MAJOR ARTICLE

Wastewater Treatment Plants Release Large Amounts of Extended-Spectrum β-Lactamase–Producing *Escherichia coli* Into the Environment

Caroline Bréchet,[1] Julie Plantin,[1] Marlène Sauget,[1] Michelle Thouverez,[1] Daniel Talon,[1] Pascal Cholley,[1] Christophe Guyeux,[2] Didier Hocquet,[1] and Xavier Bertrand[1]

[1]Service d'Hygiène Hospitalière, UMR 6249 Chrono-environnement, Centre Hospitalier Régional Universitaire, Université de Franche-Comté, Besançon; and [2]Département DISC, Institut FEMTO-ST, UMR 6174 CNRS, Université de Franche-Comté, Belfort, France

(See the Editorial Commentary by Griffiths and Barza on pages 1666–7.)

Background. The determinants of the spread of extended-spectrum β-lactamase–producing *Escherichia coli* (ESBLEC) in the community remain unclear. To evaluate its dissemination in the environment, we analyzed the ESBLEC population throughout an urban wastewater network.

Methods. Samples were collected weekly, over a 10-week period, from 11 sites throughout the wastewater network of Besançon city (France). Total *E. coli* and ESBLEC loads were determined for each sample. As a control, we analyzed 51 clinical ESBLEC isolates collected at our hospital. We genotyped both environmental and clinical ESBLEC by pulsed-field gel electrophoresis and multilocus sequence typing and identified their bla_{ESBL} genes by sequencing.

Results. The *E. coli* load was higher in urban wastewater than in hospital wastewater (7.5×10^5 vs 3.5×10^5 CFU/mL, respectively). ESBLEC was recovered from almost all the environmental samples and accounted for 0.3% of total *E. coli* in the untreated water upstream from the wastewater treatment plant (WWTP). The ESBLEC load was higher in hospital wastewater than in community wastewater (27×10^3 vs 0.8×10^3 CFU/mL, respectively). Treatment by the WWTP eliminated 98% and 94% of total *E. coli* and ESBLEC, respectively. The genotyping revealed considerable diversity within both environmental and clinical ESBLEC and the overrepresentation of some clonal complexes. Most of the sequence types displayed by the clinical isolates were also found in the environment. CTX-M enzymes were the most common enzymes whatever the origin of the isolates.

Conclusions. The treatment at the WWTP led to the relative enrichment of ESBLEC. We estimated that >600 billion of ESBLEC are released into the river Doubs daily and the sludge produced by the WWTP, used as fertilizer, contains 2.6×10^5 ESBLEC per gram.

Keywords. sequence types; WWTP; multidrug-resistant bacteria; environmental risk; sludge.

The increasing prevalence of extended-spectrum β-lactamase (ESBL) Enterobacteriaceae worldwide is a source of particular concern [1]. Since the beginning of the 2000s, ESBLs of the CTX-M type have superseded TEM and SHV derivatives, and *Escherichia coli* has become the most prevalent species among ESBL-producing Enterobacteriaceae [1]. The epidemiology of ESBL-producing *E. coli* (ESBLEC) is complex, as it covers spread in the community, nosocomial acquisition, and the horizontal transfer of plasmids carrying bla_{ESBL} genes. Human intestinal carriage of ESBLEC is frequent, in both hospital and community settings. For instance, 6% of healthy subjects living in the Paris area in 2011 harbored ESBLEC in their gut [2]. These ESBLEC are thus released into the wastewater network and subjected to treatment at wastewater treatment plants (WWTPs). Quantitative data about

Received 25 November 2013; accepted 31 January 2014; electronically published 1 May 2014.

Correspondence: Didier Hocquet, PhD, Service d'Hygiène Hospitalière, Centre Hospitalier Universitaire, 3 boulevard Fleming, Besançon, Cedex 25030, France (dhocquet@chu-besancon.fr).

Clinical Infectious Diseases 2014;58(12):1658–65

DOI: 10.1093/cid/ciu190

antimicrobial drug-resistant *E. coli* in hospital or community effluent discharge, in effluent entering the WWTP, and in WWTP-treated outflow are scarce [3–6]. The extent to which the discharge of ESBLEC into the environment contributes to the global spread of these drug-resistant *E. coli* remains uncertain [7, 8]. Quantitative data would therefore be useful to evaluate this potential risk [4]. The objectives of this study were (*i*) to quantify ESBLEC throughout the wastewater network of the city of Besançon, in eastern France, (*ii*) to compare ESBLEC loads between hospital and community wastewater, (*iii*) to assess the effect of wastewater treatment on ESBLEC load, and (*iv*) to assess the clonal diversity of ESBLEC and the diversity of bla_{ESBL} throughout the wastewater network.

MATERIALS AND METHODS

Study Setting

This study was carried out in the city of Besançon, in eastern France. The WWTP studied serves 120 000 people and had a mean hydraulic load of 30 000 m^3 per day. The effluent treated by the plant includes effluents from 2 university hospital sites, urban wastewater and rainwater, but contains no effluent from livestock farming. The water is subjected to a sequence of 3 typical treatments (sedimentation, biological content degradation, and effluent polishing) before sludge production and the discharge of the treated effluent into the River Doubs. Each year, 7500 metric tons of sludge are produced and used as fertilizer. Samples were collected from 11 sites distributed throughout the wastewater network of the city (Figure 1). Each collecting point was sampled weekly, over a 10-week period, between January and April 2011.

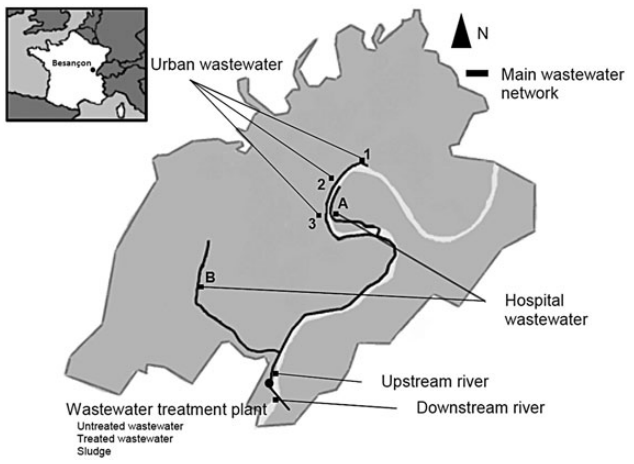

Figure 1. Map of the study area and location of the sampling sites. The large map indicates the precise location of the sampling sites, with the inset map indicating the location of the area in France. We collected wastewater from the 2 sites (A and B) of our hospital (containing only hospital effluent and rainwater), and 3 urban wastewater samples independent of hospitals (1, 2, and 3). We also collected samples within the wastewater treatment plant (WWTP): untreated inflow (n = 1), treated outflow water T1 and T2 before its discharge into the river (n = 2; the daily samples consisted of pools of aliquots taken from each 10 m^3 volume of water), and the anaerobically digested sludge ready for spreading on farm fields (n = 1). We also collected samples of river water upstream and downstream from the WWTP.

Escherichia coli Load Determination and Antimicrobial Susceptibility Testing

Total *E. coli* load was assessed by the most probable number method (MUG/EC, Biokar Diagnostics, Beauvais, France). We also quantified ESBLEC by the serial dilution method or by the membrane filtration method (depending on the type of sample) followed by culture on selective chromogenic agar plate chromID ESBL (bioMérieux, Marcy l'étoile, France). We selected at random 5 colony-forming units (CFU) per plate with a color suggestive of *E. coli* (pink to burgundy), and confirmed as *E. coli* by analysis with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Microflex, Bruker Daltonics, Bremen, Germany) [9]. The clearance rate at the WWTP was determined as follows: [(mean bacterial load in untreated water – mean bacterial load in treated water)/mean bacterial load in untreated water] × 100. For each *E. coli* colony, we checked for the presence of an ESBL by a synergy test [10]. We also assessed the activity of 9 antibiotics (Table 1) against the ESBLEC isolates according to the 2013 recommendations of the European Committee on Antimicrobial Susceptibility Testing [11]. For each sample, we kept 1 representative of each resistance phenotype for further analysis.

Escherichia coli Clinical Isolates

Within the same time period (from January to April 2011), we collected all clinical and screening ESBLEC isolated from patients hospitalized at Besançon University Hospital (Supplementary Table 2).

Genotyping

We investigated the clonality of all strains by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) as previously described [12–14] Clonal complexes (CCs) are defined as a group of sequence types (STs) sharing at least 5 loci in analyses with START2 software [15].

ESBL Identification

The extended-spectrum β-lactamases were identified by polymerase chain reaction and sequencing. After screening all samples with consensus primers targeting $bla_{\text{CTX–M}}$, we carried out more specific amplifications, beginning with primers targeting different groups of CTX-M ($bla_{\text{CTX–M group 1}}$, $bla_{\text{CTX–M group 9}}$, $bla_{\text{CTX–M group 2}}$). We then tested for the presence of bla_{SHV} or bla_{TEM} genes [16–18].

Table 1. Antimicrobial Susceptibility of Extended-Spectrum β-Lactamase–Producing *Escherichia coli* Found in the Wastewater Network of Besançon, France, and in Patients Hospitalized at the University Hospital of Besançon During the Same Time Period (January–April 2011)

Sampling Sites or Origin	No. of Isolates	Susceptibility Rate, %[a]								
		Caz	Ctx	Tzp	Fox	Ipm	Nal	Oflo	Amk	Fos
Urban wastewater	59	[b] **46**	12	97	100	100	53	[b] **66**	100	93
Hospital wastewater	45	[b] **11**	20	91	98	100	33	[b] **33**	100	87
Wastewater treatment plant										
Untreated inflow	26	42	8	100	96	100	69	73	100	92
Treated outflow	58	31	2	91	93	100	55	59	100	90
River										
Upstream	10	80	0	100	100	100	70	70	90	100
Downstream	27	30	9	96	85	100	70	74	100	85
Sludge	17	53	18	94	94	100	71	76	100	71
Inpatients	51	24	4	88	80	100	41	39	100	94

Values for which the difference was statistically significant are shown in bold text.

Abbreviations: Amk, amikacin; Caz, ceftazidime; Ctx, cefotaxime; Fos, fosfomycin; Fox, cefoxitin; Ipm, imipenem; Nal, nalidixic acid; Oflo, ofloxacin; Tzp, piperacillin-tazobactam.

[a] Susceptibility was defined according to the 2013 European Committee on Antimicrobial Susceptibility Testing recommendations [11].

[b] $P < .001$.

Ethical Considerations

This study was approved by the Ethics Committee of University Hospital of Besançon, Besançon, France. All the water and sludge samples came from public areas and facilities. All necessary permits were obtained by the Water Supply and Water Treatment Service of the City of Besançon. The location is not privately owned or protected in any way, and the field studies did not involve endangered or protected species.

RESULTS

Total *E. coli* and ESBLEC Loads of the Environmental Samples

Over the study period, we processed 110 water or sludge samples. All these samples tested positive for *E. coli* and 96% tested positive for ESBLEC, but with variable relative proportions, according to time and location. We present the total *E. coli* and ESBLEC loads at the sampling sites in Figure 2*A* (see also Supplementary Table 1 for details). The mean *E. coli* load of urban wastewater was higher than that of hospital wastewater (752 847 vs 353 635 CFU/mL, respectively; $P = .013$). The loads of *E. coli* in the inflow (untreated water) immediately upstream from the WWTP and in its outflow were stable during the study period (160 320 and 3705 CFU/mL, respectively). ESBLEC loads were lower in urban wastewater than in hospital wastewater (751 vs 27 447 CFU/mL, respectively; $P < .001$). Despite a lower amount of ESBLEC in the treated water than in the untreated water (22 vs 481 CFU/mL, respectively), the proportion of these antibiotic-resistant bacteria was significantly higher in the outflow than in the inflow (0.6% vs 0.3%, respectively; $P = .017$; Figure 2*B* and Supplementary Table 1). Hence, the WWTP clearance rates were 98% for total *E. coli* and 94% for ESBLEC. We estimated that 6×10^{11} ESBLEC were released daily into the River Doubs, and that the sludge contained 2.6×10^5 ESBLEC per gram.

Isolate Collection and Antibiotic Resistance

We selected 243 ESBLEC isolates from environmental samples for further testing: 45 were isolated from hospital wastewater, 60 from urban wastewater, 101 from the WWTP, and 37 from the river (Supplementary Table 2). In addition, we collected 51 nonduplicate ESBLEC isolates from patients hospitalized in our university hospital over the same period: 45 from clinical samples (mostly urine, n = 32) and 6 from screening samples. Table 1 details the susceptibility of the ESBLEC to antimicrobial drugs, by isolate origin. The ESBLEC isolates found in hospital wastewater were more resistant than those from the urban wastewater, particularly to ceftazidime ($P < .001$) and ofloxacin (<.001). Although ESBLEC isolates from the WWTP outflow were more resistant to β-lactams and fluoroquinolones than those from the WWTP inflow, the difference did not reach statistical significance (Table 1).

Genotyping of Environmental Isolates

We genotyped 224 of the 243 environmental isolates by PFGE (19 were nontypable). We obtained 145 different pulsotypes (PTs), 108 of which were unique (Supplementary Table 2). As PFGE typing is more discriminatory than MLST, we assumed that all the isolates with an identical PT shared the same ST. We therefore determined the MLST profiles of 1 isolate for each of the 145 PTs and of the 19 nontypable isolates. We

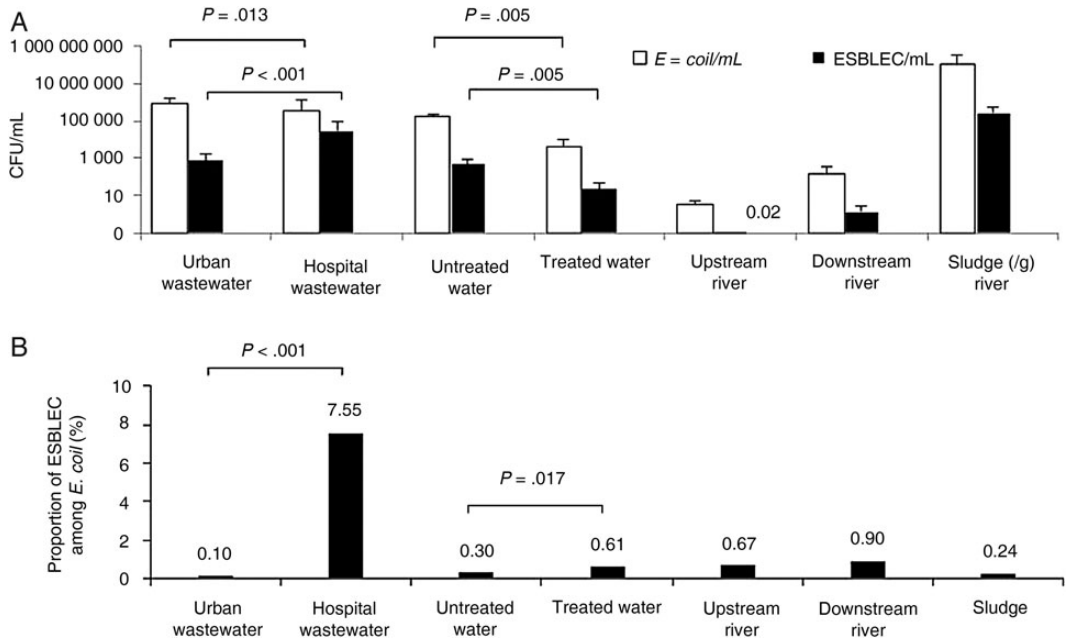

Figure 2. Loads of *Escherichia coli*, extended-spectrum β-lactamase (ESBL) *E. coli* (ESBLEC), and the proportion of ESBLEC at the various sampling points of the wastewater network of Besançon, France. *A*, Loads of total *E. coli* (white bars) and ESBLEC (black bars). For each sample, ESBLEC quantification was carried out by dividing the number of confirmed ESBLEC by the number of tested colonies resembling *E. coli* on the chromID ESBL plate, and multiplying by the *E. coli* load on the chromID ESBL plate. The bacterial load in the sludge is expressed in colony-forming units (CFU) per gram. Mean ± SEM, n = 10. *B*, Proportion of ESBLEC among total *E. coli*. Mean, n = 10. The data were compared in either nonparametric Kruskal-Wallis test or Wilcoxon signed-rank test, as appropriate. All tests were 2-tailed, and *P* values <.05 were considered statistically significant.

identified 28 CCs, including 186 isolates in total. The 57 remaining isolates were from 27 singleton STs. Five major CCs were represented by >10 isolates each. CC10, CC23, CC38, CC361, and CC155 were represented by 37, 27, 19, 16, and 11 isolates respectively, obtained from the various points of the wastewater network (Figure 3). Figure 3 shows the maximum likelihood tree of the CCs and isolated STs with the distribution in number and of their sampling locations. Seven of the 243 environmental isolates were ST131. The 74 ESBLEC isolates from the WWTP outflow or sludge belonged to 1 of 32 CCs or STs (CC10, n = 13; CC23, n = 11; CC38, n = 6; CC361, n = 6; CC45, n = 3; CC155, n = 3; CC405, n = 3), and none belonged to the extraintestinal pathogenic *E. coli* (ExPEC) B2 phylogroup.

Genotyping of Clinical Isolates

The 51 clinical isolates were distributed into 44 different PTs (Supplementary Table 2). Ten PTs were recovered from both patients and the wastewater network. These 51 isolates clustered into 22 CCs or STs, of which CC23 and CC10 were the most common, with 7 and 6 isolates, respectively. CC69 and ST131 were each represented by 4 isolates. Most (17 of 22) of the CCs or STs identified in patients were also found in environmental samples (Figure 3).

Phylogenetic Analysis

We constructed the maximum likelihood tree based on the alignment of the 3423-bp sequences, which yielded 265 informative sites (Figure 3). We then assigned each ST or CC to phylogenetic groups A, B1, B2, and D with the University of Cork database [14]. The dendrogram identified these 4 major phylogroups (Figure 3). Environmental and clinical isolates were scattered throughout the dendrogram. However, the proportion of isolates belonging to the phylogroup B2 was significantly higher in clinical isolates (10 of 51 [19.6%]) than in environmental isolates (13 of 243 [5.3%]; *P* < .001).

ESBL Produced by Environmental and Clinical Isolates

Most of the environmental isolates (215 of 243 [88%]) produced CTX-M–type ESBL (Figure 4). CTX-M-1 was the most frequent CTX-M type enzyme (50%), followed by CTX-M-15 (25%) and CTX-M-14 (10%) (Figure 4). All the SHV enzymes were SHV-12. There was no obvious link between the nature of the ESBL and the sampling site in the wastewater network. Most of the CC23 isolates (21 of 27) produced CTX-M-1. The 7 ST131 isolates produced highly diverse CTX-M–type enzymes: CTX-M-1 (n = 3), CTX-M-27 (n = 2), CTX-M-14 (n = 1), and CTX-M-15 (n = 1).

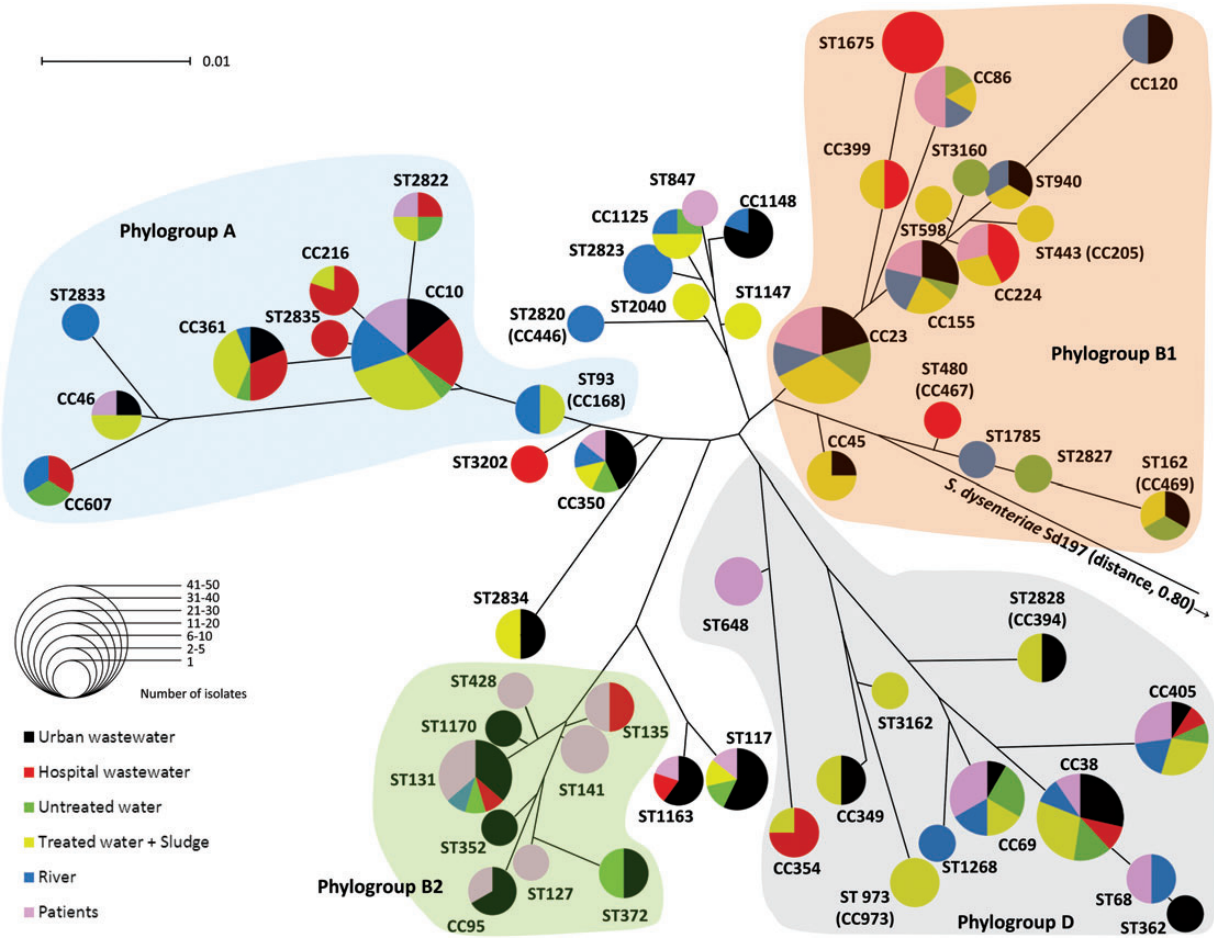

Figure 3. Dendrogram based on sequence types (STs) and clonal complexes (CCs) of *Escherichia coli* identified in the various sampling points of the urban wastewater network and in patients hospitalized in the University Hospital of Besançon, France. Each diagram corresponds to 1 ST or CC; the size of each diagram is a function of the number of isolates; the various wastewater sampling points are shown in different colors. To build a dendrogram of all the STs retrieved in this study, we concatenated the sequences of the *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* genes to form a 3423-bp sequence alignment. The best-fit nucleotide substitution model for this data was GTR + G + I, as determined with jModelTest 0.1.1 [19]. A maximum likelihood tree was constructed with RAxML 7.2.8 [20] and visualized with Dendroscope [21]. We used *Shigella dysenteriae* Sd197 as the outgroup strain. In all cases, bootstrapping percentages of >90% were obtained for most branches, with 1000 bootstrap repetitions. The bootstrap values are intentionally hidden for clarity. Based on the *E. coli* multilocus sequence typing database of the University of Cork [14], classic phylogenetic groups (phylogroup A in blue, B1 in pink, B2 in green, and D in gray) are identified on the dendrogram.

The distribution of the ESBLs produced by the clinical isolates was very similar to that of the ESBLs produced by environmental isolates (Figure 4). All the clinical ST131 isolates (n = 4) produced CTX-M-15 (Supplementary Table 2).

DISCUSSION

Municipal wastewater is a complex mixture of everything that is flushed down a toilet or that is sent down a drain. Besançon's wastewater also includes industrial waste (albeit in limited amounts) and stormwater, but contains no effluent from livestock or agriculture. The results of this study were not affected by rain, because there was very little rainfall during the study period (data not shown). To the best of our knowledge, this is the first study to quantify and characterize ESBLEC throughout an urban wastewater network over a long time period. Total *E. coli* load was higher in the urban wastewater than in hospital wastewater (Figure 3*A*). This is probably due to the higher dilution of wastewater in hospitals, in which water consumption reaches 700 L per bed and per day. The large concentration of ESBLEC present in the wastewater network results from ESBLEC carriage in the human gut. ESBLEC accounted for 0.1% of all *E. coli* in urban effluent and a much higher proportion (7.6%) in hospital effluent (Figure 2*B*). This difference certainly reflects the high proportion of inpatients infected with

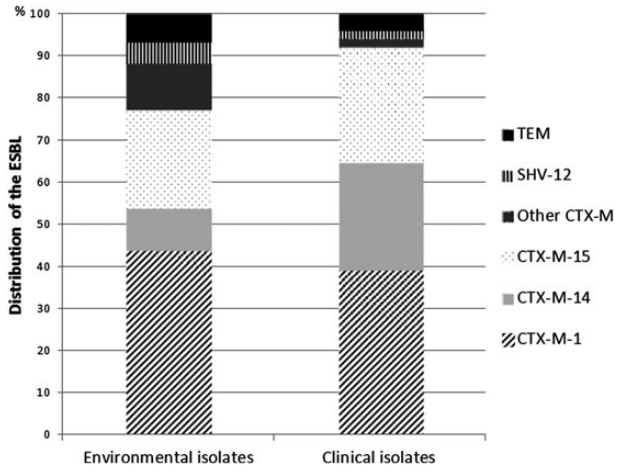

Figure 4. Comparison of the distribution (%) of extended-spectrum β-lactamases (ESBLs) between environmental and clinical isolates.

ESBLEC, together with the higher loads of these multidrug-resistant bacteria in hospitalized patients than in carriers within the community [22]. In addition, hospital effluents contain massive quantities of antibiotics and antiseptic residues that might favor the development of antibiotic-resistant clones [23].

The WWTP clearance rate of *E. coli* in this study (98%) is similar to that previously reported in France [24]. Water treatment greatly decreased ESBLEC load (from 3705 CFU/mL in the inflow to 22 CFU/mL in the outflow), but the proportion of ESBLEC among the *E. coli* present increased significantly with treatment (0.3% in the inflow vs 0.6% in the outflow) (Figure 2*B*). We then assume that ESBLEC has no significant disadvantage with respect to non-ESBL–producing *E. coli* in the treatment plant. Such ESBL-producing isolate enrichment has also been shown in Ireland [4]. Czekalski et al also showed, with a culture-independent approach, that *sul* resistance gene clearance was poor during treatment at the WWTP [25]. This is presumably due to the presence in the wastewater of antibiotics used in human medicine. These compounds exert a selective pressure, favoring antimicrobial resistant bacteria, even when present at very low concentrations [26–28]. In our town, as elsewhere in France, β-lactams are the most widely used group of antibiotics, followed by fluoroquinolones [29]. However, β-lactams are rarely detected in WWTPs, presumably because the β-lactam ring is readily cleaved by hydrolysis [30]. By contrast, high concentrations of fluoroquinolones are found in WWTPs and downstream from the WWTP, in the river [30, 31]. In our treatment plant, as elsewhere, the ESBLEC from the outflow was more resistant to fluoroquinolones than the ESBLEC from the inflow (Table 1) [32]. This supports the hypothesis that fluoroquinolones exert selection pressure within the WWTP, although other antimicrobials may also play a role.

In total, we estimate that 6×10^{11} ESBLEC are released daily into the River Doubs and that the sludge, which is used as fertilizer, contains 2.6×10^{5} ESBLEC per gram. The extent to which this discharge of ESBLEC into the environment contributes to the global epidemiology of this pathogen and, more particularly, to the acquisition of ESBLEC by individuals, is unclear. However, the improvement of elimination of antimicrobial drug–resistant microorganisms within WWTPs would appear to be a reasonable policy. There should be conceptual differences in the design of municipal WWTPs, which are designed to protect surface water against accidental exposure to pathogenic microorganisms, and analogous facilities designed to control the spread of antimicrobial drug–resistant bacteria. Disinfection technologies could be used but are complex. Chlorine produces carcinogenic by-products and alternative disinfection technologies, such as ultraviolet treatment, are substantially more costly and not necessarily more efficient [27, 33]. Other technologies, such as sand filtration, membrane filtration, constructed wetlands, ozonization of the WWTP outflow, or thermophilic anaerobic digestion for sludge, should also be explored. There is a need to assess the ability of these processes to control the load of antimicrobial drug resistance in wastewater, which should be seen as an "environmental pollutant" [34].

Genotyping revealed a high degree of genetic diversity, with no specific link between CC and a sampling site. We found only 2 exceptions among CCs represented by >2 isolates in this study. ST1675 was recovered only from the wastewater of 1 hospital site (9 of the 10 samples taken from this site contained ST1675), potentially accounting for the very high prevalence of ESBLEC at this site. Finally, ST648 isolates were recovered only from patients (Figure 3). The principal CCs recovered in our study (CC10, CC23, CC38, CC361, CC155) have been described in previous studies as predominant lineages in total populations of ESBLEC from humans, livestock or companion animals, and even wildlife [35].

In the virulent ExPEC B2 lineage, the association with ESBL genes proved a great success in the pandemic ST131 clone [36]. ST131, which is primarily associated with CTX-M-15, has diversified its ESBL repertoire [35]. Other ExPEC B2 lineages, such as CC95, CC73, ST127, and ST141, harbor only marginally ESBL-encoding genes. The reason for which these well-established and clinically successful lineages are less prone to the acquisition of ESBL than the related ST131 lineage remains to be determined. We found ExPEC B2 lineages (ST131, CC95, ST127, and ST141) in hospitalized patients, but none were released in the WWTP-treated outflow. This confirms the decrease in the prevalence of virulent *E. coli* during wastewater treatment processes reported by Frigon et al [37]. However, ST131 has recently been detected in the Thames (London, United Kingdom) and Llobregat (Barcelona, Spain) rivers, and in WWTP outflow in Brno (Czech Republic) [6, 38, 39]. The underrepresentation of

ST131 in the environment contrasts with its high prevalence in humans and merits further exploration.

The distribution of the ESBLs in clinical and wastewater isolates was similar (Figure 4). CTX-M-1 was the most frequent ESBL in our series, consistent with the findings of a Dutch study that reported CTX-M-1 to be the most prevalent ESBL common to human patients, healthy carriers, poultry, and retail chicken meat [40]. However, ESBLs appear to be unevenly distributed in Europe, because a previous survey reported CTX-M-15 and CTX-M-14 to be the most prevalent ESBLs among clinical ESBLEC [1].

Our sampling method (1 sampling session per week) provided only a snapshot of a highly complex and dynamic process. We cannot, therefore, claim that our results are representative of the ESBLEC load throughout the period. For instance, only 78% of the ESBLEC STs recovered from inpatients were retrieved in the wastewater network. However, the stability of the *E. coli* and ESBLEC loads over the 10-week period studied suggests that our data are reliable. Moreover, the WWTP outflow sampling, which involved collecting pools of aliquots from each 10 m^3 volume of water, provided representative values for the number and diversity of ESBLEC discharge into the environment.

In conclusion, we show here that our wastewater network is highly contaminated with ESBLEC, with higher ESBLEC loads in hospital effluent than in urban effluent. The production of an ESBL seems to confer a significant advantage on *E. coli* in the WWTP. The ESBLEC displayed a high degree of clonal diversity, but some lineages previously described in humans or animals were overrepresented. Our results suggest that there is a need for improvements in the monitoring of antibiotic-resistant microorganisms of human origin in effluent.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Christian Imperas, Laurent Coty, Frédéric Pothin, and the staff of the water supply facilities and wastewater treatment plant of the city of Besançon, France.

Author contributions. Conceived of and designed the experiments: M. S., D. H., X. B. Performed the experiments: C. B., J. P., P. C. Analyzed the data: M. T., C. G., D. H., X. B. Wrote the paper: C. B., D. H., X. B.

Disclaimer. This study was approved by the 'Comité d'Etude Clinique' ethics committee of Besançon University Hospital, Besançon, France. All the water and sludge samples came from public areas and facilities. All necessary permits were obtained by the Water Supply and Water Treatment Service of the City of Besançon. We confirm that the location is not privately-owned or protected in any way. We confirm that the field studies did not involve endangered or protected species.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support. This work was supported by a grant from Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (Eaux Usées *E. coli* producteurs de BLSE, number 2011-01-010).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Canton R, Novais A, Valverde A, et al. Prevalence and spread of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in Europe. Clin Microbiol Infect **2008**; 14(suppl 1):144–53.
2. Nicolas-Chanoine MH, Gruson C, Bialek-Davenet S, et al. 10-fold increase (2006–11) in the rate of healthy subjects with extended-spectrum β-lactamase-producing *Escherichia coli* faecal carriage in a Parisian check-up centre. J Antimicrob Chemother **2013**; 68:562–8.
3. Jakobsen L, Sandvang D, Hansen LH, et al. Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the waste water environment. Environ Int **2008**; 34:108–15.
4. Galvin S, Boyle F, Hickey P, Vellinga A, Morris D, Cormican M. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. Appl Environ Microbiol **2010**; 76:4772–9.
5. Mesa RJ, Blanc V, Blanch AR, et al. Extended-spectrum β-lactamase-producing *Enterobacteriaceae* in different environments (humans, food, animal farms and sewage). J Antimicrob Chemother **2006**; 58:211–5.
6. Colomer-Lluch M, Mora A, Lopez C, et al. Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain. J Antimicrob Chemother **2013**; 68:758–65.
7. Guenther S, Ewers C, Wieler LH. Extended-spectrum β-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? Front Microbiol **2011**; 2:246.
8. Manges AR, Johnson JR. Food-borne origins of *Escherichia coli* causing extraintestinal infections. Clin Infect Dis **2012**; 55:712–9.
9. Wieser A, Schneider L, Jung J, Schubert S. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond. Appl Microbiol Biotech **2012**; 93:965–74.
10. Comité de l'antibiogramme de la Société Française de Microbiologie. Recommendations 2013. Available at: http://sfm-microbiologie.org/UserFiles/file/CASFM/CASFM_2013.pdf. Accessed 1 September 2013.
11. European Committee on Antimicrobial Susceptibility Testing (EUCAST 2013). Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1. Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf. Accessed 1 September 2013.
12. Bertrand X, Hocquet D, Boisson K, Siebor E, Plesiat P, Talon D. Molecular epidemiology of Enterobacteriaceae producing extended-spectrum β-lactamase in a French university-affiliated hospital. Int J Antimicrob Agents **2003**; 22:128–33.
13. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol **1995**; 33:2233–9.
14. University College Cork. MLST databases at the ERI. Available at: http://mlst.ucc.ie/mlst/dbs/Ecoli. Accessed 1 September 2013.
15. Jolley KA, Feil EJ, Chan MS, Maiden MC. Sequence type analysis and recombinational tests (START). Bioinformatics **2001**; 17:1230–1.
16. Brasme L, Nordmann P, Fidel F, et al. Incidence of class A extended-spectrum β-lactamases in Champagne-Ardenne (France): a 1 year prospective study. J Antimicrob Chemother **2007**; 60:956–64.
17. Cao V, Lambert T, Nhu DQ, et al. Distribution of extended-spectrum β-lactamases in clinical isolates of Enterobacteriaceae in Vietnam. Antimicrob Agents Chemother **2002**; 46:3739–43.

18. Mabilat C, Courvalin P. Development of "oligotyping" for characterization and molecular epidemiology of TEM β-lactamases in members of the family Enterobacteriaceae. Antimicrob Agents Chemother **1990**; 34:2210–6.
19. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol **2008**; 25:1253–6.
20. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **2006**; 22:2688–90.
21. Huson DH, Scornavacca C. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst Biol **2012**; 61:1061–7.
22. Ruppe E, Lixandru B, Cojocaru R, et al. Relative fecal abundance of extended-spectrum β-lactamases-producing *Escherichia coli* and their occurrence in urinary-tract infections in women. Antimicrob Agents Chemother **2013**; 57:4512–17.
23. Coutu S, Rossi L, Barry DA, Rudaz S, Vernaz N. Temporal variability of antibiotics fluxes in wastewater and contribution from hospitals. PLoS One **2013**; 8:e53592.
24. Garcia-Armisen T, Servais P. Respective contributions of point and non-point sources of *E. coli* and *enterococci* in a large urbanized watershed (the Seine river, France). J Environ Manage **2007**; 82:512–8.
25. Czekalski N, Berthold T, Caucci S, Egli A, Burgmann H. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. Front Microbiol **2012**; 3:106.
26. Rizzo L, Manaia C, Merlin C, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Environ **2013**; 447:345–60.
27. Rizzo L, Fiorentino A, Anselmo A. Advanced treatment of urban wastewater by UV radiation: effect on antibiotics and antibiotic-resistant *E. coli* strains. Chemosphere **2013**; 92:171–6.
28. Gullberg E, Cao S, Berg OG, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog **2011**; 7:e1002158.
29. Gbaguidi-Haore H, Dumartin C, L'Heriteau F, et al. Antibiotics involved in the occurrence of antibiotic-resistant bacteria: a nationwide multilevel study suggests differences within antibiotic classes. J Antimicrob Chemother **2013**; 68:461–70.
30. Zhou L-J, Ying G-G, Liu S, et al. Occurrence and fate of eleven classes of antibiotics in two typical wastewater treatment plants in south China. Sci Total Environ **2013**; 452–3:365–76.
31. Gibs J, Heckathorn HA, Meyer MT, Klapinski FR, Alebus M, Lippincott RL. Occurrence and partitioning of antibiotic compounds found in the water column and bottom sediments from a stream receiving two wastewater treatment plant effluents in northern New Jersey, 2008. Sci Total Environ **2013**; 458–460:107–16.
32. Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol Ecol **2007**; 60:166–76.
33. Chen H, Zhang M. Effects of advanced treatment systems on the removal of antibiotic resistance genes in wastewater treatment plants from Hangzhou, China. Environ Sci Technol **2013**; 47:8157–63.
34. Pruden A, Pei R, Storteboom H, Carlson KH. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ Sci Technol **2006**; 40:7445–50.
35. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect **2012**; 18:646–55.
36. Rogers BA, Sidjabat HE, Paterson DL. *Escherichia coli* O25β-ST131: a pandemic, multiresistant, community-associated strain. J Antimicrob Chemother **2011**; 66:1–14.
37. Frigon D, Biswal BK, Mazza A, Masson L, Gehr R. Biological and physicochemical wastewater treatment processes reduce the prevalence of virulent *Escherichia coli*. Appl Environ Microbiol **2013**; 79:835–44.
38. Dhanji H, Murphy NM, Akhigbe C, et al. Isolation of fluoroquinolone-resistant O25b:H4-ST131 *Escherichia coli* with CTX-M-14 extended-spectrum β-lactamase from UK river water. J Antimicrob Chemother **2011**; 66:512–6.
39. Dolejska M, Frolkova P, Florek M, et al. CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella* spp. isolates in municipal wastewater treatment plant effluents. J Antimicrob Chemother **2011**; 66:2784–90.
40. Overdevest IT, Heck M, van der Zwaluw K, et al. Comparison of SpectraCell RA typing and multilocus sequence typing for extended-spectrum-β-lactamase-producing *Escherichia coli*. J Clin Microbiol **2012**; 50:3999–4001.