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Persistence and prevalence of pathogenic and extended-spectrum beta-lactamase-producing *Escherichia coli* in municipal wastewater treatment plant receiving slaughterhouse wastewater

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ABSTRACT

We compared the prevalence of pathogenic and extended-spectrum beta-lactamase (ESBL) – producing *Escherichia coli* in effluents of a municipal wastewater treatment plant (WWTP) receiving wastewater from a slaughterhouse. A total of 1248 isolates were screened for the presence of virulence genes associated with enterohemorrhagic *E. coli* (EHEC) (stx_1, stx_2, and *eae*) and extraintestinal pathogenic *E. coli* (ExPEC) (*sfa/focDE*, *kpsMT* K1, *hlyA*, *papEF*, *afa/draBC*, *clbN*, *f17A* and *cnf*). The prevalence of atypical enteropathogenic *E. coli* (EPEC) was 0.7%, 0.2% and 0.5% in city wastewater, slaughterhouse wastewater and in the treated effluent, respectively. One stx_{1a} and stx_{2b}-positive *E. coli* isolate was detected in city wastewater. The prevalence of ExPEC was significantly higher in city wastewater (8.4%), compared to slaughterhouse wastewater (1.2%). Treatment in the WWTP did not significantly impact the prevalence of ExPEC in the outlet effluent (5.0%) compared to city wastewater. Moreover, the most potentially pathogenic ExPEC were isolated from city wastewater and from the treated effluent. ESBL-producing *E. coli* was also mainly detected in city wastewater (1.7%), compared to slaughterhouse wastewater (0.2%), and treated effluent (0.2%). One ESBL-producing *E. coli*, isolated from city wastewater, was *eae*-β1 positive. These results showed that pathogenic and/or ESBL-producing *E. coli* were mainly detected in human wastewater, and at a lesser extend in animal wastewater. Treatment failed to eliminate these strains which were discharged into the river, and then these strains could be transmitted to animals and humans *via* the environment.

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1. Introduction

Waterborne pathogens are frequently associated with fecal pollution from diverse sources such as wastewater, agricultural fecal wastes and wildlife fecal droppings (Leclerc et al., 2002). Domestic and slaughterhouse wastewater contains important microbiological contaminations. Wastewater treatment has an impact on the microbial quality of surface waters. Most wastewater treatment plants (WWTP) are designed to eliminate organic matter, which allows elimination of pathogens at the same time (Curtis, 2003). *Escherichia coli* is the most used indicator to monitor the microbial quality of water (Wu et al., 2011). *E. coli* are natural habitants of the digestive tract of humans and animals. However, certain strains can be pathogenic for humans (Kaper et al., 2004). Moreover, antibiotic resistant *E. coli*, and in particular extended-spectrum beta-lactamase (ESBL)-producing *E. coli*, had been detected in effluents from human and animal origin (Dolejska et al., 2011; Galvin et al., 2010; Mesa et al., 2006; Sabate et al., 2008).

ESBL mediate resistance to extended-spectrum beta-lactam antibiotics, which are among the most clinically important antibiotics in human medicine (Poole, 2004). However, to date, studies of the detection of pathogenic and/or resistant *E. coli* in sewage from human or animal origin tended to address the issue in terms of the presence or absence of these bacteria. There are few reports of quantitative data on the presence of pathogenic and/or resistant *E. coli* in sewage (Anastasi et al., 2010; Blanch et al., 2003; Galvin et al., 2010; Garcia-Aljaro et al., 2004). As quantitative microbial risk assessment is increasingly used, quantitative data are needed to assess the impact of the effluent process in removing pathogenic and/or resistant *E. coli*. Moreover, the identification of the source of such *E. coli* discharged into the river after treatment is important to establish proper risk assessment and abatement procedures for human and animal wastewater.

Pathogenic *E. coli* can be categorized as intestinal pathogenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). Among the intestinal pathogenic *E. coli*, enterohemorrhagic *E. coli* (EHEC) are responsible for severe clinical symptoms, such as hemorrhagic colitis and the potential lethal hemolytic uremic syndrome (Karmali et al., 2010). EHEC strains are zoonotic pathogens as domestic ruminants, mainly cattle, sheep, and goats have been established as major natural reservoirs for EHEC (Ferens and Hovde, 2011). Several outbreaks attributed to *E. coli* O157:H7 were associated with recreational water and drinking water (Craun et al., 2005; Hunter, 2003), underlying the importance of eliminating these pathogens from human and animal wastewater.

ExPEC strains have the ability to cause extraintestinal infections such as urinary tract infections, neonatal meningitis and sepsis, and wound infections which can lead to serious complications and death (Bonacorsi and Bingen, 2005; Ron, 2006; Wiles et al., 2008). The host's own digestive tract is the conventionally recognized source of the ExPEC that cause human infections (Yamamoto et al., 1997). Although it is difficult to retrace the origin of ExPEC that had established residence in the intestinal tract of humans, it is possible that ExPEC from animals could contaminate humans (Belanger et al., 2011; Manges and Johnson, 2012). Indeed, ExPEC are widespread in animal reservoirs, where they also cause diseases (Dho-Moulin and Fairbrother, 1999; Johnson et al., 2008a). Moreover, human and animal pathogenic strains share common genetic backgrounds (Clermont et al., 2011; Girardeau et al., 2003). Whether animals are a source for human ExPEC is still a matter of debate. Nevertheless, *E. coli* strains with uropathogenic virulence characteristics have been detected in sewage of animal origin (Sabate et al., 2008).

Finally, transmission of antimicrobial-resistant bacteria from animals to humans can also occur (Marshall and Levy, 2011). In particular, there is a growing concern about the impact of the presence ESBL-producing *E. coli* isolated from animals on public health (Ewers et al., 2012; Smet et al., 2009). ESBLs of the CTX-M type are the most prevalent. CTX-M producing *E. coli* isolates have been recognized as the cause of hospital but also community onset infections (Pitout et al., 2005). These bacteria have been also isolated from livestock (Trott, 2012). In a 'One Health' perspective, epidemiological studies are required to precise a potentially zoonotic transmission of ESBL-producing *E. coli*.

The objective of the present study was to evaluate and to compare the prevalence of EHEC, ExPEC and ESBL-producing *E. coli* in effluents of a WWTP receiving wastewater from a slaughterhouse. The goals were to identify the human or animal origin of such strains, and to evaluate the impact of the treatment on their prevalence. Pathogenic and/or ESBL-producing *E. coli* were further characterized, in order to precise their ability to cause illness in humans, and to allow comparisons between isolates from different origins.

2. Materials and methods

2.1. Wastewater treatment plant and sampling

Samples were taken from a municipal wastewater treatment plant (WWTP) located in France in April 2009. The WWTP employed activated sludge process. It serviced population equivalent to 60,000 and wastewater from a hospital were included. It also received wastewater from a multi-species slaughterhouse. The slaughterhouse plant slaughtered cattle, sheep, pigs and horses up to 9000 tons per year. Wastewater had received a pre-treatment before entering into the WWTP. City wastewater had been screened. Slaughterhouse wastewater had been screened, sieved and treated with ferric chloride (coagulation-flocculation). Samples were taken from (i) city wastewater, (ii) slaughterhouse wastewater, and (iii) treated effluent released into the river. The outlet effluent was taken three days after the inlet effluents in order to take into account the hydraulic residence time. Flows were recorded for wastewater from slaughterhouse and for the outlet treated effluent. For each effluent, an average 24-h sample was performed using automatic samplers. Concerning city wastewater, the automatic sampler was programmed in order to take into account the variations observed in the flows during the 24-h period. Due to the presence of buffer tank in the slaughterhouse and in the WWTP, slaughterhouse wastewater and the outlet effluent were not subjected to these variations. Five liters of slaughterhouse wastewater and the

outlet effluent, and 0.5 L of city wastewater were then transported to the laboratory in isothermal containers with eutectic plates, and processed upon arrival at the laboratory.

2.2. Enumeration and isolation of E. coli

Samples were concentrated stepwise to a final volume of 5 mL. Decimal dilutions of the concentrated samples were then plated onto selective medium for *E. coli* (Petrifilm™ Select *E. coli* Count Plate, Grosseron, Saint Herblain, France). The plates were incubated at 42 °C for 24 h; and bacterial enumeration was performed. In a second stage, for each sample, about 20 Petrifilm™ Select *E. coli* Count Plates were inoculated with the dilution giving about 20 well isolated colonies. After incubation, 416 isolates were randomly picked for each sample and were grown separately with agitation at 37 °C in LB broth. They were then stored at −80 °C in LB broth containing 30% glycerol in 96-well microtiter plates for further analysis. In all, 1248 isolates were saved, as 416 isolates were saved for each of the three sampling points.

2.3. Screening for potentially pathogenic E. coli

The presence of 3 EHEC-associated genetic markers (*eae*, stx_1 and stx_2) and 8 ExPEC-associated genetic markers (*sfa/focDE*, *kpsMT* K1, *hlyA*, *papEF*, *afa/draBC*, *clbN*, *f17A* and *cnf*) was investigated in the collection of 1248 isolates. DNA was extracted from overnight cultures using NaOH. It was then subjected to multiplex and uniplex PCR sets. A triplex PCR was performed to detect *eae*, stx_1 and stx_2 genes as already described (China et al., 1996). EHEC O157:H7 RIMD 050992 (Sakaï) was used as a positive control (Hayashi et al., 2001). Concerning the ExPEC, one triplex PCR was performed to detect *sfa/focDE* (Le Bouguenec et al., 1992), *kpsMT* K1 (Johnson and Stell, 2000) and *hlyA* (Johnson and Stell, 2000), one duplex PCR to detect *papEF* (Yamamoto et al., 1995) and *afa/draBC* (Johnson and Stell, 2000), and three single PCR assays to detect *clbN* (Johnson et al., 2008b), *f17A* (Bertin et al., 1996b) and *cnf* genes (Yamamoto et al., 1995). Two duplex PCR sets allowed to identify *f17c-A* and *f17a-A*, *f17b-A* and *f111* (Bertin et al., 1996b); and two simplex PCR sets allowed to identify *cnf1* and *cnf2* (Toth et al., 2003). Furthermore, the presence of *fuyA* (Johnson and Stell, 2000) and *hlyF* (Moulin-Schouleur et al., 2007) genes was investigated in isolates positive for at least one of the 8 screened genes associated with ExPEC. Control strain J96 was used as positive control for *sfa/focDE*, *hlyA*, *papEF* and *fuyA* genes (Johnson et al., 1997), SP15 for *kpsMT* K1 and *hlyF* (Johnson et al., 2002), A30 for *afa/draBC* (Johnson and Stell, 2000), IHE3034 for *clbN* (Korhonen et al., 1985), 25KH9 for *f17a-A* (Girardeau et al., 1988), S5 for *f17b-A* and *cnf2* (Smith, 1974), 31A for *f17c-A* (Bertin et al., 1996a), 111KH86 for *f111* (Bertels et al., 1989) and 28c for *cnf1* (Dozois et al., 1997). Based on the PCR results, *E. coli* isolates were classified as ExPEC when they carried at least three virulence genes among the 10 tested genes associated with ExPEC. The PCR were performed on two apparatus (PCR System 9700 Gene Amp® and PTC-200 Peltier thermal cycler MJ Research). Isolates that tested positive for at least one virulence gene were confirmed to be *E. coli* with an API 20E test (bioMérieux, Marcy l'Etoile, France).

2.4. Screening for ESBL-producing E. coli and detection of β-lactamase genes

In order to detect ESBL-producing *E. coli*, an initial screening was performed on the 1248 isolates. They were inoculated onto Mueller Hinton agar (Biorad Laboratories) containing 1 μg/ml cefotaxime and onto Mueller Hinton agar containing 1 μg/ml ceftazidime, and incubated at 37 °C for 24 h. Isolates growing on plates containing cefotaxime or ceftazidime were confirmed to be *E. coli* with an API 20E test. ESBL-producing *E. coli* isolates were then identified by Etest for cefotaxime and ceftazidime including clavulanic acid, according to manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). *E. coli* ATCC 25922 and *K. pneumonia* ATCC 700603 were used as control strains. The presence of bla_{TEM} bla_{SHV} and $bla_{CTX\text{-}M}$ genes was investigated by PCR, using primers already described for bla_{TEM} and $bla_{CTX\text{-}M}$ genes (Bibbal et al., 2009; Saladin et al., 2002). For the detection of bla_{SHV} genes, forward and reverse primers were 5′-AGATCGGCGACAACGTCAC-3′ and 5′TCATTCAGTTCCGTTTCCCAG-3′, respectively. The bla_{TEM} genes were amplified and sequenced, as already described (Coque et al., 2002). Concerning $bla_{CTX\text{-}M}$ genes, an initial sequencing was performed, in order to identify the CTX-M group (Saladin et al., 2002). Further, gene identification was performed using specific primers and sequencing as described elsewhere (Dutour et al., 2002; Simarro et al., 2000). PCR products were purified using the Min Elute PCR Purification KIT (Qiagen, The Netherlands). Sequences were obtained using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) with an ABI 3730 sequencer (Applied Biosystems, USA). *E. coli* JS238(pOFX326) provided by Olivier Fayet (CNRS-LMGM-UMR5100, France), *E. coli* 6UZ4 (from our collection), *E. coli* 149 77-3018-11 and *S. Virchow* 75-22438-1 provided by Lisbeth Andersen (National Food Institute, Denmark), were used as control strains.

2.5. Isolation of the five major EHEC

To improve the sensitivity of detection of the five major EHEC (O157:H7, O26:H11, O145:H28, O111:H8 and O103:H2), a complementary isolation procedure was performed. Concentrated samples (1 mL) were enriched in 15 mL of modified Trypticase soy broth, supplemented with novobiocin at 20 mg/L and acriflavin at 10 mg/L, at 42 °C for 6 h. Immunomagnetic separation of *E. coli* O157, O26, O145, O103, O111 and O103 were performed according to the manufacturer's instructions (Dynabeads, Invitrogen, Cergy Pontoise, France). The beads were plated onto cefixime-tellurite-sorbitol-MacConkey agar (Oxoid, Dardilly, France) for *E. coli* O157, and onto REBECCA® (Rapid Enterobacteria *E. coli* coliform Agar) (AES, Bruz, France) for the other serogroups. Presumed *E. coli* O157, O26, O145, O103, O111 were confirmed with serogroup specific antisera (Statens Serum Institut, Copenhagen, Danemark) by slide agglutination.

2.6. Phylogenetic group classification

E. coli isolates positive for at least one virulence gene and ESBL-producing *E. coli* were tested for phylogenetic groups using multiplex PCR with the *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 according the method described by

Clermont et al. (Clermont et al., 2000). Representative *E. coli* Reference Collection strains were used as template control.

2.7. Antimicrobial susceptibility testing

E. coli isolates positive for at least one virulence gene and ESBL-producing *E. coli* were subjected to antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed by using a disk diffusion method according to the CLSI standards (CLSI, 2009a) on Mueller-Hinton agar (Bio-Rad Laboratories). *E. coli* ATCC 25922 was used as the control strain. The 16 antibiotic disks (Bio-Rad Laboratories) used in this study were ampicillin, amoxicillin plus clavulanic acid, cephalothin, ceftazidime, cefotaxime, cefepime, cefuroxime, streptomycin, gentamicin, kanamycin, nalidixic acid, ciprofloxacin, tetracycline, chloramphenicol, trimethoprim and sulfamethoxazole. Resistance to imipenem was tested for ESBL-producing *E. coli* isolates. The susceptibility breakpoints for all antimicrobials were those recommended by CLSI (CLSI, 2008, 2009b). Isolates were classified as multiresistant when they exhibited resistance to three or more classes of antimicrobial agents (Schwarz et al., 2010). The presence of major resistance genes to beta-lactam antibiotics (bla_{TEM}), sulfonamides (*sulI*, *sulII*, *sulIII*), phenicols (*floR*, *cmlA*, *catI*, *catII* and *catIII*); streptomycin- spectinomycin (*strA-strB*, *aadA1*), tetracycline (*tetA* and *tetB*) and the presence of the integrase genes *intI1* and *intI2* was investigated in the resistant strains, using specific primers and the *E. coli* control strains described elsewhere (Bibbal et al., 2009).

2.8. Characterization of stx- and eae-positive E. coli isolates

Subtyping of stx_1 and stx_2 genes was performed using specific primers and control strains as described by Scheutz et al. (Scheutz et al., 2012). The presence of *eae* $\beta 1$, $\gamma 1$, ε and $\gamma 2/\theta$ variants was screened in *eae*-positive *E. coli* by real-time PCR, using primers and control strains previously described (Madic et al., 2011). In addition, potential EPEC were PCR-tested for the presence of *bfpA* and EPEC adherence factor (EAF) plasmid as already described (Franke et al., 1994; Gunzburg et al., 1995). E2348/69 was used as control strain (Levine et al., 1985). Based on PCR results, *E. coli* isolates were classified as STEC or atypical EPEC. *E. coli* isolates positive for stx_1 and/or stx_2 genes were classified as STEC, Isolates positive for *eae* gene, and negative for stx_1, stx_2, *bfpA* and EPEC adherence factor (EAF) plasmid were classified as atypical EPEC (Trabulsi et al., 2002). The five major serotypes associated with EHEC (O157:H7, O26:H11, O145:H28, O111:H8 and O103:H2) were investigated in STEC and potential EPEC isolates, by real-time PCR with conditions and control strains described elsewhere (Madic et al., 2011; Perelle et al., 2004, 2005).

2.9. Statistical analysis

A Pearson's Chi-squared test was used to test if STEC, potential EPEC, ExPEC, ESBL producing *E. coli* and virulence genes associated with ExPEC were evenly distributed among sampling points (null hypothesis). The statistical significance was set at a P value <0.05. When the null hypothesis was rejected, sampling points were compared in pairs using a chi-square test, with significance set at a P value <0.017, to take into account multiplicity. Statistical tests were performed using Excel 2010.

3. Results

3.1. Characteristics of the effluents of the WWTP

The flow of the outlet treated effluent was 17,323 m^3/24 h. Slaughterhouse wastewater contributed up to 1.7% to this outlet effluent. Bacterial counts showed that city wastewater and slaughterhouse wastewater contained 1.9×10^4 CFU/mL and 7.7×10^1 CFU/mL *E. coli* respectively. After treatment in the WWTP, the effluent released in the river contained 2.9×10^2 CFU/mL *E. coli*.

3.2. Prevalence and characteristics of ExPEC

ExPEC associated genetic markers (*papEF*, *sfa/focDE* *afa/draBC*, *cnf*, *f17A*, *hlyA*, *clbN*, and *kpsMT* K1) were screened in the collection of 1248 isolates. ExPEC associated genetic markers were detected in 57 *E. coli* isolated from city wastewater, 7 *E. coli* from slaughterhouse wastewater and 27 *E. coli* from the outlet effluent (Table 1). The prevalence of these virulence genes was lower than 7%, and *papEF*, *clbN*, *kpsMT* K1 *sfa/focDE*, *hlyA* were the most identified genes (Table 2). Moreover, the prevalence of these 5 virulence genes was significantly higher in city wastewater compared to slaughterhouse wastewater ($P < 0.017$). Concerning the remaining genes, *afa/draBC*, *cnf1*, *f17a-A*, *f17c-A* and *f111* were only detected in city wastewater and/or in the treated effluent; whereas *cnf2* was only detected in the inlet effluent from the slaughterhouse. Isolates positive for at least one of the 8 genes associated with ExPEC were screened for *fyuA* and *hlyF* genes in a second stage. These two genes were only tested in a second stage because preliminary results had shown that their prevalence was very high in *E. coli* isolated from the three effluents. Isolates carrying at least three EHEC-associated

Table 1 – Prevalence of pathogenic and ESBL-producing *E. coli* isolated from city wastewater, slaughterhouse wastewater and treated effluent.

	City wastewater ($n = 416$)	Slaughterhouse wastewater ($n = 416$)	Treated effluent ($n = 416$)
E. coli positive for at least one ExPEC associated genetic marker[a,b,c]	13.7% (57)	1.7% (7)	6.5% (27)
ExPEC[a,c]	8.4% (35)	1.2% (5)	5.0% (21)
Atypical EPEC	0.7% (3)	0.2% (1)	0.5% (2)
STEC	0.2% (1)	0.0% (0)	0.0% (0)
ESBL-producing *E. coli*	1.7% (7)	0.2% (1)	0.2% (1)

Statistically significant differences ($P < 0.017$) in prevalence indicated by superscript letters.
a Between city wastewater and slaughterhouse wastewater.
b Between city wastewater and treated effluent.
c Between slaughterhouse wastewater and treated effluent.

Table 2 – Prevalence of virulence genes in city wastewater, slaughterhouse wastewater and treated effluent.

Virulence genes	City wastewater ($n = 416$)	Slaughterhouse wastewater ($n = 416$)	Treated effluent ($n = 416$)
ExPEC-associated genetic markers			
papEF[a,c]	6.3% (26)	0.7% (3)	3.1% (13)
clbN[a,c]	5.0% (21)	0.5% (2)	2.9% (12)
kpsMTK1[a,b]	5.3% (22)	0.5% (2)	1.4% (6)
sfa/focDE[a]	3.8% (16)	0.5% (2)	2.2% (9)
hlyA[a,b]	3.6% (15)	0.2% (1)	0.7% (3)
f17A[a,c]	1.4% (6)	0.0% (0)	1.9% (8)
f17a-A	0.5% (2)	0.0% (0)	0.0% (0)
f17c-A	1.0% (4)	0.0% (0)	0.7% (3)
f111	0.0% (0)	0.0% (0)	1.0% (4)
afa/draBC	1.0% (4)	0.0% (0)	1.0% (4)
cnf	0.7% (3)	0.2% (1)	0.7% (3)
cnf1	0.7% (3)	0.0% (0)	0.7% (3)
cnf2	0.0% (0)	0.2% (1)	0.0% (0)
EHEC-associated genetic markers			
eae	0.7% (3)	0.2% (1)	0.5% (2)
stx	0.2% (1)	0.0% (0)	0.0% (0)

Statistically significant differences ($P < 0.017$) in prevalence indicated by superscript letters.
a Between city wastewater and slaughterhouse wastewater.
b Between city wastewater and treated effluent.
c Between slaughterhouse wastewater and treated effluent.

genetic markers were defined as ExPEC. The prevalence of ExPEC was 8.4% in city wastewater, 1.2% in slaughterhouse wastewater and 5.0% in the outlet effluent (Table 1). The prevalence of ExPEC was significantly higher in city wastewater compared to slaughterhouse wastewater ($P < 0.017$). No significant difference was observed between the prevalence of ExPEC in city wastewater compared to the treated effluent. A significant difference was observed between the prevalence of ExPEC in slaughterhouse wastewater compared to the treated effluent ($P < 0.017$).

ExPEC isolates mainly belonged to phylogroup B2 (82.0%), and to a lesser extent, to phylogroup D (13.1%) and A (4.9%). Concerning the antimicrobial resistance, the results showed that 57.4% of the ExPEC isolates were resistant to at least one antibiotic; and that 31.1% were multiresistant. ExPEC isolates were mainly resistant to ampicillin (44.2%), sulfonamide (36.1%), streptomycin (26.2%) and tetracycline (26.2%). Moreover, 13.1% were resistant to the association amoxicillin plus clavulanic acid; and 4.9% were resistant to ciprofloxacin. Finally, the characteristics of the 14 most potential pathogenic ExPEC, *i.e.* harboring more than five genes associated with ExPEC, are shown in Table 3. Eleven of these strains were isolated from city wastewater and three from the treated effluent. The main association of virulence genes was the combination of *fyuA*, *papEF*, *hlyA*, *sfa/focDE*, and *clbN* genes. This combination of genes was detected in 9 strains. In addition, all the strains belonged to phylogenetic group B2; and 8 strains were resistant to at least one of the tested antibiotics.

3.3. Prevalence and characteristics of atypical EPEC and STEC

No isolate harboring both *eae* and *stx* genes was detected. Six *eae*-positive *E. coli* isolates were isolated (Table 2). They were negative for *bfpA* and EPEC adherence factor (EAF) plasmid (Table 4), which justified their classification as atypical EPEC (Kaper et al., 2004). Their prevalence was 0.7%, 0.2% and 0.5%, in city wastewater, slaughterhouse wastewater and in the outlet effluent, respectively (Table 1). No significant difference was observed according to the sampling point. A unique STEC isolate was detected in city wastewater.

Table 3 – Characteristics of ExPEC carrying at least five virulence genes isolated from the inlet and outlet effluents of the wastewater treatment plant.

Strain	Location	Combination of virulence genes	Phylogenetic group	Resistance phenotype[a]	Combination of resistance genes
M41	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*, *kpsMT* K1	B2	AMC AMP SMX	bla_{TEM}, *sulII*
M46	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*	B2	AMC AMP STR SMX	bla_{TEM}, *sulII*
M50	City wastewater	*fyuA*, *hlyA*, *sfa/focDE*, *clbN*, *kpsMT* K1, *hlyF*, *cnf1*	B2	AMC AMP STR SMX TET	bla_{TEM}, *aadA*, *sulI*, *tetA*, *intI1*
M52	City wastewater	*fyuA*, *hlyA*, *sfa/focDE*, *clbN*, *cnf1*	B2	S	
M60	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*, *kpsMT* K1	B2	AMC AMP	bla_{TEM}
M61	City wastewater	*fyuA*, *papEF*, *hlyA*, *clbN*, *kpsMT* K1	B2	S	
M64	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*	B2	S	
M66	City wastewater	*fyuA*, *papEF*, *sfa/focDE*, *clbN*, *kpsMT* K1	B2	AMP	
M75	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*, *kpsMT* K1	B2	AMP KAN STR SMX TET	bla_{TEM}, *strA-strB*, *sulII*, *tetA*
M83	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*	B2	S	
M94	City wastewater	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*, *cnf1*	B2	S	
M116	Treated effluent	*fyuA*, *papEF*, *sfa/focDE*, *clbN*, *cnf1*	B2	AMP STR SMX	bla_{TEM}, *sulII*
M120	Treated effluent	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*, *cnf1*	B2	S	
M122	Treated effluent	*fyuA*, *papEF*, *hlyA*, *sfa/focDE*, *clbN*	B2	AMP	bla_{TEM}

a AMC, amoxicillin + clavulanic acid, AMP, ampicillin; KAN, kanamycin, STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline; S, susceptible to all tested antibiotics.

Table 4 – Characteristics of STEC and atypical EPEC isolated from the inlet and outlet effluents of the wastewater treatment plant.

Strain	Location	Presence of[a]:				Serotype[c]	Phylogenetic group	Resistance phenotype[d]
		eae (variant[b])	*stx* (subtype)	*bfpA*	EAF			
M1	City wastewater	+ (ND)	–	–	–	ND	B2	S
M2	City wastewater	+ (ND)	–	–	–	ND	B2	S
M3	City wastewater	–	+ (stx_{1a}–stx_{2b})	NA	NA	ND	B1	S
M4	City wastewater	+ ($\beta1$)	–	–	–	ND	A	AMP CEF CXM STR CHL NAL SMX TMP TET
M6	Slaughterhouse wastewater	+ ($\beta1$)	–	–	–	O103:H2	B1	S
M7	Treated effluent	+ (ND)	–	–	–	ND	B2	S
M8	Treated effluent	+ (ND)	–	–	–	ND	B2	S

a +, present; –, absent; NA, Not analyzed.
b The presence of *eae* variants $\beta1$, $\gamma1$, ε and $\gamma2/\theta$ was screened in *eae*-positive isolates; ND, Not detected.
c Serotypes O157:H7, O26:H11, O145:H28, O11:H8 and O103:H2 were investigated; ND, Not detected.
d S, susceptible to all tested antibiotics; AMP, ampicillin; CEF, cephalothin; CXM, cefuroxime; STR, streptomycin; CHL, chloramphenicol; NAL, nalidixic acid; SMX, sulfamethoxazole, TMP, trimethoprim; TET, tetracycline.

In order to precise the ability of *E. coli* strains positive for virulence genes to cause illness in humans, these isolates were better characterized. Among the seven atypical EPEC isolated, *eae*-$\beta1$ was detected in two *E. coli* isolated from wastewater (Table 4). One *eae*-$\beta1$-positive *E. coli* was isolated from city wastewater (strain M4). The second *eae*-$\beta1$-positive *E. coli*, isolated from slaughterhouse wastewater, belonged to the serotype O103:H2 (strain M6). The other isolates were not assigned to the five major serotypes frequently associated with EHEC in France. Atypical EPEC mainly belonged to phylogenetic group B2 (4/6). Concerning the antimicrobial resistance, 5 of the 6 atypical EPEC were susceptible to all of the tested antibiotics. The only resistant atypical EPEC strain, belonging to phylogenetic group A, was resistant to 9 antibiotics and produced ESBL (strain M4). The only STEC, isolated from the inlet effluent from the city, carried both stx_{1a} and stx_{2b} genes; and was susceptible to all tested antibiotics (strain M3).

The specific detection, after enrichment and immunomagnetic separation, of *E. coli* belonging to the five major serogroups associated with EHEC in France (O157, O26, O145, O103, O111), failed to isolate EHEC.

3.4. *Prevalence and characteristics of ESBL-producing E. coli*

Among the 1248 tested isolates, nine ESBL-producing *E. coli* were detected. Their prevalence was 1.7% in city wastewater, 0.2% in slaughterhouse wastewater and 0.2% in the treated effluent (Table 1). No significant difference was observed between the sampling points. From the seven ESBL-producing *E. coli* isolated from city wastewater, the gene bla_{TEM-52} was detected in six isolates, while $bla_{CTX-M-14}$ was detected in one isolate (Table 5). The two ESBL-producing *E. coli* isolated from slaughterhouse wastewater and treated effluent harbored $bla_{CTX-M-1}$ gene (strains M30 and M19). All ESBL-producing *E. coli* harbored bla_{TEM-1} gene, and none of these isolates harbored bla_{SHV} gene. Eight isolates were multiresistant, and one isolate was resistant to ciprofloxacin. Moreover, all ESBL-producing *E. coli* were susceptible to imipenem. Class 1 integron was detected in eight ESBL-producing *E. coli*. The distribution of the ESBL-producing *E. coli* according to the phylogenetic groups shown that eight of the nine isolates belonged to group A. The remaining strain belonged to group B1. Finally, screenings of virulence genes associated with EHEC and ExPEC only detected the presence of *eae*-$\beta1$ gene in one of the 9 tested ESBL-producing *E. coli* (strain M4).

4. Discussion

We sampled outlet and inlet effluents, taken into account the residence time, in order to evaluate the impact of the treatment on the prevalence of pathogenic and/or resistant *E. coli*. The treatment in the WWTP eliminated about 99% of the load of the inlet effluents. However, up to 10^2 CFU/mL *E. coli* were released into the river, and these values are consistent with what has already been described (Harwood et al., 2005; Pignato et al., 2009; Reinthaler et al., 2003). Among these *E. coli* isolates, we detected pathogenic and/or resistant *E. coli* which had been disseminated in the environment. As the WWTP received inlet effluents from the city and from a slaughterhouse, we were also able to compare the prevalence of pathogenic and/or resistant *E. coli* according to human or animal origin. Nevertheless, isolates from city wastewater were not strictly from human sources, as feces from companion animals or birds might be released into domestic wastewater. However, this contribution should be very low in terms of flow and number of *E. coli*. Moreover, isolates from slaughterhouse wastewater might have a human origin as toilets of workers were released into this effluent. But this contribution to the final effluent should be very low too.

The most prevalent and the most pathogenic isolated *E. coli* strains for humans were ExPEC. The prevalence of ExPEC was significantly higher in city wastewater, compared to slaughterhouse wastewater. After treatment, no significant difference

Table 5 – Characteristics of ESBL-producing *E. coli* isolated from the inlet and outlet effluents of the wastewater treatment plant.

Strain	Location	Type of β-lactamase genes[a]		Resistance phenotype[b]	Combination of resistance genes	Phylogenetic group
		ESBL	Non-ESBL			
M4	City wastewater	bla_{TEM-52}	bla_{TEM-1}	AMP CEF CXM STR CHL NAL SMX TMP TET	*sulI, sulII, catI, tetA, intI1*	A
M16	City wastewater	bla_{TEM-52}	bla_{TEM-1}	AMP CEF CXM STR CHL NAL SMX TMP TET	*aadA1, sulI, sulII, catI, tetA, intI1*	A
M18	City wastewater	bla_{TEM-52}	bla_{TEM-1}	AMP CEF CXM CTX STR CHL NAL SMX TMP TET	*aadA1, sulI, sulII, catI, tetA, intI1*	A
M20	City wastewater	bla_{TEM-52}	bla_{TEM-1}	AMP CEF CXM STR CHL NAL SMX TMP TET	*aadA1, sulI, sulII, catI, intI1*	A
M24	City wastewater	bla_{TEM-52}	bla_{TEM-1}	AMP CEF CXM STR CHL NAL SMX TMP TET	*aadA1, sulI, sulII, catI, tetA, intI1*	A
M25	City wastewater	bla_{TEM-52}	bla_{TEM-1}	AMP CEF CXM STR CHL NAL SMX TMP TET	*aadA1, sulI, sulII, catI, tetA, intI1*	A
M32	City wastewater	$bla_{CTX-M-14}$	bla_{TEM-1}	AMP CEF CXM STR CHL NAL CIP SMX TMP TET	*aadA1, sulIII, cmlA, intI1*	B1
M30	Slaughterhouse wastewater	$bla_{CTX-M-1}$	bla_{TEM-1}	AMP CEF CXM SMX TMP	*sulII, intI1*	A
M19	Treated effluent	$bla_{CTX-M-1}$	bla_{TEM-1}	AMP CEF CXM CTX		A

a PCR detection and gene identification were performed for bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes.
b AMP, ampicillin; CEF, cephalothin; CXM, cefuroxime; CTX, cefotaxime; STR, streptomycin; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin; SMX, sulfamethoxazole, TMP, trimethoprim; TET, tetracycline.

was observed compared to city wastewater. On the other hand, the outlet prevalence was significantly higher compared to slaughterhouse wastewater. This might be explained by the fact that slaughterhouse wastewater contributed little to the final effluent, in terms of flow and number of *E. coli*. Regarding the prevalence of genetic markers associated with ExPEC, we showed that the prevalence of the main virulence genes (*papEF*, *clbN*, *kpsMT* K1, *sfa/focDE*, *hlyA*) was significantly higher in wastewater from human origin compared to animal origin. This result is consistent with what have been observed by Escobar-Paramo et al. (Escobar-Paramo et al., 2006) comparing the presence of extraintestinal virulence genes (*pap*, *sfa*, *hly* and *aer*) in collections of commensal isolates from human and animal origin. They showed that human isolates exhibited a clearly higher proportion of virulence genes than animal isolates. The *clbN* gene was shown to be significantly associated with bacteremia (Johnson et al., 2008b). Here, we showed that *clbN* gene was detected in *E. coli* isolated from city wastewater, and to a lesser extend in *E. coli* isolated from slaughterhouse wastewater. Concerning the less identified genes, *cnf*1 was only detected in city wastewater and in the outlet effluent; whereas *cnf*2 was only detected in slaughterhouse wastewater. This result is in agreement with other studies showing that *cnf*1-positive isolates have mostly been isolated from extra-intestinal infections from humans, and *cnf*2-positive isolates have been isolated from calves with septicemia or diarrhea (De Rycke et al., 1999). Finally, *f17a-A*, *f17c-A*, *f111* and *afa/draBC* were only detected in city wastewater and/or in the outlet effluent. The fact that we detected the *f17a-A* gene in two *E. coli* isolated from city wastewater is surprising, as *f17a-A* is known to be detected in pathogenic *E. coli* isolated from bovine with diarrhea (Le Bouguenec and Bertin, 1999).

The screened virulence genes have been detected in *E. coli* isolated from extraintestinal infections (Johnson, 2003; Johnson and Russo, 2005). They encode for virulence factors. The presence of additional virulence factors in the same ExPEC isolate acts in additive fashion, enhancing the cumulative impact of pathogenicity in urinary tract infections (Yamamoto et al., 1996). We identified 14 ExPEC in effluents (city wastewater and treated effluent) harboring more than five virulence genes, and they all belonged to phylogroup B2. Clinical isolates of ExPEC typically belong to phylogenetic groups B2 and, at a lesser extend D (Picard et al., 1999). It is worthy of note that the most potentially pathogenic ExPEC for humans were all isolated in wastewater from human origin and treated effluent. ExPEC released into the river might persist in the environment. In fact, isolates, with the same virulence characteristics that we detected, were isolated from surfaces waters in St. Clair River and Detroit River areas, from recreational waters collected in Great Lakes in Canada, from estuarine waters collected in Australia (Hamelin et al., 2006, 2007; Masters et al., 2011). Recently, Anastasi et al. detected identical *E. coli* isolates with uropathogenic characteristics in final effluents of WWTP and in the environment near these effluents had been released (Anastasi et al., 2012). They suggested that *E. coli* strains surviving wastewater treatment can also survive in the environment.

The screening of EHEC associated genetic markers revealed that the prevalence of these genes was very low in effluents. None of these 1248 isolates harbored both *eae* and *stx* genes.

Atypical EPEC were detected in city wastewater, slaughterhouse wastewater and in the treated effluent. Other studies showed the presence of *eae*-positive *E. coli* isolates in effluents from human and animal origin (Awais et al., 2007; Garcia-Aljaro et al., 2005; Holler et al., 1999; Vernozy-Rozand et al., 2004). Loukiadis et al. detected 54 *eae*-positive *E. coli* isolates among 5001 isolates from various effluents from 12 slaughterhouses in France (Loukiadis et al., 2006). Here, we confirmed that the prevalence of *eae*-positive *E. coli* isolates in effluents was very low. Among the five isolated EPEC, two were *eae*β1-positive, which a subtype frequently detected in atypical EPEC in humans isolates (Hernandes et al., 2009). Hamilton et al. detected *eae*-positive *E. coli* isolates with the same characteristics in Avalon Bay, CA. Most of these isolates were atypical EPEC and also carried the β intimin subtype (Hamilton et al., 2010). These results suggest that, as ExPEC, atypical EPEC released by WWTP might persist in the environment. The pathogenic potential of atypical EPEC remains controversial, but recently atypical EPEC have been linked to outbreaks of human diseases (Ochoa and Contreras, 2011).

We only detected one STEC in city wastewater. Using colony hybridization, Garcia-Aljaro et al. (2004) detected one *stx*2-positive *E. coli* isolate per 1000 coliform colonies in municipal sewage. These results are in agreement with what we observed. It is worthy of note that we did not isolate STEC in slaughterhouse wastewater, whereas Garcia-Aljaro et al.detected one *stx*2-positive *E. coli* isolate per 100 coliform colonies in wastewater from animal origin (Garcia-Aljaro et al., 2004). Moreover, we did not detect any STEC in the outlet effluent. This might be explained by the low prevalence of STEC in the studied effluents, and this is consistent with the fact that we did not isolate EHEC after enrichment and immunomagnetic separation of *E. coli* belonging to the five major serogroups.

Finally, we detected ESBL-producing *E. coli* in inlet and outlet effluents at a low prevalence. These isolates were multiresistant, as observed for the majority of clinical ESBL producers (Livermore et al., 2007). They did not harbor virulence genes, with the exception of one isolate which was *eae*-β1-positive. ESBL-producing *E. coli* were mainly detected in city wastewater. Amongst these strains, the gene $bla_{\text{TEM-52}}$ was predominant. Only one isolate harbored a $bla_{\text{CTX-M-14}}$ gene. This distribution differs from that published for ESBL-producing *E. coli* isolated from healthy subjects and patients in France, where CTX-M-producing isolates were predominant (Arpin et al., 2009; Janvier et al., 2011; Nicolas-Chanoine et al., 2013). It is worthy of note that differences in the distribution of $bla_{\text{CTX-M}}$ were also observed between these studies, confirming the fact that many factors may affect the digestive carriage of ESBL-producing *E. coli*. In our study, it would be interesting to evaluate the genetic relatedness of *E. coli* isolates carrying the gene $bla_{\text{TEM-52}}$, in order to precise if a clonal isolate was widespread in city wastewater. One CTX-M-1-producing *E. coli* was isolated from slaughterhouse effluent. This is in agreement with other studies showing that $bla_{\text{CTX-M}}$ genes are predominant in ESBL-producing *E. coli* isolated from cattle and pig in France (Madec et al., 2008; Meunier et al., 2006; Valat et al., 2012). Finally, one CTX-M-1-producing *E. coli* was isolated from the treated effluent and was released in the river. Here, we confirmed the fact that WWTP failed to eliminate ESBL-producing *E. coli*, in agreement with other studies (Dolejska et al., 2011; Galvin et al., 2010).

5. Conclusion

- In conclusion, we have shown that STEC, atypical EPEC, ExPEC and/or ESBL-producing *E. coli* were present in effluents of a WWTP, receiving slaughterhouse effluents.
- Among the investigated strains, the most prevalent and the most potentially pathogenic were ExPEC. The prevalence of ExPEC was significantly higher in wastewater from the city compared to the slaughterhouse. Moreover, the prevalence of ExPEC was not impacted by treatment in the WWTP.
- ESBL-producing *E. coli* had mainly a human origin too.
- The prevalence of atypical EPEC was very low, and a unique STEC was isolated from human wastewater.
- ExPEC, atypical EPEC and ESBL-producing *E. coli*, discharged into the river might persist in the environment and could be transmitted to humans and animals.

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REFERENCES

Anastasi, E.M., Matthews, B., Gundogdu, A., Vollmerhausen, T.L., Ramos, N.L., Stratton, H., Ahmed, W., Katouli, M., 2010. Prevalence and persistence of *Escherichia coli* strains with uropathogenic virulence characteristics in sewage treatment plants. Applied and Environmental Microbiology 76, 5882–5886.

Anastasi, E.M., Matthews, B., Stratton, H.M., Katouli, M., 2012. Pathogenic *Escherichia coli* found in sewage treatment plants and environmental waters. Applied and Environmental Microbiology 78, 5536–5541.

Arpin, C., Quentin, C., Grobost, F., Cambau, E., Robert, J., Dubois, V., Coulange, L., Andre, C., 2009. Nationwide survey of extended-spectrum {beta}-lactamase-producing *Enterobacteriaceae* in the French community setting. Journal of Antimicrobial Chemotherapy 63, 1205–1214.

Awais, R., Miyanaga, K., Unno, H., Tanji, Y., 2007. Occurence of virulence genes associated with enterohemorrhagic *Escherichia coli* in raw municipal sewage. Biochemical Engineering Journal 33, 53–59.

Belanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E., Dozois, C.M., 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunology and Medical Microbiology 62, 1–10.

Bertels, A., Pohl, P., Schlicker, C., Van Driessche, E., Charlier, G., De Greve, H., Lintermans, P., 1989. Isolatie van het Att111 fimbrieel antigen op *Escherichia coli* geisoleerd uit kalverdiaree: karakterizatie en evaluatie van de noodzaak tot aanpassing

van de vaccins ter bestrijding van neonatale colidiarree. Vlaams Diergeneeskundlg Tijdschrift 58, 118–122.
Bertin, Y., Girardeau, J.P., Darfeuille-Michaud, A., Contrepois, M., 1996a. Characterization of 20K fimbria, a new adhesin of septicemic and diarrhea-associated *Escherichia coli* strains, that belongs to a family of adhesins with N-acetyl-D-glucosamine recognition. Infection and Immunity 64, 332–342.
Bertin, Y., Martin, C., Oswald, E., Girardeau, J.P., 1996b. Rapid and specific detection of F17-related pilin and adhesin genes in diarrheic and septicemic *Escherichia coli* strains by multiplex PCR. Journal of Clinical Microbiology 34, 2921–2928.
Bibbal, D., Dupouy, V., Prère, M.F., Toutain, P.L., Bousquet-Mélou, A., 2009. Relatedness of *Escherichia coli* strains with different susceptibility phenotypes isolated from swine feces during ampicillin treatment. Applied and Environmental Microbiology 75, 2999–3006.
Blanch, A.R., Garcia-Aljaro, C., Muniesa, M., Jofre, J., 2003. Detection, enumeration and isolation of strains carrying the *stx2* gene from urban sewage. Water Science and Technology 47, 109–116.
Bonacorsi, S., Bingen, E., 2005. Molecular epidemiology of *Escherichia coli* causing neonatal meningitis. International Journal of Medical Microbiology 295, 373–381.
China, B., Pirson, V., Mainil, J.G., 1996. Typing of bovine attaching and effacing *Escherichia coli* by multiplex in vitro amplification of virulence-associated genes. Applied and Environmental Microbiology 62, 3462–3465.
Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Applied and Environmental Microbiology 66, 4555–4558.
Clermont, O., Olier, M., Hoede, C., Diancourt, L., Brisse, S., Keroudean, M., Glodt, J., Picard, B., Oswald, E., Denamur, E., 2011. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. Infection, Genetics and Evolution 11, 654–662.
CLSI, 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals-third Edition. Document M31-A3. CLSI, Wayne, PA.
CLSI, 2009a. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard-tenth Edition. CLSI document M02-A10. CLSI, Wayne, PA.
CLSI, 2009b. Performance Standards for Antimicrobial Testing; 19th Informational Supplement. Document M100-S19. CLSI, Wayne, PA.
Coque, T.M., Oliver, A., Perez-Diaz, J.C., Baquero, F., Canton, R., 2002. Genes encoding TEM-4, SHV-2, and CTX-M-10 extended-spectrum beta-lactamases are carried by multiple *Klebsiella pneumoniae* clones in a single hospital (Madrid, 1989 to 2000). Antimicrobial Agents and Chemotherapy 46, 500–510.
Craun, G.F., Calderon, R.L., Craun, M.F., 2005. Outbreaks associated with recreational water in the United States. International Journal of Environmental Health Research 15, 243–262.
Curtis, T., 2003. Bacterial pathogen removal from wastewater treatment plants. In: Duncan, M., Horan, N. (Eds.), The Handbook of Water and Wastewater Microbiology. Elsevier, pp. 477–490.
De Rycke, J., Milon, A., Oswald, E., 1999. Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. Veterinary Research 30, 221–233.
Dho-Moulin, M., Fairbrother, J.M., 1999. Avian pathogenic *Escherichia coli* (APEC). Veterinary Research 30, 299–316.
Dolejska, M., Frolkova, P., Florek, M., Jamborova, I., Purgertova, M., Kutilova, I., Cizek, A., Guenther, S., Literak, I., 2011. CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella* spp. isolates in municipal wastewater treatment plant effluents. Journal of Antimicrobial Chemotherapy 66, 2784–2790.
Dozois, C.M., Clement, S., Desautels, C., Oswald, E., Fairbrother, J.M., 1997. Expression of P, S, and F1C adhesins by cytotoxic necrotizing factor 1-producing *Escherichia coli* from septicemic and diarrheic pigs. FEMS Microbiology Letters 152, 307–312.
Dutour, C., Bonnet, R., Marchandin, H., Boyer, M., Chanal, C., Sirot, D., Sirot, J., 2002. CTX-M-1, CTX-M-3, and CTX-M-14 beta-lactamases from *Enterobacteriaceae* isolated in France. Antimicrobial Agents and Chemotherapy 46, 534–537.
Escobar-Paramo, P., Le Menac'h, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B., Skurnik, D., Denamur, E., 2006. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environmental Microbiology 8, 1975–1984.
Ewers, C., Bethe, A., Semmler, T., Guenther, S., Wieler, L.H., 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clinical Microbiology and Infection 18, 646–655.
Ferens, W.A., Hovde, C.J., 2011. *Escherichia coli* O157:H7: animal reservoir and sources of human infection. Foodborne Pathogens 8, 465–487.
Franke, J., Franke, S., Schmidt, H., Schwarzkopf, A., Wieler, L.H., Baljer, G., Beutin, L., Karch, H., 1994. Nucleotide sequence analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor probe and development of PCR for rapid detection of EPEC harboring virulence plasmids. Journal of Clinical Microbiology 32, 2460–2463.
Galvin, S., Boyle, F., Hickey, P., Vellinga, A., Morris, D., Cormican, M., 2010. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. Applied and Environmental Microbiology 76, 4772–4779.
Garcia-Aljaro, C., Muniesa, M., Blanco, J.E., Blanco, M., Blanco, J., Jofre, J., Blanch, A.R., 2005. Characterization of Shiga toxin-producing *Escherichia coli* isolated from aquatic environments. FEMS Microbiology Letters 246, 55–65.
Garcia-Aljaro, C., Muniesa, M., Jofre, J., Blanch, A.R., 2004. Prevalence of the *stx2* gene in coliform populations from aquatic environments. Applied and Environmental Microbiology 70, 3535–3540.
Girardeau, J.P., Der Vartanian, M., Ollier, J.L., Contrepois, M., 1988. CS31A, a new K88-related fimbrial antigen on bovine enterotoxigenic and septicemic *Escherichia coli* strains. Infection and Immunity 56, 2180–2188.
Girardeau, J.P., Lalioui, L., Said, A.M., De Champs, C., Le Bouguenec, C., 2003. Extended virulence genotype of pathogenic *Escherichia coli* isolates carrying the afa-8 operon: evidence of similarities between isolates from humans and animals with extraintestinal infections. Journal of Clinical Microbiology 41, 218–226.
Gunzburg, S.T., Tornieporth, N.G., Riley, L.W., 1995. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. Journal of Clinical Microbiology 33, 1375–1377.
Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T.a., Bekal, S., Fairbrother, J.M., Harel, J., Maynard, C., Masson, L., Brousseau, R., 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Great Lakes recreational waters. Applied and Environmental Microbiology 72, 4200–4206.
Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T.a., Fairbrother, J., Harel, J., Maynard, C., Masson, L., Brousseau, R., 2007. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River areas. Applied and Environmental Microbiology 73, 477–484.

Hamilton, M.J., Hadi, A.Z., Griffith, J.F., Ishii, S., Sadowsky, M.J., 2010. Large scale analysis of virulence genes in *Escherichia coli* strains isolated from Avalon Bay, CA. Water Research 44, 5463–5473.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., Rose, J.B., 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Applied and Environmental Microbiology 71, 3163–3170.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M., Shinagawa, H., 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Research 8, 11–22.

Hernandes, R.T., Elias, W.P., Vieira, M.A., Gomes, T.A., 2009. An overview of atypical enteropathogenic *Escherichia coli*. FEMS Microbiology Letters 297, 137–149.

Holler, C., Koschinsky, S., Witthuhn, D., 1999. Isolation of enterohaemorrhagic *Escherichia coli* from municipal sewage. Lancet 353, 2039.

Hunter, P.R., 2003. Drinking water and diarrhoeal disease due to *Escherichia coli*. Journal of Water and Health 1, 65–72.

Janvier, F., Merens, A., Delaune, D., Soler, C., Cavallo, J.D., 2011. Fecal carriage of third-generation cephalosporins-resistant *Enterobacteriaceae* in asymptomatic young adults: evolution between 1999 and 2009. Pathologie Biologie 59, 97–101.

Johnson, J.R., 2003. Microbial virulence determinants and the pathogenesis of urinary tract infection. Infectious Disease Clinics of North America 17, 261–278. viii.

Johnson, J.R., Clabots, C., Kuskowski, M.A., 2008a. Multiple-host sharing, long-term persistence, and virulence of *Escherichia coli* clones from human and animal household members. Journal of Clinical Microbiology 46, 4078–4082.

Johnson, J.R., Oswald, E., O'Bryan, T.T., Kuskowski, M.A., Spanjaard, L., 2002. Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in the Netherlands. Journal of Infectious Diseases 185, 774–784.

Johnson, J.R., Owens, K., Gajewski, A., Clabots, C., 2008b. *Escherichia coli* colonization patterns among human household members and pets, with attention to acute urinary tract infection. Journal of Infectious Diseases 197, 218–224.

Johnson, J.R., Russo, T.A., 2005. Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli*. Journal of Medical Microbiology 295, 383–404.

Johnson, J.R., Russo, T.A., Scheutz, F., Brown, J.J., Zhang, L., Palin, K., Rode, C., Bloch, C., Marrs, C.F., Foxman, B., 1997. Discovery of disseminated J96-like strains of uropathogenic *Escherichia coli* O4:H5 containing genes for both PapG(J96) (class I) and PrsG(J96) (class III) Gal(alpha1-4)Gal-binding adhesins. Journal of Infectious Diseases 175, 983–988.

Johnson, J.R., Stell, a.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. Journal of Infectious Diseases 181, 261–272.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. Nature Reviews Microbiology 2, 123–140.

Karmali, M.A., Gannon, V., Sargeant, J.M., 2010. Verocytotoxin-producing *Escherichia coli* (VTEC). Veterinary Microbiology 140, 360–370.

Korhonen, T.K., Valtonen, M.V., Parkkinen, J., Vaisanen-Rhen, V., Finne, J., Orskov, F., Orskov, I., Svenson, S.B., Makela, P.H., 1985. Serotypes, hemolysin production, and receptor recognition of *Escherichia coli* strains associated with neonatal sepsis and meningitis. Infection and Immunity 48, 486–491.

Le Bouguenec, C., Archambaud, M., Labigne, A., 1992. Rapid and specific detection of the *pap*, *afa*, and *sfa* adhesin-encoding operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. Journal of Clinical Microbiology 30, 1189–1193.

Le Bouguenec, C., Bertin, Y., 1999. AFA and F17 adhesins produced by pathogenic *Escherichia coli* strains in domestic animals. Veterinary Research 30, 317–342.

Leclerc, H., Schwartzbrod, L., Dei-Cas, E., 2002. Microbial agents associated with waterborne diseases. Critical Reviews In Microbiology 28, 371–409.

Levine, M.M., Nataro, J.P., Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.L., O'Brien, A.D., 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. Journal of Infectious Diseases 152, 550–559.

Livermore, D.M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G.M., Arlet, G., Ayala, J., Coque, T.M., Kern-Zdanowicz, I., Luzzaro, F., Poirel, L., Woodford, N., 2007. CTX-M: changing the face of ESBLs in Europe. Journal of Antimicrobial Chemotherapy 59, 165–174.

Loukiadis, E., Kerouredan, M., Beutin, L., Oswald, E., Brugere, H., 2006. Characterization of Shiga toxin gene (*stx*)-positive and intimin gene (*eae*)-positive *Escherichia coli* isolates from wastewater of slaughterhouses in France. Applied and Environmental Microbiology 72, 3245–3251.

Madec, J.Y., Lazizzera, C., Chatre, P., Meunier, D., Martin, S., Lepage, G., Menard, M.F., Lebreton, P., Rambaud, T., 2008. Prevalence of fecal carriage of acquired expanded-spectrum cephalosporin resistance in *Enterobacteriaceae* strains from cattle in France. Journal of Clinical Microbiology 46, 1566–1567.

Madic, J., Vingadassalon, N., de Garam, C.P., Marault, M., Scheutz, F., Brugere, H., Jamet, E., Auvray, F., 2011. Detection of Shiga toxin-producing *Escherichia coli* serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 in raw-milk cheeses by using multiplex real-time PCR. Applied and Environmental Microbiology 77, 2035–2041.

Manges, A.R., Johnson, J.R., 2012. Food-borne origins of *Escherichia coli* causing extraintestinal infections. Clinical Infectious Diseases 55, 712–719.

Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clinical Microbiology Reviews 24, 718–733.

Masters, N., Wiegand, A., Ahmed, W., Katouli, M., 2011. *Escherichia coli* virulence genes profile of surface waters as an indicator of water quality. Water Research 45, 6321–6333.

Mesa, R.J., Blanc, V., Blanch, A.R., Cortes, P., Gonzalez, J.J., Lavilla, S., Miro, E., Muniesa, M., Saco, M., Tortola, M.T., Mirelis, B., Coll, P., Llagostera, M., Prats, G., Navarro, F., 2006. Extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in different environments (humans, food, animal farms and sewage). Journal of Antimicrobial Chemotherapy 58, 211–215.

Meunier, D., Jouy, E., Lazizzera, C., Kobisch, M., Madec, J.Y., 2006. CTX-M-1- and CTX-M-15-type beta-lactamases in clinical *Escherichia coli* isolates recovered from food-producing animals in France. International Journal of Antimicrobial Agents 28, 402–407.

Moulin-Schouleur, M., Répérant, M., Laurent, S., Brée, A., Mignon-Grasteau, S., Germon, P., Rasschaert, D., Schouler, C., 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. Journal of Clinical Microbiology 45, 3366–3376.

Nicolas-Chanoine, M.H., Gruson, C., Bialek-Davenet, S., Bertrand, X., Thomas-Jean, F., Bert, F., Moyat, M., Meiller, E.,

Marcon, E., Danchin, N., Noussair, L., Moreau, R., Leflon-Guibout, V., 2013. 10-Fold increase (2006-11) in the rate of healthy subjects with extended-spectrum beta-lactamase-producing *Escherichia coli* faecal carriage in a Parisian check-up centre. Journal of Antimicrobial Chemotherapy 68, 562–568.

Ochoa, T.J., Contreras, C.A., 2011. Enteropathogenic *escherichia coli* infection in children. Current Opinion In Infectious Diseases 24, 478–483.

Perelle, S., Dilasser, F., Grout, J., Fach, P., 2004. Detection by 5'-nuclease PCR of Shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. Molecular and Cellular Probes 18, 185–192.

Perelle, S., Dilasser, F., Grout, J., Fach, P., 2005. Detection of *Escherichia coli* serogroup O103 by real-time polymerase chain reaction. Journal of Applied Microbiology 98, 1162–1168.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., Denamur, E., 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infection and Immunity 67, 546–553.

Pignato, S., Coniglio, M.A., Faro, G., Weill, F.X., Giammanco, G., 2009. Plasmid-mediated multiple antibiotic resistance of *Escherichia coli* in crude and treated wastewater used in agriculture. Journal of Water and Health 7, 251–258.

Pitout, J.D., Nordmann, P., Laupland, K.B., Poirel, L., 2005. Emergence of *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBLs) in the community. Journal of Antimicrobial Chemotherapy 56, 52–59.

Poole, K., 2004. Resistance to beta-lactam antibiotics. Cellular and Molecular Life Sciences 61, 2200–2223.

Reinthaler, F.F., Posch, J., Feierl, G., Wust, G., Haas, D., Ruckenbauer, G., Mascher, F., Marth, E., 2003. Antibiotic resistance of *E. coli* in sewage and sludge. Water Research 37, 1685–1690.

Ron, E.Z., 2006. Host specificity of septicemic *Escherichia coli*: human and avian pathogens. Current Opinion in Microbiology 9, 28–32.

Russo, T.A., Johnson, J.R., 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. Journal of Infectious Diseases 181, 1753–1754.

Sabate, M., Prats, G., Moreno, E., Balleste, E., Blanch, A.R., Andreu, A., 2008. Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. Research In Microbiology 159, 288–293.

Saladin, M., Cao, V.T., Lambert, T., Donay, J.L., Herrmann, J.L., Ould-Hocine, Z., Verdet, C., Delisle, F., Philippon, A., Arlet, G., 2002. Diversity of CTX-M beta-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiology Letters 209, 161–168.

Scheutz, F., Teel, L.D., Beutin, L., Pierard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Celsa, A.R., Sanchez, M., Persson, S., O'Brien, A.D., 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. Journal of Clinical Microbiology 50, 2951–2963.

Schwarz, S., Silley, P., Simjee, S., Woodford, N., van Duijkeren, E., Johnson, A.P., Gaastra, W., 2010. Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. Journal of Antimicrobial Chemotherapy 65, 601–604.

Simarro, E., Navarro, F., Ruiz, J., Miro, E., Gomez, J., Mirelis, B., 2000. *Salmonella enterica* serovar virchow with CTX-M-like beta-lactamase in Spain. Journal of Clinical Microbiology 38, 4676–4678.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Herman, L., Haesebrouck, F., Butaye, P., 2009. Broad-spectrum beta-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. FEMS Microbiology Reviews 34, 295–316.

Smith, H.W., 1974. A search for transmissible pathogenic characters in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated, or identical, with colicine V. Journal of general microbiology 83, 95–111.

Toth, I., Herault, F., Beutin, L., Oswald, E., 2003. Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV). Journal of Clinical Microbiology 41, 4285–4291.

Trabulsi, L.R., Keller, R., Tardelli Gomes, T.A., 2002. Typical and atypical enteropathogenic *Escherichia coli*. Emerging Infectious Diseases 8, 508–513.

Trott, D., 2012. beta-lactam resistance in gram-negative pathogens isolated from animals. Current Pharmaceutical Design 19, 239–249.

Valat, C., Auvray, F., Forest, K., Metayer, V., Gay, E., Peytavin de Garam, C., Madec, J.Y., Haenni, M., 2012. Phylogenetic grouping and virulence potential of extended-spectrum-beta-lactamase-producing *Escherichia coli* strains in cattle. Applied and Environmental Microbiology 78, 4677–4682.

Vernozy-Rozand, C., Montet, M.P., Bertin, Y., Trably, F., Girardeau, J.P., Martin, C., Livrelli, V., Beutin, L., 2004. Serotyping, stx2 subtyping, and characterization of the locus of enterocyte effacement island of shiga toxin-producing *Escherichia coli* and *E. coli* O157:H7 strains isolated from the environment in France. Applied and Environmental Microbiology 70, 2556–2559.

Wiles, T.J., Kulesus, R.R., Mulvey, M.A., 2008. Origins and virulence mechanisms of uropathogenic *Escherichia coli*. Experimental and Molecular Pathology 85, 11–19.

Wu, J., Long, S.C., Das, D., Dorner, S.M., 2011. Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. Journal of Water and Health 9, 265–278.

Yamamoto, S., Nakata, K., Yuri, K., Katae, H., Terai, A., Kurazono, H., Takeda, Y., Yoshida, O., 1996. Assessment of the significance of virulence factors of uropathogenic *Escherichia coli* in experimental urinary tract infection in mice. Microbiology and Immunology 40, 607–610.

Yamamoto, S., Terai, A., Yuri, K., Kurazono, H., Takeda, Y., Yoshida, O., 1995. Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. FEMS Immunology & Medical Microbiology 12, 85–90.

Yamamoto, S., Tsukamoto, T., Terai, A., Kurazono, H., Takeda, Y., Yoshida, O., 1997. Genetic evidence supporting the fecal-perineal-urethral hypothesis in cystitis caused by *Escherichia coli*. Journal of Urology 157, 1127–1129.