible value of 120 mg/L. Dissolved oxygen concentration limits are between 5.5 and 8.0 mg/L (Gúzman-Colis et al., 2011). BOD and COD concentrations limits are 150 and 100 mg/L respectively according to Mexican norms NOM-003-ECOL-1997 and NOM-001-ECOL-1996.

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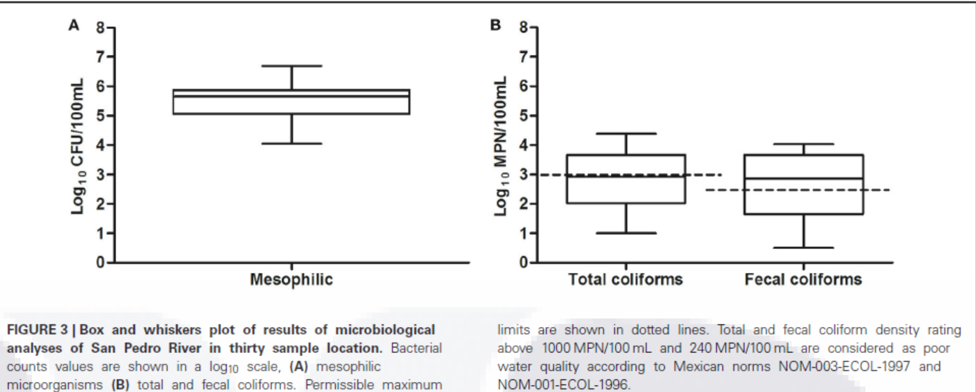

FIGURE 3 | Box and whiskers plot of results of microbiological analyses of San Pedro River in thirty sample location. Bacterial counts values are shown in a $\log_{10}$ scale, **(A)** mesophilic microorganisms **(B)** total and fecal coliforms. Permissible maximum limits are shown in dotted lines. Total and fecal coliform density rating above 1000 MPN/100 mL and 240 MPN/100 mL are considered as poor water quality according to Mexican norms NOM-003-ECOL-1997 and NOM-001-ECOL-1996.

ANTIBIOTIC RESISTANCE PHENOTYPES OF *E. coli* ISOLATES

The antimicrobial susceptibility of 150 *E. coli* isolated from the San Pedro River to 13 antimicrobial agents was measured by the disc diffusion method (CLSI, 2010). Fifty-two percent (79/150) of the isolates were resistant to at least one antimicrobial agent; 37.3% (56/150) were resistant to at least two and 30.6% (46/150) were multi-drug-resistant. A total of 59 isolates (39.3%) were resistant to ampicillin (**Figure 4**). The second most prevalent antibiotic resistance was toward trimethoprim-sulfamethoxazole (28.6%, 43/150 isolates), followed by carbenicillin (26%, 39/150 isolates), chloramphenicol (22%, 33/150 isolates), and cephalothine (17.3%, 26/150 isolates). Few isolates (1.33%; 2/150) had a resistance toward cephotaxim, netilmicin and amikacin. Interestingly, it was noticed that 7.3% (11/150) of the isolates were resistant to pefloxacin and 4% (6/150 isolates) were resistant to levofloxacin (**Figure 4**). These antibiotics are second and third-generation quinolones widely used in Mexico against intestinal and UTIs (Guajardo-Lara et al., 2009). Furthermore, 12 strains were FQ-resistant and presented a multiresistant phenotype. Most *E. coli* isolates with resistance to FQ were found in the sample locations close to farms, agricultural areas, urban runoff and industrial sewage.

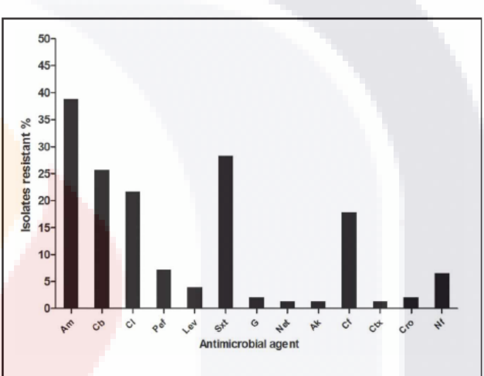

FIGURE 4 | Antimicrobial resistance (%) of *E. coli* isolated from San Pedro River. A total of 150 isolates were characterized by antimicrobial susceptibility assay. [a]Am, ampicillin; Cb, carbenicillin; Cl, chloramphenicol; Pef, pefloxacin; Lev, levofloxacin; Sxt, trimethoprim-sulfamethoxazole; G, gentamicin; Net, netilmicin; Ak, amikacin; Cf, Cephalotine; Ctx, cephatoxim; Cro, ceftriaxone; Nf, nitrofuratoin.

Among isolates with a multi-resistant phenotype, 1.3% (2/150) was resistant to seven antimicrobial agents; 3.3% (5/150) were resistant to six antimicrobial agents; 5.3% (8/150) were resistant to five antimicrobial agents, 7.3% (11/150) were resistant to four antimicrobial agents and 13% (20/150) were resistant to three antimicrobial agents. The location sites close to discharges from urban runoff and industrial sewage had a more important proportion of isolates that were resistant to multiple antibiotics (15 isolates and 10 isolates respectively, **Figure 5**). Wastewater treatment plant effluent and agricultural locations had the lowest proportion of antibiotic resistant bacteria with only two multiresistant and one resistant bacteria in each location. Urban runoff locations as well as industrial sewage, open space and slaughterhouse sample locations had the most important counts of antibiotic resistant bacteria. Urban runoff sample locations were also the locations with highest density of total and fecal coliforms. Additionally, even when farm locations presented low proportion of antibiotic resistant bacteria, these locations presented multidrug resistance patters to more antibiotic classes (**Figure A1**). Non-resistant bacteria were found in samples isolates from farms (site locations No. 8 and 30), urban runoff (site location No. 14) and agricultural (site location No. 21) locations (**Figure 5**). Furthermore, density of multiresistant bacteria was negatively-correlated with nitrogen concentration ($r_s = -0.78$, $p = 0.04$).

A noticeable result shows that a co-resistance to beta-lactams and sulfonamides was frequently observed because most of sulfonamide-resistant isolates were also resistant to beta-lactams (**Table 5**). Resistance phenotype to quinolones was associated

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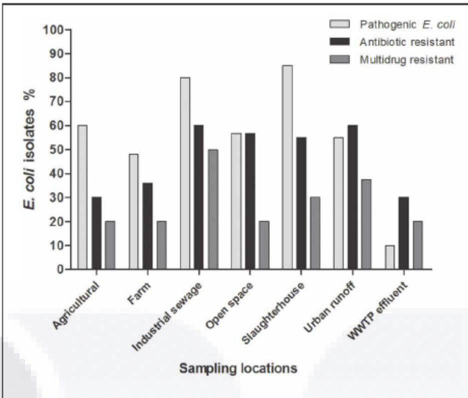

FIGURE 5 | Antimicrobial resistant, multidrug resistant, and potentially pathogenic *Escherichia coli* isolates found according to the sample location. aWWTP-E, wastewater treatment plant effluent.

with beta-lactams and sulfonamides resistance ($0.05 \geq p \geq 0.01$), and beta-lactams and phenicols resistance ($0.01 \geq p \geq 0.001$).

Wastewater treatment plant effluent sample, which was considered as less polluted based on tested parameters (**Figures 2, 3**) presented proportion of antibiotic resistant bacteria similar to that of the agricultural sector (30%). Nevertheless, samples from industrial sewage had the highest proportion of multidrug resistant bacteria (50%) suggesting that industrial sewage might have an impact on the presence of multidrug resistant bacteria.

PATHOTYPE AND VIRULENCE GENES DETERMINATION OF *E. coli* ISOLATES

Sixty percent (91/150) of the strains were PCR positive for at least one virulence gene (Aranda et al., 2007). Eighty-six isolates were identified as InPEC, including 44.6% (67/150) EAEC, 6.6% (10/150) EPEC, and 6% (9/150) ETEC (**Table 4**). Only 5 (3.3%) isolates were identified as incomplete ExPEC because these isolates were positive for the virulence genes *fyuA*, *kpsMII*, *sfa*, and *afa/dra* (Johnson and Stell, 2000). EIEC and Shiga-toxigenic *E. coli* were not detected. In addition, slaughterhouse (85%, 17/20 isolates), industrial sewage (80%, 16/22 isolates) and agricultural (60%, 6/10 isolates) sample locations had the most important proportion of pathogenic bacteria.

Furthermore, strains belonging to the pathotypes EPEC ($n = 7$), ETEC ($n = 4$), EAEC ($n = 37$) and incomplete ExPEC ($n = 4$) were at least resistant to one antimicrobial agent. Among strains with a multi-antimicrobial resistance phenotype, 22 were EAEC, 4 were ETEC isolates, 6 were EPEC, and 1 was an incomplete ExPEC. Most pathogenic *E. coli* that had multi-resistant phenotype were resistant to beta-lactams and trimethoprim-sulfamethoxazole. Amongst the commensal *E. coli*, multi-resistance was also detected in 22% (13/59) of the isolates.

Significative differences between number of pathogenic isolates ($p = 0.02$) and isolates with resistance to one antimicrobial agent ($p = 0.04$) were found when comparing polluted samples with that of less polluted sample sites. Non-statistical differences were found compared multidrug resistance ($p > 0.05$). This suggests that in polluted water there are more pathogenic bacteria (**Figure 5**).

CHARACTERIZATION OF QUINOLONE RESISTANCE IN *E. coli* ISOLATES

The genotypes associated with the quinolone resistance (including intermediate resistance) phenotype were characterized for seven pathogenic *E. coli* and 11 commensal *E. coli*. Three of 18 isolates were resistant to second generation quinolones (levofloxacin and pefloxacin), nine isolates were resistant to one of the two quinolones and six showed intermediate resistance to pefloxacin (6/18 isolates). Resistance to quinolones was usually observed in strains with a multi-drug resistance phenotype (16/18 isolates).

The sequencing results for the QRDR of *gyrA* and *parC* are summarized in **Table 6**. The Ser-83 → Leu and Asp-87 → Asn substitution in *gyrA* and the Ser-80 → Ile substitution in *parC* were found in isolates resistant to both levofloxacin and pefloxacin ($n = 2$), to levofloxacin alone ($n = 2$), and to pefloxacin alone ($n = 3$). *E. coli* isolates with only the Ser-83 → Leu and Asp-87 → Asn substitution in *gyrA* showed resistance and intermediate resistance to pefloxacin. A single mutation in *gyrA* at Ser-83 → Leu ($n = 2$) was found in one strain resistant to both (levofloxacin and pefloxacin), and one with intermediate resistance to pefloxacin. An isolates with a single mutation (Ser-80 → Ile) in *parC* exhibited an intermediate resistance toward pefloxacine. Three strains had one or more *qnr* genes. Five *E. coli* isolates possessed *qnrA*, seven isolates had *qnrS* and two isolates had *qnrB*. Overall, eight isolates had chromosomal mutations in *gyrA*, *parC* or both as well as horizontally acquired *qnr* genes. The *qnr* genes were found in two strains with a triple mutation profile (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*) that were resistant to both levofloxacin and pefloxacin. Quinolone resistance isolates ($n = 3$) with one mutation Ser-83 → Leu in *gyrA*, or, Ser-80 → Ile in *parC* possessed also *qnr* genes. The three strains with no chromosomal mutation possessed one or two *qnr* genes. One of the isolates did not have either a chromosomal mutations or *qnr* genes. In addition, the ETEC pathotype isolate 18A (**Table 6**) exhibited a multi-resistance phenotype and had three substitutions (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*), *qnrS* and the newly described *aac (6′)-Ib-cr* gene. Additionally, five isolates that had the triple mutation profile (Ser-83 → Leu, Asp-87 → Asn in *gyrA* and Ser-80 → Ile in *parC*) in the QRDR carried also one *qnr* gene (*qnrA* or *qnrS*) and at least one beta-lactamase gene.

VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES AMONG QUINOLONE RESISTANT *E. coli* ISOLATES

Microarray analysis was done on 17 quinolone resistant *E. coli* isolates. Based on the microarray analysis, nine isolates were classified as commensal and six were classified as InPEC, ExPEC, or potentially pathogenic *E. coli*. The isolates belonged to the phylotype group A (14 isolates), group D (1 isolate) and B1 (2 isolate).

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Table 4 | Antimicrobial resistance found in potentially pathogenic and commensal *Escherichia coli*.

Antimicrobial class	Antimicrobial agent	No. (%)[a] of pathogenic *E. coli* [b]				Commensal ($n = 59$)
		EPEC ($n = 10$)	ETEC ($n = 9$)	EAEC ($n = 67$)	Incomplete ExPEC ($n = 5$)	
Aminoglycosides	Gentamicin	0 (0)	0 (0)	3 (4)	0 (0)	0 (0)
	Netilmicin	0 (0)	0 (0)	1 (1)	0 (0)	1 (2)
	Amikacin	0 (0)	0 (0)	0 (0)	0 (0)	2 (3)
Phenicols	Chloramphenicol	3 (30)	3 (33)	16 (24)	1 (20)	10 (17)
Quinolones	Pefloxacin	1 (10)	1 (11)	3 (4)	0 (0)	7 (12)
	Levofloxacin	1 (10)	1 (11)	3 (4)	1 (20)	0 (0)
Sulfonamides	Trimethoprim-sulfamethoxazole	3 (30)	3 (33)	22 (33)	0 (0)	14 (24)
Beta-lactams	Ampicillin	6 (60)	4 (44)	29 (43)	1 (20)	17 (29)
	Carbenicillin	5 (50)	3 (33)	19 (28)	1 (20)	12 (20)
Nitrofurans	Nitrofuratoin	1 (10)	0 (0)	5 (7)	1 (20)	3 (5)
Cephalosporins	Cephalotine	2 (20)	1 (11)	12 (18)	0 (0)	7 (12)
	Cephatoxim	1 (10)	0 (0)	1 (1)	0 (0)	0 (0)
	Ceftriaxone	1 (10)	0 (0)	2 (3)	0 (0)	0 (0)

[a] *Percentages were calculated as follows: number of isolates with resistance phenotype/total number of E. coli isolates per pathotype per 100.*
[b] *EPEC, enteropathogenic E. coli. ETEC, enterotoxigenic E. coli. EAEC, enteroaggregative E. coli. Incomplete ExPEC, incomplete extra-intestinal pathogen E. coli.*

Table 5 | Association between antimicrobial resistance phenotypes of *E. coli* isolates from stream water.

Antimicrobial agent		Phenicols	Quinolones		Sulfonamides	Beta-lactams		Cephalosporin
		Chloramphenicol	Pefloxacin	Levofloxacin	Trimethoprim-sulfamethoxazole	Ampicillin	Carbenicillin	Cephalotine
Quinolones	Pefloxacin	++						
	Levofloxacin	–	+					
Sulfonamides	Trimethoprim-sulfamethoxazole	++	++	++				
Beta-lactams	Ampicillin	++	+++	++	–			
	Carbenicillin	++	++	–	+	+		
Nitrofurans	Nitrofuratoin	+	+	–	–	–	+++	
	Cephalotine	–	+++	++	+++	+++	+++	
Cephalosporin	Cephatoxim	–	–		–	+	–	–
	Ceftriaxone	++	–	–	–	++	–	+

Only the antimicrobial multi-resistant phenotypes that exhibited an association with another phenotype at the $p < 0.05$ level are shown. The levels of significance of the association as assessed by the chi-square exact test were as follows: –, $p > 0.05$; +, $0.05 \geq p \geq 0.01$; ++, $0.01 \geq p \geq 0.001$; +++, $0.001 \geq p$.

The most frequent antimicrobial resistance genes were *aph3strA* (10/17), bla_{TEM} (10/17) and *tet(B)* (5/17), which code for resistance to streptomycin, ampicillin, and tetracycline respectively (**Table 6**). Class 1 integron markers were also found in ten isolates. A bla_{TEM} and *aph3(strA)* combination was observed in nine isolates, and a bla_{TEM}, *aph3(strA)* and *tet(B)* combination was observed in six isolates. In our study, 10 quinolone resistance isolates also carried bla_{TEM} gene. Among these 10 isolates, nine isolates were positive for the beta-lactamase gene and a plasmid acquired resistance gene.

Isolates with *qnr* genes and the triple mutation patter in the QRDR were found in sample locations close to farm and agricultural sector (**Figure 1**, **Table 6**). Nevertheless, isolates with intermediate-resistance to FQ phenotypes were found in sample locations close to urban runoff, industrial sewage, slaughterhouses, open space and wastewater treatment plant. Interestingly, samples with resistance to one or other FQ phenotype were sampled in locations close to urban runoff, industrial sewage, open space, farm, and agricultural sectors (**Table 6**, **Figures 1**, **5**).

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Table 6 | Summary of the pathotype, antimicrobial resistance patterns, QRDR mutation and presence of *qnr* resistance genes for the 18 *E. coli* isolates selected for their resistance to fluoroquinolones of second (pefloxacin) and third (levofloxacin) generation.

Strains ID	MDR phenotype[a]	Diffusion disc[b]		QRDR mutation		PMQR genes	Other resistance genes	*E. coli* type[c]
		LEV	PEF	→GyrA	→ParC			
16C	AmCbClSxtLevPef	R	R	S83→L, D87→N	S80→I	*qnrA*	*aph3(strA)*, *bla*$_{TEM}$, class 1 integron	Incomplete ExPEC
18A	AmCfCtxCroLevPefStx	R	R	S83→L, D87→N	S80→I	*aac-(6')-Ib-cr*, *qnrS*	*aph3(strA)*, *aph6(strB)*, *bla*$_{TEM}$, *tet(B)*, *tet(M)*	InPEC
18C	AmCfCtxCroLevPefStx	R	R	S83→L	None	*aac-(6')-Ib-cr*, *qnrS*	*aph3(strA)*, *aph6(strB)*, *bla*$_{TEM}$, *dhfrVII*, *tet(B)*	InPEC
17A	AmGLevSxt	R	S	S83→L, D87→N	S80→I	None	*bla*$_{TEM}$, *aac(3)-IIa(aacC2)*, *aph3(strA)*, *mphA*, *sulI*, class 1 and 2 integron	InPEC
17D	AmCbGLev	R	S	S83→L, D87→N	S80→I	None	*aph3(strA)*, *bla*$_{TEM}$, *aac(3)-IIa(aacC2)*, *mphA*, *sulI*, *sulII*, *tet(B)*, class 1 integron	InPEC
17E	AmCbLev	R	S	None	None	None	*aph3(strA)*, *bla*$_{TEM}$, *dhfrXII*, *sulII*, *tet(A)*	Incomplete ExPEC
24A	AmCbClCfPefSxt	S	R	S83→L, D87→N	S80→I	*qnrA*	*aph3(strA)*, *bla*$_{TEM}$, *dhfrXII*, *mphA*, *sulII*, *tet(B)*, class 1 integron	Commensal
16D	AmCbClPefSxt	S	R	S83→L, D87→N	S80→I	*qnrA*	*aph3(strA)*, *bla*$_{PSE}$, *bla*$_{TEM}$, *catI*, *dhfrXII*, *mphA*, *tet(A)*, *tet(B)*, *sulI*, *sulII*, class 1 and 2 integron	Commensal
24C	AmCbClPefSxt	S	R	S83→L, D87→N	S80→I	None	*cmlAI*, *dhfrXII*, class 1 integron	Commensal
24E	AmCbClPefSxt	S	R	S83→L, D87→N	S80→I	None	*dhfrXII*, *cmlAI*.	Commensal
1A	CfNfPef	S	R	S83→L, D87→N	None	*qnrS*	*aph3(strA)*	Commensal
22E	Pef	S	R	None	None	*qnrS*	ND	Commensal
3A	AmClStxPef	S	I	None	None	*qnrA*	*aph3(strA)*, *bla*$_{PSE}$, *dhfrVII*, *sulII*, *tet(A)*, class 2 integron	InPEC
2E	AmClStxPef	S	I	S83→L, D87→N	None	None	Class 1 and 2 integron.	Commensal
29A	AmCbNfSxtPef	S	I	None	S80→I	*qnrA*, *qnrB*	*bla*$_{PSE}$, *dhfrXII*, class 1 integron	InPEC
20A	AmPef	S	I	None	None	*qnrB*, *qnrS*	*aph3(strA)*, *bla*$_{TEM}$	Commensal
29E	AmCfCbSxtPef	S	I	None	None	*qnrS*	*aadA (1)*, *bla*$_{TEM}$, *dhfrI*, *sulI*, class 1and 2 integron, transposon *Tn21*	InPEC
25A	AmCbPef	S	I	S83→L	None	*qnrB*, *qnrS*	Transposon *Tn21*	Commensal

[a]*Multi-drug resistance (MDR) phenotype, Am, ampicillin; Cb, carbenicillin; Cl, chloramphenicol; Pef, pefloxacin; Lev, levofloxacin; Sxt, trimethoprim-sulfamethoxazole; G, gentamicin; Net, netilmicin; Ak, amikacin; Cf, Cephalotine; Ctx, cephatoxim; Cro, ceftriaxone; Nf, nitrofuratoin. ND, not determined.*

[b]*R, resistant; I, intermediate resistance; S, susceptible.*

[c]*InPEC, intestinal E. coli; ExPEC, extraintestinal E. coli.*

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DISCUSSION

Aquatic environments such as rivers and streams are considered ideal reservoirs for antibiotic resistance dissemination, since antimicrobials and antimicrobial resistant bacteria are often directly released in the environment (Roe et al., 2003; Zhang et al., 2009b; Lupo et al., 2012). In developing countries, the contribution of sewage and wastewater as point sources, as well as animal farming without adequate outlet control, represent extra loads of contamination for aquatic systems. Most of the water from these activities is subsequently used for irrigation, without any treatment. This can represent potential risks of contamination (Mazari-Hiriart et al., 2008; Zhang et al., 2009c; Jang et al., 2013). In the State of Aguascalientes in Mexico, this is of great importance because river water is directly used for irrigation and in rural communities as a source of drinking water for livestock. Our results showed that the concentration of coliforms in San Pedro River and its major creeks was exceeding by more than one order of magnitude the WHO tolerance limits (**Figure 3**). The physicochemical values and microbiological counts found in the San Pedro River are consistent with an important pollution originating from municipal and livestock wastewater (**Figures 1, 2**). Furthermore, these conditions provide a favorable environment for microbial growth (**Figures 2, 3**). Given that the water from the San Pedro River is used for irrigation, the water may constitute a source of bacterial contamination that could infect humans or animal through direct contact, aerosol, or consumption of vegetables.

In Mexico, antibiotics are medical drugs with high rate of consumption and their consumption is associated with a high rate of misuse (Dreser et al., 2008). Misuse is caused by unwarranted prescription, inappropriate choice of treatment, self-prescription, lack of adherence by consumers, as well as lax regulation on the use of antibiotics (Zaidi et al., 2003; Amábile-Cuevas et al., 2010). The misuse and overuse of antibiotics is particularly important because it may contribute to selection and increased occurrence of antimicrobial resistant bacteria.

Anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes and antimicrobial resistant bacteria usually relevant in clinical environments (Tacão et al., 2012). Our results indicated that samples from industrial and urban runoff sewage had an important presence of antibiotic resistance bacteria (**Figure 5**). Moreover, the highest number of multi-resistant isolates was found in samples from wastewater discharges from human sewage, industrial sector, and farms. This suggests the importance of wastewater discharges in the dissemination of antimicrobial resistance strains. In the multi-resistant isolates, resistance to FQs such as pefloxacin and levofloxacin was significatively associated with resistance to trimethoprim-sulfamethoxazol and ampicillin ($p < 0.05$). This association is likely due to the presence of *qnr* genes and beta-lactams resistance genes in the multi-resistant isolates (Wang et al., 2001).

At least two thirds of all *E. coli* isolates were resistant to beta-lactams such as ampicillin and carbenicillin, and at least one third of the isolates were resistant to trimethoprim-sulfamethoxazole and chloramphenicol (**Figure 4**). The presence of antibiotic resistant *E. coli* was also observed in other studies from human and animal fecal sources, wastewater treatment plant and surface water (Sayah et al., 2005; Ibekwe et al., 2011; Mokracka et al., 2011; Sun et al., 2012). Similar resistance levels were found in *E. coli* isolated from children and adults in Latin America and the 53.2 and 57.7% isolates were resistant to ampicillin and trimethoprim-sulfamethoxazol, respectively (Estrada-Garcia et al., 2005, 2009).

Most *E. coli* identified as of pathogenic or potentially pathogenic were classified as intestinal pathogens (61%, 91/150). EAEC was the most prevalent intestinal pathogenic *E. coli* (44% of the isolates), and this is consistent with previous studies conducted in Mexico (Estrada-Garcia et al., 2005), since in clinical settings, EAEC and ETEC are the most prevalent pathotypes in Mexico (Estrada-Garcia et al., 2005, 2009). Furthermore, several intestinal pathogens with multiple-resistance to antimicrobials were isolated from the River. Thus, the occurrence of pathogenic *E. coli* with multiple antimicrobial resistances in the San Pedro River represents a great concern due to possible transfer of resistant genes and may increase the probability of infections with a higher cost of treatment.

In our study, most of the isolates resistant to levofloxacin and pefloxacin had a multi-resistant phenotype and some were potentially pathogenic *E. coli*. Similar results were found in a Mexican study on the prevalence of FQ resistance among *E. coli* isolates from urinary tract infection (Zaidi et al., 2003; Llanes et al., 2012) as well as from an environmental study (Amábile-Cuevas et al., 2010). In addition, we showed that the triple mutation profile (Ser-83 → Leu, Asp-87 → Asn in *gyrA*, and Ser-80 → Ile in *parC*) was the most prevalent. The point mutations Ser-83 → Leu and Asp-87 → Asn found in *gyrA* and Ser-80 → Ile in *parC*, have been observed in other studies (Namboodiri et al., 2011; Sun et al., 2012). Previous studies have shown that *E. coli* with a single mutation (Ser-83 → Leu) in the *gyrA* subunit are resistant to nalidixic acid, a first generation quinolone (Vila et al., 1994; Sun et al., 2012). In addition, most fluoroquinolone resistance isolates carried horizontally acquired quinolone resistance genes and these were found primarily in combination with QRDR mutations. Qnr proteins may supplement resistance to quinolones due to altered quinolone target enzyme, efflux pump activation, or deficiencies in outer-membrane porins (Martinez-Martinez et al., 2003; Poirel et al., 2012). The presence of Qnr determinants facilitates the selection of low-level of resistance to quinolones encoded on the chromosome-encoded and the selection of higher-level resistance mutation (Jacoby, 2005). Several strains that harbored *qnrS* also carried bla_{TEM} gene. It was reported that, *qnrS* genes are associated with transposons containing TEM-1 type -lactamases (Hernandez et al., 2011; Dalhoff, 2012). Tetracycline and streptomycin resistance genes were also detected along with FQ resistance genes (Roberts, 2005; Zhang et al., 2009a).

Our results revealed the presence of pathogenic *E. coli* in the river with present mobile elements as integrons, and multidrug resistance characteristics, including FQ resistance, an antibiotic highly used in humans and animals worldwide, mostly found in locations of the river that have been impacted by industrial sewage and urban runoff. This situation highlights the risk of multidrug resistance pathogens dissemination (Allou et al., 2009; Dalhoff, 2012; Sun et al., 2012). Furthermore, our study was conducted in

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a densely populated area, a setting that is often encountered in developing countries and that must be taken into account (Mazari-Hiriart et al., 2008). This poses a potential risk for human infections because water is used for consumption or for recreation (Maynard et al., 2005; Hamelin et al., 2006, 2007).

The permanent influx of pollutants such as antimicrobial agents, detergents, disinfectants, heavy metals, livestock waste, and watershed may contribute to the emergence of antibiotic resistant bacteria in water as well as the spread of antimicrobial resistance genes and virulence bacteria in San Pedro River. The results show that it is urgent to evaluate the management of wastewater and the water quality in San Pedro River and if necessary, implement a local wastewater treatment to prevent the emergence of infectious outbreaks.

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Antimicrobial resistance: the role of aquatic environments

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KEYWORDS

Antimicrobial resistance, multi-drug resistance, environment, wastewater, biofilms

A B S T R A C T

Antimicrobial resistance is a major concern worldwide. Antibiotics are one of the most used drugs in humans and animals. The intensive use of antimicrobial agents is one of the main factors for the generation of resistant bacteria. Infections with resistant organisms and the emergence of multi-drug resistant pathogenic and non-pathogenic bacteria, represents an urgent global challenge since increases the incidence of morbidity and mortality and decreases the effectiveness of infectious diseases treatments. Many of the encoding antimicrobial resistant genes from human pathogens have originated in natural environments. Anthropogenic activities on aquatic environments, sediments and soils, such as hospital, pharmaceuticals and municipals discharges into rivers and lakes increase the generation of new resistance genes and the spread of resistant bacteria in the environment as well as in public health. This highlights the importance of the environment as a reservoir of resistance genes and dispersal vector. It is necessary to take measures to reduce the introduction and spread of resistant bacteria and its determinants in the environment, which could contain diverse chemicals, antibiotic residues or quaternary ammonium compounds. These measures include the management of wastewater treatment.

Introduction

An antibiotic is a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi, or protozoa (Kümmerer, 2009a). They could be

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natural (produced by micro-organisms), semi-synthetics (derivatives of natural antibiotics with structural modifications) and fully synthetics. Several antibiotics are produced by environmental microorganisms, including bacteria within soils and water (Finley et al., 2013). Thus, genes for antibiotic resistance must also have emerged in non-clinical habitats (Martínez, 2008).Antibiotics are widely used for prevent infections in human being and animals, as growth promoters in animal's husbandry and as prophylactic treatment in aquaculture. Thus, it is a priority conserves the antibiotic efficacy. Nevertheless, resistance to all classes of antibiotics has emerged, and there are no antibiotics for which resistance does not exist (Wright, 2010). For decades, clinicians and scientist have asked to make rational use of antibiotics, in an effort to avoid antibiotic resistance and its dispersion in the environment. However, the intensive use, misuse and the selection pressure applied by antibiotics used in clinical and agricultural settings have promoted the evolution and spread of genes that confer resistance (Allen et al., 2010).

After the administration, antibiotics are excreted unchanged and/or not fully metabolized into the environment (Kümmerer, 2003). Therefore, a mixture of pharmaceuticals and their metabolites will enter municipal sewage and wastewater treatment plants (WWTP) (Kümmerer, 2004), and can reach the environment. In recent years, antibiotics contamination is recognized as an emerging environmental pollution in aquatic environments, because of their potential adverse effects on the ecosystem and human health (Huang et al., 2001; Kümmerer, 2009a). The anthropogenic activities (including sewage discharge, wastewater treatment, drug manufacturing and intensive agricultural livestock) are changing environmental reservoirs of resistance genes and their precursors: "*the resistome*" (D`Costa et al., 2006; Finley et al., 2013). Thus, the probability of recruitment of resistance genes into clinically relevant pathogens will increase (Peak et al., 2007; Knapp et al. 2011; Finley et al., 2013)because environmental bacteria act as an unlimited source of genes that might be resistance genes when entering in pathogenic organisms (Baquero et al., 2008). Furthermore, as pharmaceuticals are constantly released into the environment, organisms will be exposed to many of these compounds for their entire lifetime, exerting their effect onendogenous environmental bacteria (Boxall, 2004). Therefore, it is a concern, the potential bioaccumulation and persistence of released pharmaceuticals (Hernandes-Coutinho et al., 2013). It is highly importance to consider the environment when is intended the management of the antibiotic resistance and their spread into public health. A better understanding of antibiotic resistance prevalence and diversity in the environment will help elucidate resistant gene movement between environmental and clinical pathogenic bacteria (Martínez, 2008).In this review, is discussed how antimicrobial resistance and their determinants arise and disseminate in natural environments, focusing in aquatic environments and the anthropogenic impact on them.

The resistome: link between clinic and environment

Antibiotics and resistance mechanisms are ancient. Environmental bacteria harboring resistance mechanisms against β-lactams, vancomycin and tetracycline may be more than 30,000 years old (D'Costa et al., 2011). Indeed, some natural antibiotic resistance bacteria appeared on the planet over 100 million years ago (Hernandes-Coutinho et al., 2010). The antibiotic "resistome" is the

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collection of all genes that directly or indirectly contribute to antibiotic resistance, in both, the environment and the clinic background (Perry et al., 2013).The "resistome" includes the genes that codify for the resistance determines as well as their precursors that could be linked to antimicrobial resistance(Figure 1)(Wright, 2007).The environmental "resistome" is a significant source of resistance genes, and it has been theorized that antibiotic producing organisms in the environment are the source of resistance genes found in clinical pathogens (Perry et al., 2013). Therefore, environmental bacteria carry genes encoding resistance to antibiotics have become critically important in public health (D'Costa et al., 2011).

One of the more distinctive characteristics of microbial genomics is the movement of genes vertically (through populations by cell division), and horizontally (across species and genera)(Perry et al., 2013). This movement is allowed by the "*mobilome,*" the genetic elements that facilitate and contribute to horizontal gene transfer (HGT) (Siefert, 2009; Gaze et al., 2013). Three principal mechanisms are involved in HGT: conjugation (direct cell-to-cell transfer), transduction (phage-assisted transfer), and natural transformation (DNA-to-cell transfer, Figure 1). These mechanisms mobilize genetic elements, such as plasmids, genetic islands, and phages that can contain resistance elements (Colomer-Lluch et al., 2011; Heuer et al., 2012; Gaze et al., 2013). Furthermore, resistance gene cassettes can be collected by integrons that drive tandem genes from a single promoter element offering stunning multidrug resistance phenotypes (Patridge et al., 2009). Moreover, resistance genes can be mobilized within the chromosome and to plasmids by transposable elements (Gaze et al., 2013). The "*mobilome*" is key to the spread of genes encoding resistance to antimicrobial drugs and heavy metals, and for pathogenic traits among bacteria which is helped by the movement through the environment (Hawkey et al., 2009). Thus, no regions are safe or can escape the introduction and movement of antimicrobial drug–resistant organisms and their genes (Gaze et al., 2013). Moreover, this mobilization is enhances by the selection pressure apply by human activities, providing the opportunity of natural bacteria and bacteria introduced by anthropogenic way into the environment for multiple-resistant phenotypes (Gaze et al., 2013).

One example of antimicrobial resistance exchange between clinical and environmental resistomes its showed in the class-A extended-spectrum β-lactamase CTX-M, found on plasmids carried by the globally important pathogens and traced to environmental *Kluyvera* spp. (Humeniuk et al., 2000), and the quinolone resistance gene *qnr*, found on a broad-host range conjugative plasmid from a ciprofloxacin–resistant strain of *Klebsiella pneumoniae* and traced to the environmental water-borne species *Vibrio*, *Shewanella*, and *Aeromonas*(Poirel et al., 2005; Baquero et al., 2008; Cattoir et al., 2008). Forsber et al. (2012) also showed evidence of resistance exchange from the two backgrounds. They showed a multi-drug resistant in Proteobacteria isolated from soil, which contain resistance cassettes against five classes of antibiotics (β-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) that have perfect nucleotide match to genes from diverse human pathogen. Thus, the environmental resistome represents a deep pool reservoir of antimicrobial drug resistance elements that can be readily mobilized into human pathogens resulting in disease that is increasingly challenging to treat (Gaze et al., 2013).

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Figure.1 Microbial resistome: resistance mechanisms and spread of drug resistance

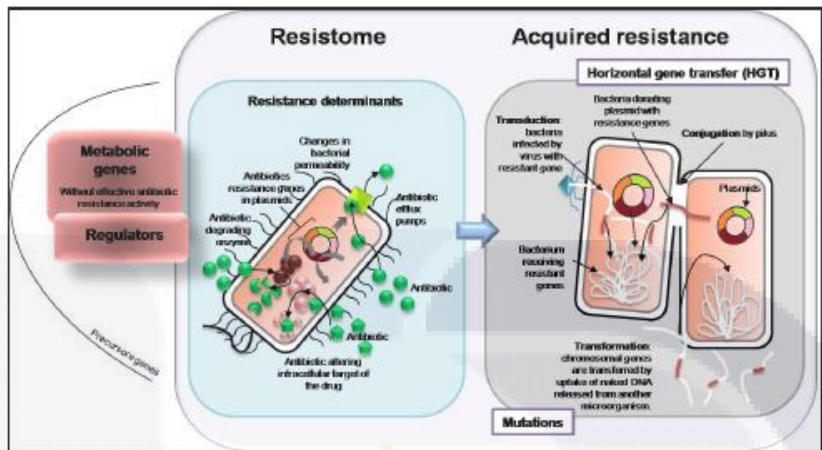

Figure.2 Presence and fate of pharmaceuticals in the environment

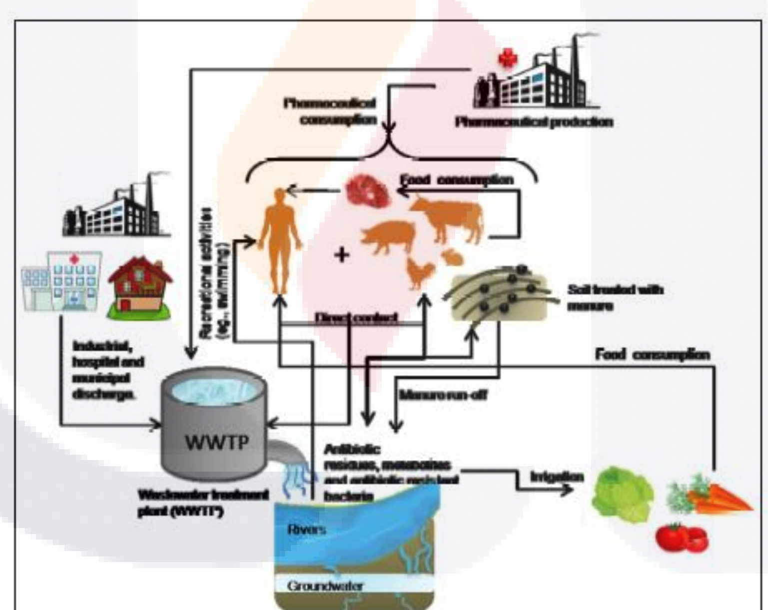

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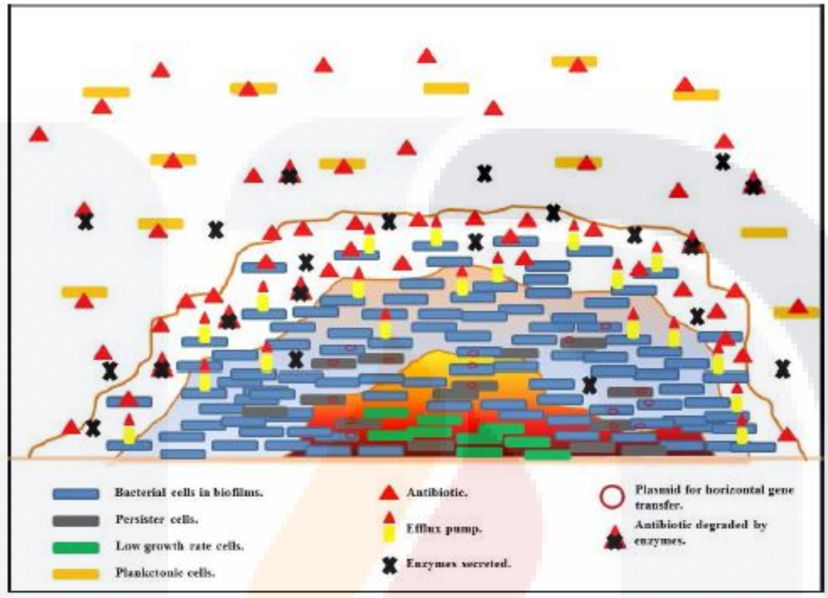
Figure.3 Antimicrobial resistance in biofilms. Resistance to antimicrobial agents is the most important cause of non effective therapy of biofilm-associated infections. The increased resistance of bacteria is due to: 1) Difficulty of diffusion of antibiotics in to them and electrostatic charge of the exopolysaccharides which attract oppositely charged antibiotics. 2) A low growth rate. 3) Changes in phenotype acquired by bacteria forming biofilms. 4) Inactivation of antibiotics by polymers and enzymes secreted by bacteria



Antibiotics spread in aquatic environments

One of the main routes of spread of pathogenic and/or antibiotic resistant bacteria is the environment, including water, soil and air. Multi-drug resistant bacteria have been detected in various water sources including rivers, lakes, groundwater and drinking water (Hamelin et al., 2006; Hamelin et al., 2007; Lindberg et al., 2007; Managaki et al., 2007; Thomas et al., 2007; Baquero et al., 2008; Gulkowska et al., 2008; Marti et al., 2013; Ramírez-Castillo et al., 2013).Consumption and handling of water, whether treated or untreated, can lead to colonization of the gastrointestinal tract of humans and animals by bacteria containing resistance genes and exchange genes with bacteria already present in the intestinal tract (Baquero et al., 2008; Coleman et al., 2012; Finley et al., 2013). After use the antimicrobial agents, antibiotics and metabolites can directly enter to the environment through discharges from antimicrobial industries producing (Fick et al., 2009; Wellington et al., 2013), slurry, manure run-off, municipal and hospital discharges and sewage system (Figure 2).

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Industrial and municipal discharges containing antibiotics generally at low concentrations, reach WWTP, and by contact with activated sludge encountered during the processing of wastewater, it may associated with these and enter into agriculture land when sludge are used as fertilizers (Kinney et al., 2006).Furthermore, irrigation with treated water and surface water is also a way for antibiotics entering agricultural soils (Kinney et al., 2006), then, contaminating products of human consumption with antibiotics (Finley et al., 2013). Veterinary pharmaceuticals and their metabolites can be released directly into the environment due to the use in aquaculture and treatment of farming animals, or indirectly during application of manure and slurry from intensive livestock (Boxall et al., 2004). Moreover, compounds that are released onto the soil and can be transported by surface water and groundwater (Blackwell et al., 2007;Topp et al., 2008) and even can be cycled and re-cycled in the environment (Wellington et al., 2013).

Indeed, more than 100 antibiotics have been detected in rivers, lakes and streams throughout the world at concentrations from nanogram(ng) until ug/L(Daughton and Ternes, 1999; Jorgensen and Halling-Sorensen, 2000; Kümmerer,2001). In surface water, antibiotics are often detected, except for the pristine sites in mountains before streams reach urban and agricultural areas (Yang and Carlson, 2003). Nevertheless, some antibiotics had been found in groundwater even as deep as more than 10 m (Batt et al., 2006).

Humans and animals can be exposed to antibiotics, antimicrobial resistance genes or resistant bacteria in the environment by different pathways (Figure 2): cultures exposed to activated and/or sludge, animals (chicken, cattle, pig, etc.,) that have been treated with antibiotics as drugs or growth promoters, groundwater and surface water containing residues of pharmaceuticals and used as drinking water or irrigation, inhalation of dust emitted by livestock facilities and food crops (Heberer et al., 2002; Hamscher et al., 2003; Kumar et al., 2005; Boxall et al., 2006; Farkas et al., 2007, Benotti et al., 2009; Wellington et al., 2013).

Fresh water also serves as an important vehicle for spreading antibiotic resistance (Lupo et al., 2012). In fact, the newly recent New Delhi metalo-β-lactamas (NDM-1) gene, which confers resistance to β-lactams, was found in high prevalence in chlorinated drinking water in India (Baquero et al., 2008; Walsh et al., 2011).

Although antibiotics may persist in the environment for long periods (months or years, e.g., tetracyclines and fluoroquinolones) and some of them may not be biodegradable (Kay et al., 2004; Boxall et al., 2006; Monteiro et al., 2010), these pollutants are not reported as toxic, persistent or bioaccumulative substances. Thus, few efforts have been made to avoid contamination by pharmaceuticals in the environment even though its prevalence is noticeable (Kümmerer, 2009; Wellington et al., 2013).

An example of the effect of antibiotic contamination is that of the quinolone resistance gene *qnr*, which is present in the chromosomes of waterborne bacteria (Poirel et al., 2005), after being integrated in plasmid, where it is constitutively expressed, *qnr* contributes to low-level of resistance to fluoroquinolones (Martinez-Martinez et al., 1998). Furthermore, other types of contaminations such as heavy metal pollutions (McArthur and Tuck field, 2000), quaternary ammonium compounds (QAC)

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(Hegstadet al., 2010) and personal care products might result in the presence of antimicrobial resistant bacteria. Stress conditions, which are usually found in polluted environments, have also the potential to increase recombination and HGT favoring the dissemination of antibiotic resistance genes (Beaber et al., 2004; Martínez, 2008). It is notice that, anthropogenic activities increase in the concentration of antibiotics in natural ecosystems influence antibiotic resistance, and may also affect the diversity of microbial population dynamics in different natural environments (Martinez, 2008).

The impact of the wastewater treatment plants in the antibiotic resistance

The importance of water environments for gene exchange is that they are mixing environmental and clinical organisms, including pathogenic bacteria (Perry et al., 2013). Antibiotics and antibiotic resistant bacteria (ARB) can enter into WWTP through various sources, such as industrial waste, hospital discharges and municipal discharges and run-off manure from intensive livestock. Both, antibiotics and ARB, are affected by the different treatment process. Untreated wastewater in the WWTP are important reservoirs for human and animals commensal and pathogenic bacteria in which antibiotic resistant organisms persist and as well as their determinants (Zhang et al., 2009). Furthermore, since microbial density is usually high, and is conform from diversity on microorganisms (commensal bacteria, environmental microorganisms, clinically pathogens, and biofilms from activated sludge), mayfacilitate the antibiotic resistant dissemination by HGT (Schluter et al., 2007; LaPara et al., 2012).

Usually, the WWTPs reduce concentrations of nutrients in the water and thus, the total number of bacteria at the end of the treatment (Zhang et al., 2007). However, even resistant bacteria are eliminated quite well from sewage in WWTP (Kümmerer, 2009b), the treatment is often not adequate for the removal of antimicrobial resistance genes (ARGs) that are release into the environment, primarily through direct discharges to rivers and lakes (Mokracka et al., 2012; Munir et al., 2013). In fact, studies have shown that antibiotics are not completely degraded during the treatment process (Marti et al., 2013). Several studies have demonstrated the presence of antibiotic residues in treated water at concentration of nanograms per liter (Andreozzi et al., 2003; Janzon et al., 2005; Zhang et al., 2011), including non-biodegradable compounds such as erythromycin, clarinthromycin, amoxicillin, and sufamethoxazole (Monteiro et al., 2010).Although antibiotics are diluted at concentrations below the minimum inhibitory concentrations (MIC) on ceriches the river might apply a pressure to the development of resistant bacteria (Marti et al., 2013). Additionally, process treatments in the WWTP may also affect to emerge ARB. Treatments such as chlorination may lead to impairment to the microbial populations of WWTP, with the selection of bacteria resistant to chlorination (e.g., *Bacillus subtilis* and *Bacillus liceniformis*), which in turn, contribute to the particular selection of resistant genes (Macauley et al., 2006; Baquero et al., 2008).However, several treatments are effective to treat the antibiotics pollutants. In a conventional wastewater treatment, which includes mechanical treatment, sedimentations, and biological treatment, may eliminate around 80% of the fluoroquinolones or tetracyclines before entering rivers (Sukul et al, 2007; Gulkowska et al., 2008; Baquero et al., 2008). Indeed, some authors have demonstrated that the adsorption, biodegradation, disinfection and membrane

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separation are the best routes for removal antibiotics in water treatment process (Zhang et al., 2011; Golet et al., 2003; Kim et al., 2005), including antibiotics such as ampicillin, norofloxacin, ciprofloxacin, ofloxacin, tetracyclien, roxithromucin and trimethoprim (Zhan et al., 2011). Water disinfection with ClO_2 may contribute to removal of β-lactam agents (Baquero et al., 2008). Applications of techniques such as coagulation, granular activated carbon, and ion treatment are promising approaches for the removal of sulfonamides (Baquero et al., 2008; Choi et al., 2008). Nevertheless, antibiotics and compounds cannot be completely eliminated and enter the environment through sewage sludge and effluents. Distribution and fate of pharmaceuticals are dependent on a range of factors, such as the physico-chemical properties of the drug (e.g., polarity and water solubility), several processes such as partitioning to soil and sediments, and degradation in the aquatic and soil environment (Boxall et al., 2004; Daughton and Ternes, 1999), as well as, environmental characteristics, such as climate and soil type (Boxall et al., 2004; Monteiro et al., 2010).

Antibiotic resistance bacteria on biofilms

Bacteria use different pathways to become resistant to antibiotics, biofilms is one of them. In biofilms, single-celled individuals gather together to form asedentary but dynamic community within a complex structure, displaying spatial and functional heterogeneity (Bordi and de Bentzmann, 2011). The biofilms are composed of bacterial cells embedded in a complex, self-produced polymeric matrix, in which the cells may be attached to biotic or abiotic surfaces, and differ radically from planktonic cells. Biofilms have been described as being present in most bacterial habitats both in the environment and in the human body, and they are highly relevant in clinical settings where they cause many types of chronic infections (Stoodleyet al., 2002; Burmølleet al., 2014).

Biofilm formation is induced by a variety of stresses, including nutrient limitation, iron limitation, and cell envelope stress. In turn, the biofilm phenotype functions as a barrier that provides protection against environmental stresses (Grant and Hung, 2013). Resistance to antimicrobial agents is the most important cause of non-effective therapy of biofilm associatedinfections (Ciofu and Tolker-Nielsen, 2011; Marcinkiewiczet al., 2013). The increase in resistance exhibited by bacteria in biofilms can be profound, rendering the cells 10–1,000 times less susceptible to specific antimicrobial agents compared with bacteria in planktonic cultures (Maddox, 2011). This phenomenon is called as biofilm-associated resistance. For example, Archambaultand cols (2012) reported that the bacteria *A. pleuropneumoniae* in a biofilm were 100 to 30,000 times more resistant to antimicrobials than their planktonic counterparts. These bacterial biofilms found in natural and pathogenic conditions are formed in the presence of multiple species and genetically distinct strains (Pereira et al., 2010). In general, the increased resistance of bacteria is due to: 1) difficulty of diffusion of antibiotics in to them and electrostatic charge of the exopolysaccharides which attract oppositely charged antibiotics; 2) a low growth rate; 3) changes in phenotype acquired by bacteria forming biofilms; and, 4) inactivation of antibiotics by polymers and enzymes secreted by bacteria (Figure 3). Bacteria in biofilms are also resistant to antibodies, bactericidal enzymes and disinfectants, such as hypochlorite and glutaraldehyde (Loera-Muroet al., 2008; Loera-Muro et al., 2012). Moreover, biofilms are uniquely suited for horizontal

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gene transfer because they sustain high bacterial density and provide a stable physical environment with cell–cell contact (Hannan et al., 2010; Burmølle et al., 2014). Horizontal gene transfer through conjugation and transformation occurs often in biofilm. The large amount of extracellular DNA (eDNA) in biofilms is likely to be an important common source of usable genetic information for members of the biofilm community (Jakubovics et al., 2013; Burmølle et al., 2014). For example, the tetracycline resistance determinant (TetM) has been shown to be spread among different bacteria within oral biofilms by transformation (Burmølle et al., 2014). In natural aquatic environments, outside animal host, it have been found the presence of pathogenic bacteria species forming biofilms, such as *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Mycobacterium ulcerans* (Hall-Stoodley et al., 2005). Moreover, important waterborne bacterial pathogens which can infect the gastrointestinal tract of humans and warm-blooded animals such as *Salmonella enteric*, *Shigella spp*., *Vibrio cholera*, pathogenic *E. coli* variants, *Yersenia enterocolitica*, *Campylobacater spp*., and *Helicobacter pylory* have the potential to become components of microbial communities in water biofilm (Wingender, 2011; Wingender et al., 2011). Biofilm producing bacteria (including pathogens) are an important factor that has to be included in risk assessment applied to water-related pathogens and dispersion or ARGs (Wingender et al., 2011).

Surveillance programs and risk assessment

The potential transmission of antimicrobial resistant bacteria among bacteria and/or their resistance determinants to humans and animals is one of the biggest concerns worldwide. Many monitoring and surveillance programs of antimicrobial resistance in several sectors have been established throughout the world. Surveillance of antimicrobial resistance tracks changes in microbial populations, permits the early detection of resistant strains of public health importance, and supports the prompt notification and investigation of outbreaks. Surveillance findings are needed to inform clinical therapy decisions, to guide policy recommendations, and to assess the impact of resistance containment interventions. The World Health Organization (WHO) recently announced a suite of policies that, if implemented, should mitigate the emergence and further dissemination of antibiotic resistant organisms (Leung et al., 2011). These initiatives have focused on antibiotic stewardship in the hospital and community settings, and reducing antibiotic use in livestock production (Finley et al., 2013). The European Antimicrobial Resistance Surveillance System (EARSS) provide data on antimicrobial resistance for public health purposes (http:// www.ecdc. europa.eu/en/ activities/ surveillance /EARS- Net/Pages /index. aspx).

In North America, Canada has the Canadian Integrated Program on The Antimicrobial Resistance Surveillance (CIPARS) that aims to provide information of organisms and environment with antimicrobial resistance in humans, animals and outbreaks of infection due to the same(http://www.phac-aspc.gc.ca/surveillance-eng.php). In United States, the National Antimicrobial Resistance Monitoring System (NARMS) is the national public health surveillance system that tracks antibiotic resistance in foodborne bacteria at collaboration with the U.S. Food and Drug Administration (FDA), the Centers for Disease Control and Prevention (CDC), and the U.S. Department

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of Agriculture (USDA).NARMS monitors antimicrobial susceptibility among enteric bacteria from humans, retail meats, and food animals. NARMS also collaborates with antimicrobial resistance monitoring systems in other countries, to work towards international harmonization of testing and reporting (http://www.fda.gov/ animal veterinary/safetyhealth/antimicrobialresistance/nationalantimicrobialresistancemonitoringsystem/default.htm).In Latin America, the SENTRY program is responsible for monitoring important pathogens and antimicrobial resistance patterns of nosocomial and community-acquired infections. This program integrates a wide network of information from various hospitals geographically distributed throughout Latin America (Sader et al., 2001). Other countries such as France, Denmark, Spain, Germany, Norway, Italy, Japan, Netherlands, Sweden, Greece, Finland, and Australia also counts with surveillance and monitoring national systems. All the surveillance systems aimed to reduce the antimicrobial resistance spread and the eliminated the generation of new antimicrobial resistance determinants. It is imperative that we obtain better information of the sources, fate and effects of both antibiotics and resistant bacteria in the environment in order to warranty the proper risk assessment and risk management. The emission of antibiotics into the environment should be educed as an important part of the risk management. For this reason, not used therapeutic drugs must to be discarding in proper containers.

Physicians must be made aware that antibiotics are not completely metabolized by patients (humans/animals) and ensure the best practice in prescription of antibiotics and the use of degradable pharmaceuticals instead persistent ones. Antibiotics have to be not able to use as growth promoters or at less been regulated and limited. Routine analyses of soil and water have to integrate the detection of antibiotics in the environment, and new technologies to investigate the detection, transport, fate on the environment of ARGs, management and removal of these pollutants must be developed, such as ozonation and membrane technology for removal of ARB, multiplex polymerase chain reaction (PCR), real-time PCR, DNA sequencing, and hybridization-based techniques including microarray (Hamelin et al., 2006, 2007; Zhang et al., 2009; Wellington et al., 2013). The improvement of policies to reduce the release of antibiotics and bacteria by anthropogenic ways is also essential. Education to the public on the importance of antibiotics and the prudent use and disposal of the antibiotics has to be made. Surveillance programs are need it worldwide and even more in developing countries were wastewater treated represents often the main source of water to food irrigation. Wastewater treatment plants have to be considerate as biological reactors for antibiotic resistant spread and where horizontal transfer often occurs (Kümmerer, 2003).

Dedicated hospital wastewater treatment must be exists. We also need to take in consideration that the use of one antibacterial agent can increase levels of multiple drugs, and not only to a specific one (cross-resistance), and transfer of resistance genes is most likely to occur in compartments with high bacterial density like in biofilms (Kümmerer, 2009).A corresponding metric for environmental antibiotic concentration could be developed based on the concept of the minimum selective concentration (MSC)(Gullberg et al., 2011), defined as the minimum concentration of an antibiotic agent that selects for resistance (Liu et al., 2011).

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Management of wastewater treatment must considerer the antibiotics as micropollutants in order to reduce the promotion of emergence of antibiotic resistant bacteria.

Conclusion

Aquatic environments are one of the mainways to spread and evolution of antimicrobial resistant bacteria. In water, especially with high density of microorganisms, bacteria of different species and from diverse origins are mixed and given arise to new antimicrobial resistant strains due to the genetic exchange between different bacterial species. All together, resistant bacteria release in the environment directly from the human and animal treated with antibiotics increase the prevalence of resistant strains. Furthermore, antibiotics, quaternary ammonium compounds, health care products and heavy metals could increase the selection of antimicrobial resistant bacteria and reduce the susceptible microbiota in the environment. Pollution by antibiotic and their determinants can enhance the possibilities of human pathogens for acquiring resistance. Several efforts have to be made in order to reduce the impact of antibiotics in the environment and public health. To reduce the impact of resistance genes containment measurements avoiding as much as possible the contact of human-linked and environmental bacteria should be evaluated (Baquero et al., 2008; Martínez et al., 2009).New polices has to be made for limiting the use and misuse of antibiotics as well as limiting the use as growth promoters in farming animals. Water quality assessment must integrate detection of antibiotics in water as a routine quality parameter. New guidelines for treatment water must to be made in order to include the management of antibiotic bacteria and resistance genes in wastewater and detection and removal of these pollutants. The dispersal of bacteria may contribute to the storage and dissemination of antibiotic resistance genes in the environment, for this reason it should has a better handle in both wastewater treatment plants and anthropological activities to control and even eradicate such dispersion and ensuring effective treatment against infectious diseases.

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FLOR YAZMÍN RAMÍREZ CASTILLO
CENTRO DE CIENCIAS BÁSICAS
DEPTO. DE MORFOLOGÍA
P R E S E N T E

En adjunto le hacemos llegar 3 ejemplares del número 54 de la revista *Investigación y Ciencia de la Universidad Autónoma de Aguascalientes.*

Agradecemos su colaboración y esperamos seguir contando con sus contribuciones en próximos números; así mismo le agradeceremos la difusión de la Revista en la comunidad académica y científica de su entorno.

Sin otro particular por el momento, reciba un cordial saludo.

A T E N T A M E N T E
"SE LUMEN PROFERRE"
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MTRA. ROSA DEL CARMEN ZAPATA
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Biopelículas multi-especie: asociarse para sobrevivir

Multi-species biofilms: association to survive

Abraham Loera Muro,[1] Flor Yazmín Ramírez Castillo,[2] Francisco Javier Avelar González,[3] Alma Lilián Guerrero Barrera[4]

Revisión Científica

Loera Muro, A.; Ramírez Castillo, F. Y.; Avelar González, F. J.; Guerrero Barrera, A. L., Biopelículas multi-especie: asociarse para sobrevivir. 54, 49-56, 2012.

RESUMEN

Las biopelículas son una estrategia de supervivencia para los microorganismos que les permite la colonización de ambientes hostiles, tejidos del hospedero o superficies inertes, aún en condiciones cambiantes y para las bacterias patógenas representan un mecanismo de dispersión de infecciones. Debido a lo anterior, el estudio de las biopelículas permite comprender nuevas formas de colonización, resistencia a antibióticos, transferencia horizontal de genes, entre otros mecanismos compartidos por los microorganismos que las conforman. Así, el propósito de la presente revisión es brindar un conocimiento general de estas comunidades, resaltando su importancia en el ambiente y las interacciones entre las especies que participan en su formación.

Palabras clave: biopelículas multi-especie, ciclo celular, matriz extracelular, resistencia antimicrobiana, quorum sensing, FISH.
Key words: multi-species biofilms, cell cycle, extracellular matrix, antimicrobial resistance, quorum sensing, FISH.

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ABSTRACT

Biofilms are an ancient survival microorganism strategy that allows the colonization of hostile environments, host tissues or inert surfaces, even under changing conditions. For pathogenic this represents, a dispersal mechanism of infections. Therefore, the study of biofilms is important to understand new colonization strategies, antibiotic resistance, and horizontal gene transfer, among other mechanisms shared by microorganisms at the consortia. The purpose of this review is to provide a general understanding of these communities, highlighting their importance in the environment and interactions among species that form them.

INTRODUCCIÓN

La formación de biopelículas es reconocida como una estrategia de supervivencia microbiana en diferentes ambientes que brinda resistencia a la desinfección, estrés ambiental y condiciones hostiles en microambientes adversos dentro de los tejidos del hospedero (Pereira *et al*., 2010; Almeida *et al*., 2011).

Las biopelículas son complejas comunidades tridimensionales de microorganismos embebidos en una matriz extracelular (MEC), en las cuales despliegan fenotipos únicos o característicos de adaptación especiales, comparados con la forma de vida libre de estos microorganismos, también conocida como planctónica (Ganguly y Mitchell, 2011; Trappetti *et al*., 2011). En la naturaleza, las biopelículas multi-especie representan

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el estilo de vida bacteriano preferido (Yang *et al.*, 2011). Su formación es dirigida por un conjunto de respuestas moduladas por la percepción de señales ambientales a través de sistemas específicos, entre los que se encuentra el *quorum sensing* (QS), que les permiten sobrevivir aún en ambientes extremadamente adversos para el desarrollo de su vida planctónica (Bordi y Bentzmann, 2011).

Figura 1. Presencia de biopelículas en la vida cotidiana. A) Se muestra su presencia en los sistemas de tratamiento de aguas residuales; B) en las redes de distribución de agua; C) en la placa dental; y D) en el baño de nuestra casa.

En nuestra vida cotidiana, las biopelículas están presentes en objetos comunes como los cepillos dentales en uso, los aires acondicionados, el sarro del cuarto de baño (Nazar, 2007) y en los muebles de cocina, así como en las tuberías de las redes de distribución de agua potable y de drenaje. También las podemos encontrar ocasionando problemas de salud importantes asociados con implantes, catéteres, sondas y otro tipo de materiales empleados de rutina en los hospitales (Yang *et al.*, 2011). Por otra parte, las biopelículas pueden ser aliados de gran utilidad como agentes de biorremediación en el tratamiento de aguas residuales y lodos activados y, en algunos casos, sirven como bioindicadores de la calidad del agua natural y en las redes de distribución (Almeida *et al.*, 2011) (figura 1).

Por lo anterior, el propósito de la presente revisión es introducir a los lectores en el estudio y comprensión de las biopelículas bacterianas, enfatizando la importancia de las comunidades multi-especie en el ambiente.

Consorcio microbiano: una ventaja contra el ambiente

De manera natural, el crecimiento dominante de los microorganismos es a través de consorcios de múltiples especies, regulados por gran variedad de interacciones intra e inter-específicas importantes en su desarrollo, composición, estructura y función (Bowen y Koo, 2011; Høiby *et al.*, 2011). Al mantener microambientes selectivos, la población no depende de la multiplicación rápida, sino aumenta la oportunidad de interacciones mono y multi-específicas; se incrementa la probabilidad de la transferencia horizontal de genes, como genes de virulencia, resistencia a antibióticos y resistencia a drogas (Bordi y Bentzmann, 2011).

Las especies que conforman una biopelícula multi-especie viven en un tipo particular de simbiosis, que ha sido denominado *sociomicrobiología*. Funcionan como un comunidad activa, coordinada con múltiples influencias sinérgicas o antagónicas entre sus integrantes, con reglas propias de "comportamiento" que permiten el éxito del consorcio (Bordi y Bentzmann, 2011; Høiby *et al.*, 2011). En estas comunidades se favorece el crecimiento, reproducción, estabilidad estructural, difusión de sustancias y reserva de energía (Bowen y Koo, 2011). Adicionalmente, la biopelícula permite la resistencia a diferentes tipos de estrés ambiental, como falta de alimento, presencia de metales pesados, luz UV, desecación, agentes bactericidas y bacteriostáticos, diferencias de temperatura y de pH, amén de resistencia a fagocitosis, anticuerpos y otras defensas de los hospederos. Finalmente, la formación del consorcio puede verse correlacionada con una mayor resistencia a agentes antimicrobianos en infecciones en animales y/o humanos (Pereira *et al.*, 2010; Almeida *et al.*, 2011).

Desarrollo de biopelículas

Las interacciones inter e intra-específicas guían la formación de una biopelícula multi-especie (Yang *et al.*, 2011) (figura 2). La etapa inicial en la formación es la adherencia sobre una superficie inerte o viva, la cual se inicia con la coagregación.

El fenómeno de coagregación actúa como estrategia para la adhesión entre bacterias asociadas, pero genéticamente distintas. Es mediada por interacciones fisicoquímicas y moléculas

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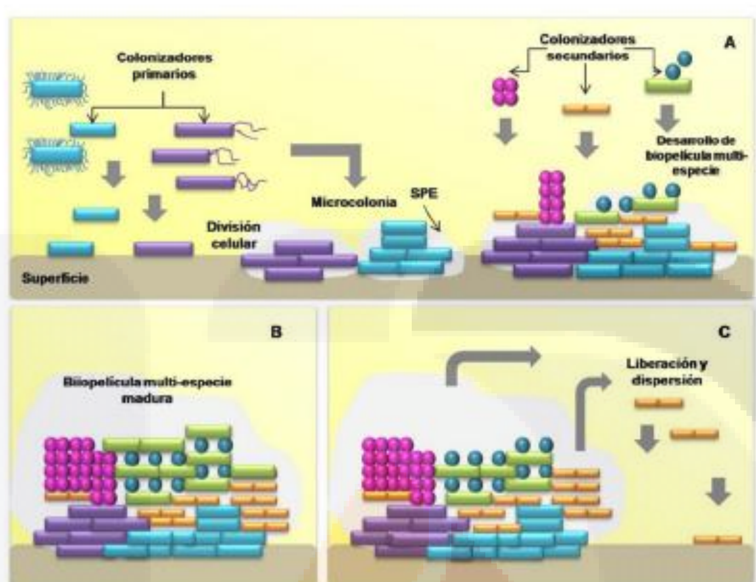

Figura 2. Formación de una biopelícula multi-especie. (A) Los colonizadores primarios cubren una superficie (viva o inerte), a manera de una biopelícula, multiplicándose y formando microcolonias, lo anterior permite la llegada de los colonizadores secundarios y su adhesión a las biopelículas; (B) maduración y formación de la biopelícula multi-especie, caracterizado por el incremento en la producción de sustancias poliméricas extracelulares (SPE); (C) promoción de la dispersión de células y cambio de células planctónica a células en biopelícula. Adaptado de Rickard *et al.* (2003).

llamadas adhesinas, que permiten la agregación secuencial y sucesiva de diferentes microorganismos a una superficie (Rickard *et al.*, 2003; Yang *et al.*, 2011). Al inicio del proceso se adhieren los colonizadores primarios y tempranos a la superficie, éstos se multiplican formando microcolonias que, finalmente, conforme con las condiciones microambientales, cubren totalmente la superficie colonizada, facilitando la llegada de los colonizadores tardíos o secundarios induciendo el desarrollo de los consorcios multi-especie (Bowen y Koo, 2011) (figura 2). El siguiente paso para el desarrollo de la biopelícula es la unión irreversible a la superficie y la multiplicación de las bacterias, seguida por el incremento de la producción de sustancias poliméricas extracelulares que refuerzan la adhesión celular y actúan como un "cemento intercelular" (Rickard *et al.*, 2003). El estadio siguiente involucra la liberación de células bacterianas que pueden propagarse hacia otros espacios, permitiendo la formación de nuevas biopelículas. La liberación puede deberse a sustancias secretadas por las bacterias (liasas de alginato, DNAsas, etc.), por la actividad de bacteriófagos dentro de la biopelícula, o bien, por mecanismos físicos (Loera *et al.*, 2008).

La formación de la matriz extracelular es clave para la biopelícula. La MEC está constituida por exopolisacáridos sintetizados por los microorganismos integrantes de la biopelícula, macromoléculas como proteínas, ácidos nucleicos y otros productos procedentes de la lisis bacteriana, que en conjunto se denominan sustancias poliméricas extracelulares (SPE). El ADN extracelular ayuda a la adhesión microbiana y aumenta la versatilidad genética del consorcio (Trappetti *et al.*, 2011). La arquitectura de la matriz no es sólida. Las bacterias en biopelículas viven en torreones celulares [figura 3] que se extienden en forma tridimensional desde la superficie a la cual están adheridas (Loera *et al.*, 2008).

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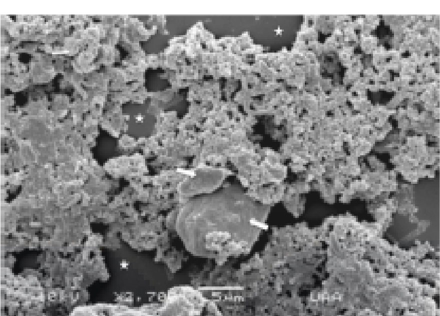

Figura 3. Microscopia electrónica de una biopelícula encontrada en agua, pues se observan los torreones celulares en forma tridimensional donde viven las bacterias. Las flechas indican bacterias inmersas en matriz extracelular formando los torreones y las estrellas la superficie.

Interacciones en biopelículas multi-especie
La comunidad bacteriana constituye una sociedad microbiana multi-especie, con "reglas y patrones de comportamiento" propios (Bordi y Betzmann, 2011); con interacciones estables múltiples entre especies, que regulan la función de la comunidad microbiana (Hansen *et al.*, 2007). Además, las biopelículas presentan interacciones de competencia por nutrientes (Rendueles *et al.*, 2011) e inhibición del crecimiento de otras especies por secreción de sustancias tóxicas (Yang *et al.*, 2011) (tabla 1). *Escherichia coli*, por ejemplo, secreta polímeros durante la formación de su biopelícula que inhiben el crecimiento de *Staphylococcus aureus* y de otras bacterias Gram positivas, pero no así a bacterias Gram negativas (Rendueles *et al.*, 2011).

Comunicación inter e intra-específica en las biopelículas multi-especie
De manera análoga a lo que sucede en la comunicación celular de las células eucariotas que forman tejidos, en las biopelículas moléculas señal pequeñas controlan la expresión de genes involucrados en una gran variedad de funciones y vías metabólicas, tanto entre los miembros de una misma especie como entre especies diferentes: producción de factores de virulencia, biosurfactantes, producción de SPE y movilidad bacteriana (Yang *et al.*, 2011).

Distintas vías de regulación complejas integran señales ambientales a través de las cuales se disparan respuestas adecuadas (figura 4), entre estas vías se encuentran: los sistemas de dos componentes (TCS: por sus siglas en inglés "two-component systems") (figura 4A), las rutas de señalización de función extracitoplásmica (ECF: por sus siglas en inglés "extracytoplasmic function") (figura 4B), los sistemas QS (figura 4C) y otras moléculas, donde se incluye al c-di-GMP (figura 4D) como segundo mensajero procariote (Bordi y Betzmann, 2011). Los TCS y el ECF son los principales mecanismos empleados por las bacterias para monitorear el medio externo e interno. Se monitorean nutrientes, iones, temperatura, estado REDOX, entre otros y se reacciona con respuestas adaptativas (Bordi y Betzmann, 2011).

El sistema de respuesta multicelular o QS coordina la expresión de genes necesarios para la formación de la biopelícula y detecta la densidad del consorcio (figura 4C), comprende el proceso de comunicación bacteriana que utiliza pequeñas moléculas denominadas autoinductoras o feromonas, que median un gran rango de comunicaciones intra e inter-específicas determinando así la densidad de la población (Bordi y Betzmann, 2011; Yang *et al.*, 2011). Por ejemplo, en *S. aureus*, la transición entre células planctónicas y la formación de biopelículas es predominantemente controlada por el sistema QS (Bordi y Betzmann, 2011).

Tabla 1. Interacciones relevantes en comunidades microbianas en diversos ambientes

Tipo de interacción	Bacteria
Antagonismo	Bacterias marinas epifíticas; bacterias entéricas.
Comensalismo	*Acinetobacter* spp/*Pseudomonas putida.*
Competencia	*Klebsiella oxytoca/Burkholderia.*
Mutualismo	Bacterias de suelo; bacterias orales; bacterias marinas epifíticas.
Neutralismo	*Candida* sp; *Schizosaccharomyces* spp; *Saccharomyces* spp.
Sinergismo	*Microbacterium phyllosphaerae; Shewanella japónica; Dokdonia donghaensis.*

Fuente: Simões *et al.* (2007).

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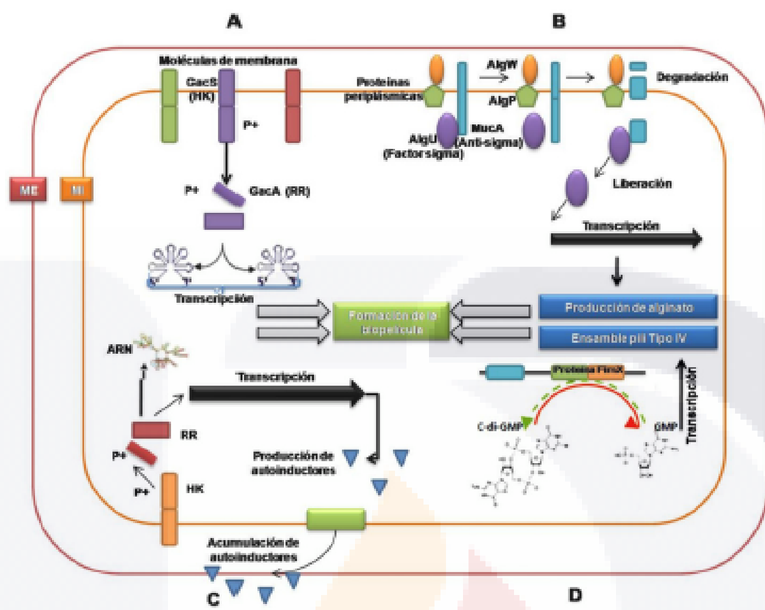

Figura 4. Vías de regulación que controlan la transición entre bacterias planctónicas y la formación de biopelículas. Las líneas representan la envoltura celular: la membrana externa (ME) es la línea púrpura y la membrana interna (MI) es la línea naranja. Se representan los mecanismos presentes en las bacterias Gram positivas (C) y Gram negativas (A, B y D). A) Formación de la biopelícula en *Pseudomonas aeruginosa* mediante la vía TCS, incluye una proteína sensor histidina cinasa (HK) y una proteína reguladora de la respuesta (RR) [GacS(HK)/GacC(RR)]. B) Control de la producción de sustancias poliméricas extracelulares de alginato en *P. aeruginosa* vía ECF, en la que interviene el factor regulador de la trascripción sigma AlgU – anti-sigma MucA – AlgP (MI) – AlgW (periplásmica). C) Control de la formación de biopelículas en *Staphylococcus aureus* a través de la vía QS. D) Control de la formación de la biopelícula en *P. aeruginosa* a través de la vía de segundos mensajeros c-di-GMP. Fuente: Bordi y Betzmann (2011).

Finalmente, la vía de señalización mediante el segundo mensajero c-di-GMP (figura 4D), se relaciona con la estimulación de la formación de biopelículas vía la producción de organelos de adhesión, síntesis de SPE y el decremento de la movilidad de las células, que se asocia a su vez, a niveles elevados de c-di-GMP (Bordi y Betzmann, 2011).

Resistencia antimicrobiana y su relación con la formación de biopelículas

Las infecciones persistentes constituyen un problema mundial para el ser humano (Chen y Wen, 2011). Se estima que 65% de las infecciones bacterianas involucra la formación de biopelículas y que causan infecciones crónicas (Chen y Wen, 2011), entre otras (Rayner *et al.*, 1998; Wagner *et al.*, 2003; Ciofu *et al.*, 2005; Mazzoli, 2010; Chen y Wen, 2011). Como se expuso, la formación de biopelículas es una estrategia microbiana común empleada por las bacterias patógenas para aumentar la resistencia a antibióticos y al sistema inmune del hospedero, haciendo uso de bombas de eflujo, adquisición de nuevas enzimas y mutaciones de drogas blanco y, por supuesto, de la MEC, que funciona como una barrera protectora ante la entrada de agentes antimicrobianos (Chen y Wen, 2011; Bordi y Bentzmann, 2011) (figura 5). Algunas interacciones en las biopelículas multi-especie promueven la resistencia a agentes antimicrobianos. Por ejemplo, *Candida albicans* induce la resistencia de *S. aureus* a vancomicina durante la formación de biopelículas (Harriott y Noverr, 2010).

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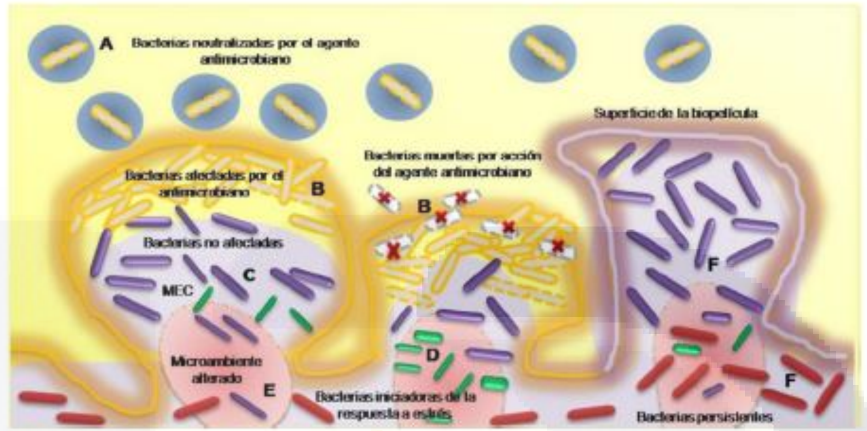

Figura 5. Resistencia a agentes antimicrobianos en una biopelícula. A) Las bacterias planctónicas son neutralizadas por los agentes antimicrobianos. B) Las bacterias en la biopelícula más cercanas a la superficie reciben el daño del agente antimicrobiano. C) La matriz extracelular (MEC) retarda la velocidad de penetración del agente antimicrobiano a la biopelícula. D) Las bacterias generan una respuesta ante el estrés, haciendo que la actividad de las células cambien como respuesta a estímulos del ambiente y E) se genera un microambiente alterado. F) Bacterias persistentes se generan, las cuales son capaces de resistir a los agentes antimicrobianos permitiendo nuevamente la colonización de la superficie por parte de las bacterias en biopelícula.

Técnicas para el estudio de la biopelícula

Existen distintas técnicas para el estudio de biopelículas, enfocándose cada una de ellas en su campo de aplicación. En el aspecto clínico, la detección oportuna de la formación de biopelículas en infecciones persistentes es un factor clave para combatir padecimientos. En este campo, las técnicas persiguen el desarrollo de antígenos y anticuerpos específicos para el diagnóstico terapéutico. Actualmente, se han desarrollado ensayos ELISA (del inglés "Enzyme-Linked ImmunoSorbent Assay", o bien, Ensayo por Inmunoabsorción Ligado a Enzimas) para anti-adhesinas de superficie, que son altamente expresadas en células en biopelículas para monitorear la formación de biopelículas, por ejemplo, de *Staphylococcus* en pacientes con injertos vasculares sintéticos (Chen y Wen, 2011).

Asimismo, se han desarrollado estrategias tales como sistemas de biopelículas artificiales, donde podemos destacar al ensayo de biopelículas en microplaca, utilizado para examinar eventos primarios en la formación de la biopelícula, detectar su dispersión y monitorear la adhesión microbiana a una superficie abiótica. La técnica utiliza una microplaca de 96 pozos, en la cual las células se cultivan por cierto periodo. Las células adheridas a los pozos son teñidas con un medio de contraste (por ejemplo, cristal violeta 0.1%), lo que permite la visualización de los patrones de adhesión de las células a la superficie. Esta técnica permite la medición de la absorbancia en tiempos determinados para obtener ensayos semicuantitativos de la formación de la biopelícula (Merritt *et al.*, 2005; Labrie *et al.*, 2010).

La hibridación fluorescente *in situ* (FISH) acoplada a microscopía, es por mucho, la técnica más usada, pues se utiliza para analizar la composición y la localización de especies microbianas específicas en la biopelícula. La técnica FISH detecta secuencias de ácidos nucleicos por un oligonucleótido altamente específico para cada bacteria, que hibrida concretamente a su secuencia blanco complementaria en la célula (Yang *et al.*, 2011). Las células hibridadas fluorescen bajo cierta longitud de onda, puesto que el oligonucleótido está marcado con un fluorocromo o una proteína fluorescente. Las células son visualizadas a través de microscopía confocal (Sternberg *et al.*, 2006). Esta técnica es ampliamente utilizada, ya que el

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equipo permite realizar cortes ópticos virtuales a lo largo de la imagen, obteniendo un mejor análisis con respecto a la localización y diseño de la biopelícula (Almeida *et al.*, 2011).

Existen también otras técnicas tales como el método de electroforesis en geles desnaturalizantes en gradiente (DGGE) y la microscopia electrónica. La primera es aplicada para describir la diversidad microbiana e identificar especies individuales en biopelículas multi-especie; este método separa comunidades bacterianas a partir de amplificados de PCR (Reacción en Cadena de la Polimerasa) de ARN ribosomal 16S con base en su contenido en guanina y citosina. La segunda es utilizada para observar la estructura de la biopelícula en forma directa (figura 3) (Yang *et al.*, 2011).

Biopelículas bacterianas en el interior de nuestras células

Las bacterias patógenas son capaces de entrar a células epiteliales humanas por procesos de internalización, causando infecciones invasivas. *Bartonella henselae*, una bacteria capaz de colonizar las válvulas del corazón, invade a las células endoteliales vía *invasoma*, es decir, formando grandes agregados que se internalizan en las células eucariotas (Dehio, 1997). *Escherichia coli* enteropatógena (EPEC), causante de diarreas agudas, y *E. coli* uropatogénica (UPEC), que provoca infecciones del tracto urinario, también pueden invadir el epitelio intestinal y el de vejiga urinaria en humanos y animales, respectivamente (Yamamoto *et al.*, 2009). Dentro de las células epiteliales de la vejiga, UPEC se mueve a través de la membrana por medio de compartimentos similares a un endosoma tardío, éste se rompe en el citosol de la célula huésped y se multiplica rápidamente, formando comunidades intracelulares tipo biopelícula que contienen hasta miles de bacterias (Wiles *et al.*, 2008).

Otros patógenos asociados con infecciones crónicas ligadas a la formación de biopelículas incluyen: *Pseudomonas aeruginosa* en la fibrosis quística con pneumonia, *Haemophilus influenzae* y *Streptococcus pneumoniae* en otitis media crónica, *Staphylococcus aureus* en rinosinusitis crónica y *Mycobacterium tuberculosis* en la tuberculosis humana, entre otras, donde la biopelícula provee una estrategia de supervivencia como un reservorio de células que pueden repoblar y recolonizar sitios que previamente han sido tratados con drogas o antibióticos (Hall-Stoodley y Stoodley, 2009; Chen y Wen, 2011).

Conclusiones y perspectivas futuras

Las biopelículas son una extraordinaria estrategia de supervivencia que las bacterias y otros microorganismos han aprovechado por millones de años, permitiéndoles habitar bajo condiciones ambientales desfavorables, una incrementada resistencia a agentes antimicrobianos y una elevada transferencia horizontal de genes. Por todo lo anterior, el conocimiento del desarrollo de una biopelícula y las interacciones que existen dentro de ella, es de suma importancia, tanto para el tratamiento eficaz de enfermedades que son causadas por patógenos en este estado, así como para su utilización en beneficio del hombre, como es el caso de la biorremediación. Gracias al desarrollo de nuevas investigaciones encaminadas a descubrir las interacciones existentes dentro de estas estructuras y los genes implicados en su formación y desarrollo, además del avance de las técnicas para su estudio, como las mencionadas anteriormente, el conocimiento de las biopelículas se ha incrementado en los últimos años, permitiéndonos una mejor comprensión de estos consorcios, que forma parte del ciclo de vida de los microorganismos.

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SUPPLEMENTARY DATA F

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