

## Extended-spectrum $\beta$ -lactamase-producing Enterobacteriaceae in different environments (humans, food, animal farms and sewage)

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**Objectives:** This study aimed to determine the presence of extended-spectrum  $\beta$ -lactamase (ESBL)-producing Enterobacteriaceae in different environments.

**Methods:** Clinical samples and stool samples from animal farms, sewage, human faecal carriers attending the emergency room and faecal carriers in the context of food-borne disease outbreaks were subcultured onto MacConkey agar supplemented with cefotaxime for the detection of ESBL-producing Enterobacteriaceae. Identification, susceptibility pattern and ERIC-PCR were used for clone delineation in each sample. Community consumption of antibiotics was also recorded.

**Results:** An ESBL-producing Enterobacteriaceae prevalence of 1.9% was observed in human infections. A cross-sectional survey of human faecal carriers in the community showed a general prevalence of 6.6% with a temporal distribution. High use of antibiotics in winter coincided with a lower prevalence in carriers. ESBL-producing Enterobacteriaceae were detected in the five samples of human sewage, in samples from 8 of 10 pig farms, 2 of 10 rabbit farms, from all 10 poultry farms and in 3 of 738 food samples studied. Faecal carriage of ESBL-producing Enterobacteriaceae was detected in samples from 19 of 61 food-borne outbreaks evaluated. All food-borne outbreaks were due to enteropathogens. The prevalence of carriers in these outbreaks ranged from 4.4% to 66.6%.

**Conclusions:** This widespread occurrence of ESBL-producing Enterobacteriaceae suggests that the community could act as a reservoir and that food could contribute to the spread of these strains.

Keywords:  $\beta$ -lactamases, drug resistance, ESBLs, food outbreaks, farms, sewage

### Introduction

Strains of Enterobacteriaceae that produce extended-spectrum  $\beta$ -lactamases (ESBLs) have emerged as significant pathogens. First reported in the mid-1980s, they were mainly found in *Klebsiella pneumoniae* and *Escherichia coli* although they can now be found in many other species.<sup>1</sup>

Reports of infection or colonization with ESBL-producing Enterobacteriaceae strains have focused mainly on hospitalized

patients or nursing home residents.<sup>1</sup> However, ESBL-producing Enterobacteriaceae have also been described in animals and in patients in the community, with and without chronic conditions.<sup>2,3</sup> As little is known about ESBL dissemination mechanisms, we undertook an extensive epidemiological analysis to determine the presence of ESBL-producing Enterobacteriaceae strains in different environments: human infections and faecal carriers, human faecal carriers in the context of food-borne outbreaks, cooked and uncooked food, animal

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farms for food production, and human sewage in Barcelona (Spain).

## Materials and methods

### Samples

**Human infections.** All clinical samples from patients attending the Hospital de la Santa Creu i Sant Pau (HSP) and Hospital de la Vall d'Hebron (HVH) in Barcelona during 2003 were included. A total of 8020 Enterobacteriaceae were isolated and screened for the presence of ESBLs. Only one isolate per patient was included.

**Human faecal carriers.** According to our previous data,<sup>2,4</sup> (assuming a prevalence of 7%, a precision of 2% and adding 50% for lost samples), to obtain a final sample of 950, we tested 160 stool samples each month from patients attending the emergency room for the presence of ESBL producers; none of these patients was from a nursing home or a healthcare centre or was involved in a food-borne outbreak. A cross-sectional survey was conducted over 6 months in 2003 (February, March, May, July, September and November). Census data for Barcelona city were used to stratify the population according to age. Subjects were divided into younger than 26 years (25%), between 25 and 65 (50%) and older than 65 (25%). Each month, 40 stools from people under 26 years of age, 80 from people 26–65, and 40 stools from people over 65 were collected. Once these figures were reached for a group, collection for that group was stopped for that month. Statistical methods included analysis of the contingency table ( $\chi^2$  test) and calculation of 95% confidence interval (CI) for the parameters. Analyses were performed using the SPSS (V14) and Epi Info (V6) software.

**Human faecal carriers in the context of food-borne disease outbreaks.** A total of 544 stool samples (one per patient) from people involved in 61 food-borne disease outbreaks were evaluated in 2003. The number of stools available for study in each outbreak ranged between 3 and 45.

**Food.** A total of 155 salads (containing lettuce and tomatoes) and 583 cooked foods from the HVH catering service were evaluated.

**Animal farms.** A total of 10 pig, 10 rabbit and 10 poultry farms in Catalonia (Spain) were evaluated. Ten samples of 10 g of floor samples of faecal material were collected and processed independently for each farm.

**Sewage.** Five samples were collected from the influent raw urban sewage at two wastewater treatment plants from August 2000 to April 2003. Treatment plant 1 is a physicochemical plant serving a large conurbation of ~1 400 000 inhabitants, while plant 2 is an aerated activated sludge-treatment plant receiving sewage from a population of ~400 000.

### Microbiological studies

**Stool samples.** Stool samples from faecal carriers and faecal carriers affected by food-borne outbreaks were inoculated onto MacConkey agar plates containing 2 mg/L cefotaxime (MacC-CTX) and incubated at 37°C for 48 h under aerobic conditions. These plates were controlled by growing: a SHV-2-producing *K. pneumoniae* strain; TEM-3 and TEM-24-producing *K. pneumoniae* strains; a CTX-M-9-producing *E. coli* strain; and *E. coli* ATCC 25922 as a susceptible control strain.

**Food samples.** Each food sample (25 g) was placed in 225 mL of peptone water. After overnight incubation at 37°C, an aliquot of 1 mL was inoculated in MacC-CTX broth and incubated overnight at 37°C. In case of turbidity or bacterial growth, a subculture was

made on MacC-CTX agar and incubated overnight at 37°C under aerobic conditions.

**Farm samples.** Floor samples of faecal material (10 g) were homogenized in 90 mL of peptone water. Aliquots of 0.1 mL were plated on MacC-CTX agar and incubated for 48 h at 37°C under anaerobic conditions to avoid overgrowth of aerobic Gram-negative bacteria.

**Sewage samples.** A sample (1 mL) was inoculated in 2 mL of trypticase soy broth with cefotaxime (2 mg/L) (TSB-CTX) and incubated at 37°C for 2 h under aerobic conditions. After the pre-enrichment procedure, 2 mL from each tube was inoculated in 10 mL of TSB-CTX. Tubes were further incubated for 18 h at 37°C under anaerobic conditions to avoid overgrowth of aerobic Gram-negative bacteria. Each culture was diluted 10-fold with Ringer solution and 0.1 mL of each dilution was plated in duplicate onto MacC-CTX. Plates were incubated using the conditions described above.

### Strain selection

Except for clinical samples, whenever possible, three colonies representing each morphological type on the MacC-CTX plates were subcultured for subsequent characterization. Identification, susceptibility pattern and ERIC-PCR were used for clone delineation. One representative per clone was included.

### ERIC-PCR

ERIC-PCR was performed with the oligonucleotide ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') as described previously.<sup>5</sup>

### Susceptibility testing

Susceptibility to  $\beta$ -lactam antibiotics was determined as described previously.<sup>6</sup> ESBL-producing isolates were defined by synergy between amoxicillin/clavulanic acid and any of cefotaxime, ceftazidime, aztreonam or cefepime, and confirmed by Etest (AB Biodisk, Solna, Sweden). The breakpoints used were those defined by CLSI for Enterobacteriaceae. For *Klebsiella oxytoca*, only those isolates showing resistance to ceftazidime were included; those susceptible to ceftazidime, but resistant to aztreonam, were considered hyperproducers of chromosomal K1  $\beta$ -lactamase.

### Antibiotic consumption

Community antibiotic use was based on ambulatory care centre data provided by the Catalan Health Institute. Antibiotics were classified in the following groups: broad-spectrum penicillins, cephalosporins, amoxicillin/clavulanic acid, quinolones, macrolides and telithromycin.

## Results

Table 1 shows the prevalence of ESBL-producing Enterobacteriaceae in the different environments.

### Human infections

The prevalence of ESBL-producing isolates (155 out of 8020 Enterobacteriaceae isolates) was 1.9%. The prevalence of ESBL per species was 127 of 5836 *E. coli* (2.2%), 1 of 46 *Citrobacter freundii* (2.2%), 14 of 742 *K. pneumoniae* (1.9%), 5 of 342 *Salmonella enterica* (1.5%), 4 of 264 *Enterobacter cloacae* (1.5%), 2 of 144 *K. oxytoca* (1.4%) and 2 of 570 *Proteus mirabilis* isolates (0.3%). These isolates were isolated from 5957

## ESBLs in different environments

**Table 1.** Prevalence of ESBL-producing Enterobacteriaceae in the different environments evaluated

	Number of samples	Positive for ESBL	Prevalence (%)	CI (95%)
Infections	8020	155 127 <i>E. coli</i> 14 <i>K. pneumoniae</i> 5 <i>S. enterica</i> 4 <i>E. cloacae</i> 2 <i>K. oxytoca</i> 2 <i>P. mirabilis</i> 1 <i>C. freundii</i>	1.9	1.63–2.23
Faecal carriers	948	63 73 <i>E. coli</i> 1 <i>K. pneumoniae</i> 1 <i>C. koseri</i>	6.6	6.26–9.8
Human sewage	5	5 <sup>c</sup>	100	47.8–100
Poultry farms	10 <sup>a</sup>	10 <sup>a,c</sup>	100	69.2–100
Pig farms	10 <sup>a</sup>	8 <sup>a,c</sup>	80	44.4–97.5
Rabbit farms	10 <sup>a</sup>	2 <sup>a</sup> 2 <i>E. coli</i> 1 <i>E. cloacae</i>	20	2.5–55.6
Foods	738	3 2 <i>E. coli</i> 1 <i>K. pneumoniae</i>	0.4	0.08–1.2
Food-borne outbreaks	61 <sup>b</sup>	19 <sup>c</sup>	31.1	19.4–44.3

CI, confidence interval.

<sup>a</sup>Number of farms studied. Ten floor samples of faecal material of each farm were evaluated.

<sup>b</sup>All patients affected by each food-borne outbreak were evaluated.

<sup>c</sup>All were *E. coli* isolates.

samples of urine, 927 of blood, 382 from the respiratory tract, 226 from stools and 528 from other samples.

### Human faecal carriers

The prevalence of ESBL-producing isolates was 6.6% (63 of 948 stool samples). A statistically significant difference was observed ( $P < 0.03$ ) in the cross-sectional survey. A detailed analysis using standard residuum showed that this difference was mainly due to a decrease in positivity in May and an increase in July (Figure 1). According to age, 9.4% (23 of 244; 95% CI: 6.1–13.8) of stool samples from younger people, 5.9% (29 of 486; 95% CI: 4–8.5) from people aged 26–65 years and 5% (11 of 218; 95% CI: 2.6–8.8) from older people were positive for ESBL producers. Eleven of these faecal carriers had more than one ESBL-producing isolate. Seventy-three out of 75 ESBL-producing strains were *E. coli* (97.3%), one *K. pneumoniae* and one *Citrobacter koseri*.

### Human faecal carriers in the context of food-borne disease outbreaks

We found ESBL-producing strains in samples from 19 of the 61 outbreaks evaluated (31.1%). The prevalence of ESBL carriers in each of these outbreaks ranged from 4.4% (2 of 45 stools

samples; CI 0.5–15.2) to 66.6% (6 of 9 stools samples; CI 29.9–92.5). A total of 66 clones of *E. coli* were isolated.

### Food samples

Three of 738 (0.4%) studied foods harboured ESBL-producing strains. Two *E. coli* strains were isolated from salads and one *K. pneumoniae* strain was isolated from a cooked chicken, all consumed in September.

### Animal farms

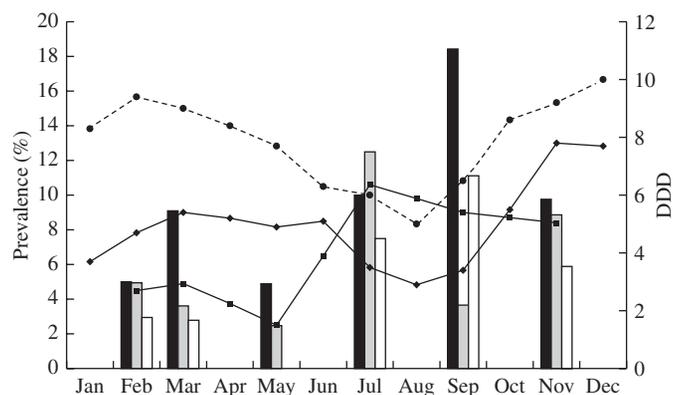
ESBL-producing isolates were isolated from floor samples of faecal material from 10 poultry, 8 pig and 2 rabbit farms. A total of 51 *E. coli* ESBL-producing strains were identified on the poultry farms, 39 *E. coli* on pig farms, and 2 *E. coli* and 1 *E. cloacae* on rabbit farms.

### Sewage

A total of 32 ESBL-producing *E. coli* clones were identified from the five human sewage samples.

### Antibiotic consumption

Antibiotic consumption for  $\beta$ -lactams and macrolides was highest between October and December 2003 and lowest in August 2003



**Figure 1.** Prevalence of ESBL-producing Enterobacteriaceae in faecal carriers during 2003, compared with antibiotic consumption (including  $\beta$ -lactams, macrolides and fluoroquinolones). Black bars, 0–25 years old; grey bars, 26–65 years old; white bars, >65 years old; filled diamonds, amoxicillin/clavulanic acid; filled circles, total consumption; filled squares, overall prevalence.

(Figure 1). Amoxicillin/clavulanic acid was the most frequently prescribed antibiotic, followed by broad-spectrum penicillins and macrolides (Figure 1).

## Discussion

ESBL-producing Enterobacteriaceae are infrequently reported in human-related environments other than the clinical setting.<sup>1,2,7</sup> The present study aimed to evaluate the prevalence of these strains in human-related environments within a single geographic area.

The prevalence of ESBLs in clinically relevant strains varies from year to year, from region to region and even from hospital to hospital.<sup>8</sup> In the Hospital de la Santa Creu i Sant Pau in 1994, the prevalence of ESBL-producing strains was 0.08% in *E. coli* and 0.6% in *K. pneumoniae*.<sup>9</sup> In 2003, however, this prevalence increased to 2.6% and 1.7%, respectively. Hospital Vall d'Hebron, in the same city, serves a different population and the prevalence in 2003 was similar: 1.8% in *E. coli*, 2% in *K. pneumoniae*. These figures are comparable to those from other studies in Spain.<sup>10</sup> The appearance of species such as *C. freundii*, *E. cloacae*, *K. oxytoca* or *S. enterica* spp. in both hospitals is of particular note.

Regarding human faecal carriers in the community, there was a temporal distribution of ESBL-producing Enterobacteriaceae. Prevalence was highest between July and November and lowest between February and May. The same fluctuation was observed in a previous study<sup>2,4</sup> performed in the same area. It thus seems realistic to take the time of year into account when comparing ESBL prevalence in faecal carriers. The prevalence was 2.1% (95% CI: 1.2–3.5) in winter–spring 2001, whereas during the same period in 2003 it increased to 4% (95% CI: 2.4–6.1). Likewise, in autumn, the prevalence increased from 7.5% (95% CI: 3.9–12.7) in 2002 to 8.7% (95% CI: 5.82–12.4) in 2003.<sup>2</sup> This increasing prevalence in faecal carriers has also been observed in other studies from Spain.<sup>11</sup>

Human sewage could probably be used as a rapid screening method to detect resistant strains that are representative of those present in the community. Indeed, the five human sewage samples screened contained ESBL-producing Enterobacteriaceae,

suggesting that the community acts as a reservoir for these strains.

Animals, including food animals and pets, are increasingly recognized as a reservoir for ESBL-producing strains. A number of reports discuss the isolation of ESBL-producing *E. coli*<sup>3,12</sup> and *S. enterica*<sup>13</sup> from animals, but most have used faeces from individual animals. When studying faeces, ESBL-producing Enterobacteriaceae represent only a low percentage of the total number of strains isolated.<sup>12,13</sup> Nevertheless, the prevalence and variety of species have increased.<sup>3</sup> We evaluated the presence of ESBL-producing Enterobacteriaceae in floor samples of faecal material as representative of a particular farm's animal community. Results showed that these strains were present in most poultry and pig farms but in few rabbit farms. Therefore, although ESBL-producing Enterobacteriaceae appear to have a low prevalence in animals, they can be found if an appropriate number of floor faecal samples are analysed. The low prevalence of these strains in floor samples of faecal material from rabbit farms stands out.

Available data suggest that food can contribute to the dissemination of resistant Enterobacteriaceae in the community.<sup>7,14</sup> The incidence was low in the present study but it should be noted that 79% of the studied foods were cooked. To our knowledge, this is the first report of an ESBL-producing *K. pneumoniae* strain isolated from food. This species was not isolated in the other non-human environments. The prevalence of these strains among food outbreaks (31.1%) and the prevalence of carriers within each outbreak (from 4.4% to 66.6%) reinforce the hypothesis that ESBL-

producing Enterobacteriaceae could be transmitted via the food supply. All food-borne outbreaks were due to enteropathogens. The role of the ESBL-producing Enterobacteriaceae in these persons was considered as commensal flora.

Several studies correlate third-generation cephalosporins with a higher prevalence of ESBL-producing *K. pneumoniae* strains in hospitals.<sup>15</sup> However, there are no data about ambulatory antibiotic prescriptions and community ESBL-producing Enterobacteriaceae. During our study we observed that the higher use of antibiotics between October and December coincided with a lower prevalence of ESBL. This could be due to the higher administration of amoxicillin/clavulanic acid, an antibiotic that is active against some ESBL-producing strains. Similarly, between July and September when use of amoxicillin/clavulanic acid fell, the prevalence of ESBL strains increased. However, other variables, such as cross-selection or summer dietary habits (a greater consumption of uncooked food), should also be taken into consideration.

In summary, the prevalence of ESBL-producing Enterobacteriaceae was notable in all the environments studied, suggesting a global expansion of these enzymes. This prevalence is likely to increase among humans worldwide in the future, as occurred with ampicillin resistance mediated by the TEM-1 enzyme.

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### Transparency declarations

None to declare.

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