Rapid Communication
Isolation of Candida auris from invasive and non-invasive samples of a patient suffering from vascular disease, Italy, July 2019
Francesca Crea, Giulia Codda, Andrea Orsi, Alberto Battaglini, Daniele Roberto Giacobbe, Emanuele Delfino, Riccardo Ungaro and Anna Marchese

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We recently isolated *Candida auris* from a blood culture and cutaneous swabs of a patient in her mid-70s. Our routine phenotypic methods failed to identify the microorganism, but it was identified by molecular tests and MALDI-TOF MS analysis. Our report, the first from Italy, further underlines the geographically wide distribution of *C. auris* and the need to confirm species identification of any suspicious colony as soon as possible to stop its spread.

*Candida auris* is a rapidly spreading, multidrug-resistant, healthcare-associated pathogen [1].

Since the first description of this novel *Candida* species, isolated from a patient in a Japanese hospital in 2009, the emergence of *C. auris* has been documented on all continents [2]. Invasive infections have been associated with high rates of treatment failure and mortality ranging from 30% to 72% [3]. *C. auris* is thus considered to pose a serious global public health threat.

*C. auris* can colonise patients for a long time, as well as persist on surfaces in healthcare environments [4]. Furthermore, *C. auris* can be misidentified in the diagnostic laboratory when traditional phenotypic methods are used. These features of *C. auris* contribute to its spread in healthcare facilities.

Here, we report a case of *C. auris* being isolated from a female in her mid-70s suffering from vascular disease.

**Case report**

On 16 June 2019 (day 1), the patient who had a history of hypertension and dyslipidaemia, was admitted to hospital for endovascular repair of an abdominal aortic aneurysm and left renal artery stenting. Because of surgical complications she was transferred to the intensive care unit (ICU). The postoperative course was further complicated and required left subclavian artery stenting on day 14. The patient first developed a fever on day 17, the same day the patient was transferred from the ICU. A computed tomography performed on day 26 for worsening condition and respiratory symptoms showed bilateral ground glass opacities. The patient was then retransferred to the ICU on day 26. Blood cultures were repeatedly collected during intermittent fever episodes that were unresponsive to antimicrobial treatment with meropenem and linezolid. Serum (1,3)-beta-D-glucan tests were performed repeatedly during fever episodes and were constantly negative. Ultimately, *C. auris* grew from blood cultures collected on day 31. The patient was immediately transferred to a single room in the infectious disease unit. *C. auris* also grew from axillary and ear swabs collected on day 41 and day 47 respectively. Treatment with caspofungin was started and all subsequent blood cultures and swabs were negative. On day 39, the patient’s improved clinical condition led to being discharged from the ICU and being admitted to the infectious disease unit. *C. auris* also grew from axillary and ear swabs collected on day 41 and day 47 respectively. Treatment with caspofungin was started and all subsequent blood cultures and swabs were negative. On day 39, the patient’s improved clinical condition led to being discharged from the ICU and being admitted to the infectious disease unit. *C. auris* also grew from axillary and ear swabs collected on day 41 and day 47 respectively. 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Identification of Candida auris and phylogenetic analysis
The pathogen was not identified by VITEK2 Advanced Expert System software version 8.01 (bioMérieux, Macy-l’Étoile, France), reporting a low discrimination between C. guillermondii (50%) and Cryptococcus laurentii (50%). It was also not identified by MALDI-TOF mass spectrometry (MALDI-TOF MS) analysis using VITEK MS software version 3 (bioMérieux). The microorganism was identified as C. auris by a species-specific PCR for GPI protein-encoding genes [5]. MALDI-TOF MS analysis using BD MALDI Biotyper System (Bruker Daltonics, Bremen, Germany) identified the yeast as C. auris (score 2.7). This result was further confirmed by D1/D2 region and internal transcribed spacer (ITS) sequencing [6,7]. The BLAST tool at the National Center for Biotechnology Information (NCBI) database was used to perform sequences similarity searches. Our sequences (GenBank accession numbers MN275234 and MN294701) showed >99% homology with C. auris.

The D1/D2 sequences were aligned with the ClustalW programme [8].

A neighbour-joining tree based on 26S rRNA gene D1/D2 domains sequences was generated using MEGA software version X [9].

Phylogenetic analyses showed that our isolate, C. auris FG GE01, clustered with the southern Asian strains (Figure).

Antifungal susceptibility
Antifungal susceptibility was determined using the Clinical and Laboratory Standards Institute (CLSI) microdilution method [10] and Sensititre YeastOne (Thermo Scientific, Waltham, Massachusetts, United States (US)). The following minimum inhibitory concentration (MIC) values were observed: >256 mg/L (fluconazole), 2 mg/L (amphotericin B), 0.5 mg/L (flucytosine), 4 mg/L (voriconazole), 0.25 mg/L (posaconazole), 0.5 mg/L (itraconazole), 0.12 mg/L (micafungin), 0.25 mg/L (anidulafungin) and 0.12 mg/L (caspofungin).

MEGAX software was used to align 26S rRNA gene D1/D2 domains sequences and to draw the bootstrap (550 replicates) unweighted pair group method. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Kimura 2-parameter method was used to compute the evolutionary distances.
Epidemiological and environmental investigations

Preliminary epidemiological and environmental investigations have not yet revealed the source of the infection. The patient had no history of recent travel abroad or hospital admission and to date, *C. auris* has not been isolated from any of the case’s close contacts. A close contact was defined as a patient who was hospitalised in the same room during the same period (at least 12 hours) of the case or a patient who occupied the same bed as the case immediately after. No contacts outside the hospital, e.g. family members, were swabbed.

In August 2019, screening was performed of the environment and medical devices of the vascular ward, ICU and operating theatre where the patient stayed during the course of hospitalisation. The 36 environmental samples tested (dynamic mattresses, \( n = 5 \); bedrails, \( n = 10 \); trolley, \( n = 5 \); ventilators, \( n = 5 \); suction apparatus, \( n = 5 \); floor, \( n = 2 \); washbasin, \( n = 2 \); bed bell, \( n = 2 \)) were negative. Additional cleaning using hydrogen peroxide and hypochlorite was implemented across the hospital.

Discussion

To our knowledge, this is the first isolation of *C. auris* in Italy. However, this is not particularly surprising given the widespread distribution of this pathogen and its previous detection in several nearby European countries, including France, Spain and Greece [11].

We were able to correctly identify the microorganism by using MALDI-TOF as well as molecular techniques, approaches that are still not available in many diagnostic laboratories. The first finding of *C. auris* in Italy should encourage a careful approach to yeast identification when non-*albicans Candida* strains are detected. Given the present scenario, confirmation of species identification of any suspicious colony is clearly essential.

Four major phylogenetically distinct clades of *C. auris* have been described: clade I (South Asian), clade II (East Asian), clade III (African) and clade IV (South American) [12]. A potential fifth clade has recently been reported in Iran [13].

The isolate in this study clusters with the southern Asian strains. Clade I has been already described in European hospitals and in hospitals in the US, and linked to outbreaks with invasive infections [14].

Since the potential source of the infection has not been identified, we currently have no basis for any speculation about the origin of *C. auris* in the hospital. The prompt isolation of the patient seems to have stopped the spread. Although specific breakpoints for *C. auris* have not been defined by international committees, susceptibility data published to date suggests that this pathogen exhibits resistance to fluconazole (MIC\( > 32 \) mg/L) and different level of susceptibility to the other azoles, echinocandins and amphotericin B. A considerable percentage of *C. auris* strains investigated had high MICs for voriconazole and amphotericin B (MIC\( > 1 \)). In general, our susceptibility results are in agreement with previous observations [15]. Adopting CLSI breakpoints for closely related species (*C. guillermondii* and *C. parapsilosis*), *C. auris* FG GE01 was categorised as susceptible to echinocandins. This finding is supported by clinical data: caspofungin treatment was effective and blood samples collected 7 days after starting the treatment were negative. However, development of resistance to echinocandins has also been described in *C. auris* and other *Candida* species [16,17].

This isolation of *C. auris* is further confirmation its intercontinental distribution, and that judicious use of antifungals coupled with strengthened infection control measures are needed to prevent and control the spread of *C. auris*.

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Conflict of interest

None declared.

Authors’ contributions

Francesca Crea: analysis and interpretation of patient’s primary cultures; isolation of *C. auris*; analysis and interpretation of conventional identification and susceptibility tests; critical revision of the manuscript.

Giulia Codda: analysis and interpretation of molecular data; GenBank deposit; contribution to critical revision of manuscript.

Anna Marchese: acquisition, analysis and interpretation of data; integration of information submitted by all contributors; drafting and revising the manuscript.

Andrea Orsi, Alberto Battaglini: epidemiological follow up of patient; revising the epidemiological aspects of the manuscript.

Daniele Roberto Giacobbe, Emanuele Delfino, Riccardo Ungaro: administration of antimicrobial therapy; follow-up of patient; contribution to the acquisition of data; and drafting and revising the clinical aspects of the manuscript.
References


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Any supplementary material referenced in the article can be found in the online version.

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Population-level surveillance of antibiotic resistance in Escherichia coli through sewage analysis

Marion Hutinel1,2, Patricia Maria Catharina Huijbers1,2, Jerker Fick3, Christina Åhrén1,2,4, Dan Göran Joakim Larsson1,2, Carl-Fredrik Flach1,2

Introduction: The occurrence of antibiotic resistance in faecal bacteria in sewage is likely to reflect the current local clinical resistance situation. Aim: This observational study investigated the relationship between Escherichia coli resistance rates in sewage and clinical samples representing the same human populations. Methods: E. coli were isolated from eight hospital (n=721 isolates) and six municipal (n=531 isolates) sewage samples, over 1 year in Gothenburg, Sweden. An inexpensive broth screening method was validated against disk diffusion and applied to determine resistance against 11 antibiotics in sewage isolates. Resistance data on E. coli isolated from clinical samples from corresponding local hospital and primary care patients were collected during the same year and compared with those of the sewage isolates by linear regression. Results: E. coli resistance rates derived from hospital sewage and hospital patients strongly correlated (r²=0.95 for urine and 0.89 for blood samples), as did resistance rates in E. coli from municipal sewage and primary care urine samples (r²=0.82). Resistance rates in hospital sewage isolates were close to those in hospital clinical isolates while resistance rates in municipal sewage isolates were about half of those measured in primary care isolates. Resistance rates in municipal sewage isolates were more stable between sampling occasions than those from hospital sewage. Conclusion: Our findings provide support for development of a low-cost, sewage-based surveillance system for antibiotic resistance in E. coli, which could complement current monitoring systems and provide clinically relevant antibiotic resistance data for countries and regions where surveillance is lacking.

Introduction

Due to increasing problems with antibiotic resistance, treatment guidelines need to be continuously adapted to the local resistance situation to secure effective empirical antibiotic therapy. A cornerstone in the guidance on first line treatment is therefore up-to-date surveillance of antibiotic resistance rates. In addition, informative surveillance can alert in case of emergence of rare or new resistance threats as well as help to prioritise actions to be taken and evaluate their outcomes [2]. Today’s clinical surveillance systems for antibiotic resistance are all dependent on the analysis of samples from a large number of individuals in order to provide epidemiologically relevant data. This resource-demanding process requires considerable infrastructure, a major reason behind the still very limited or complete lack of surveillance in large parts of the world [3].

Sewage contains pooled urine and faeces from a large number of individuals and, in many aspects, reflects the population connected to the sewage system. Hence, sewage analysis has emerged as an attractive means for different population-based surveillance purposes. Such an approach referred to as sewage/wastewater epidemiology [4] has, for instance, provided estimations of pharmaceutical [5] and illicit drug consumption [6,7], as well as been employed for the surveillance of viral pathogens [8,9]. With regard to antibiotic-resistant bacteria, in several studies similar strains were isolated from both sewage and clinical samples [10-14].

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Additionally, results from a few studies have indicated that antibiotic resistance rates in sewage bacteria have increased over time, which may reflect an increased prevalence of resistant bacteria in the human population [15,16]. Altogether, this suggests that analyses of sewage samples have potential to serve as a resource-efficient complement to today’s clinical surveillance systems of antibiotic-resistant bacteria. For that purpose, the relationship between resistance rates in sewage and clinical isolates needs to be established.

The overall aim of the study was to contribute to the development of a sewage monitoring system for the surveillance of antibiotic-resistant pathogens in human populations. Specifically, we aimed to investigate the relationship between *E. coli* resistance rates in sewage and clinical samples collected from both a hospital and a broader municipal population. In the interest of facilitating antibiotic susceptibility testing (AST) of a large number of sewage isolates, we also evaluated a resource-efficient broth screening methodology by comparing it to standardised disk diffusion tests.

### Methods

#### Sewage samples

Hospital sewage was sampled on eight occasions in 2016 at the Sahlgrenska University Hospital in Gothenburg, the largest hospital in Sweden (1,950 beds), from the principal sewage line of the hospital’s main site. Each occasion consisted of a period of 24 hours, with subsamples taken every 9th minute over the period (n=160). Municipal sewage was sampled, during the same year, on six occasions from the inlet to the Ryaverket (Gryaab AB, Gothenburg, Sweden) wastewater treatment plant (WWTP), serving at that time 746,882 persons from the larger Gothenburg area. At each municipal sewage sampling occasion, a minimum of 224 subsamples were taken flow-proportionally over 24 hours.

### Identification and isolation of *Escherichia coli*

Sewage samples were kept at 4 °C and processed within 3 hours after collection. The samples were serially diluted 10-fold with sterile 0.85% NaCl before plating on ECC (CHROMagar, Paris, France) chromogenic media in triplicates and incubated at 37 °C for 20 to 24 hours. The *E. coli* concentration was assessed by counting the blue colonies on the ECC plates. Well-isolated, presumed *E. coli* colonies were randomly picked from all plating replicates of two or three dilutions and stored at − 80 °C in lysogeny broth (LB) with 20% glycerol. All isolates were subjected to confirmatory species identification by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (VITEK, Biomerieux, Marcy l’Étoile, France). Only verified *E. coli* were included in subsequent analyses.

#### Broth resistance screening of *Escherichia coli* isolated from sewage samples

Antibiotic stock solutions were prepared as described in Supplement S1, filter sterilised (0.2 µm, VWR, Radnor, Pennsylvania (PA) United States (US)), aliquoted and stored at − 80 °C. The concentrations of the antibiotic stock solutions were verified experimentally before the first and after the last use of the antibiotic stock solutions. Minimum inhibitory concentration (MIC) determinations for the *E. coli* ATCC 25922 and

### Table 1

Annual means of the resistance rates in *Escherichia coli* isolated from hospital and municipal sewage, Gothenburg, Sweden, 2016 (n = 1,252)

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>Mean resistance rates a, %</th>
<th>Hospital sewage (8 sampling occasions; 721 isolates)</th>
<th>Municipal sewage (6 sampling occasions; 531 isolates)</th>
<th>p value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>19.4</td>
<td>9.7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>8.8</td>
<td>5.7</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>5.5</td>
<td>2.0</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>5.2</td>
<td>1.4</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>11.6</td>
<td>4.7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Mecillinam</td>
<td>2.0</td>
<td>4.2</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0.9</td>
<td>0.0</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>0.9</td>
<td>0.3</td>
<td>0.316</td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>5.1</td>
<td>0.4</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>21.7</td>
<td>11.7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>19.6</td>
<td>10.8</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>ESBLs</td>
<td>5.5</td>
<td>1.8</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

ESBLs: extended spectrum beta-lactamase-producing *E. coli*.

a Mean of the resistance rates measured for the different sampling occasions in 2016. The rates at each sampling occasion are detailed in Tables 2 and 3 for hospital and municipal sewage, respectively.

b p value of Fisher’s exact test comparing overall resistance rates (cumulative data for all sampling occasions) from hospital and municipal sewage.
ATCC 35218 control strains were performed by broth microdilution following the recommendations from the European Committee on antimicrobial susceptibility testing (EUCAST) for internal quality control [17,18]. The resistance profile of 1,252 E. coli isolates was determined for 11 antibiotics (amoxicillin-clavulanic acid, cefadroxil, cefotaxime, ceftazidime, ciprofloxacin, mecillinam, nitrofurantoin, piperacillin-tazobactam, tobramycin, trimethoprim, trimethoprim-sulfamethoxazole) at EUCAST clinical breakpoint concentrations [19]. The panel of antibiotics tested was chosen to match the tests routinely performed on E. coli isolated from urine or blood samples in the local clinical setting.

In order to facilitate high throughput testing, an AST methodology based on a 96-well-plate screening in broth was applied. A plate was prepared for each antibiotic by diluting the antibiotic stock solutions in cation-adjusted Mueller–Hinton broth to the appropriate breakpoint concentration. An extra plate with unsupplemented broth served as positive growth control. Half of the wells of each plate were inoculated with sewage isolates (pre-grown overnight on horse blood agar) to a final concentration of ca 5 x 10⁵ CFU/mL (leaving every second well on the plates without inoculum to enable detection of accidental contaminations). Resistance/susceptibility was determined by visual assessment of growth after overnight culture at 37°C.

Isolates susceptible to cefadroxil were considered susceptible to all cephalosporins, whereas isolates resistant to cefadroxil were subsequently tested for resistance to cefotaxime and ceftazidime by disk diffusion as well as extended-spectrum beta-lactamases (ESBL) production by double-disk synergy test [20]. Due to high frequency of de novo mutations providing mecillinam resistance in vitro, isolates found resistant to mecillinam in broth were subjected to disk diffusion. Disk diffusion tests were performed with Oxoid disks (Thermo Fisher, Waltham, Massachusetts (MA) US) following EUCAST guidelines [21].

### Collection of clinical data on antibiotic resistance

Aggregated resistance data were supplied from the clinical laboratories, which isolated bacteria from blood and/or urine samples and determined the species of the isolates as part of their routine work. AST was performed by the laboratories with different sets of antibiotics depending on the type of sample (urine or blood), by disk diffusion using EUCAST guidelines and breakpoints [19,21]. Cephalosporin-resistant isolates were tested for the ESBL phenotype as described for sewage isolates above. The resistance rates were calculated for E. coli isolated in 2016 from, on the one hand, patients of the hospital wards connected to the sampling point for the hospital sewage, and on the other hand, primary care patients from the municipalities connected to the sampled WWTP. Only the first urine and/or blood isolate from each patient was included to avoid bias due to repeated sampling.

### Table 2

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>Sampling occasion (number of E. coli tested)</th>
<th>p valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 Jan (96)</td>
<td>22 Mar (8a)</td>
</tr>
<tr>
<td></td>
<td>n²</td>
<td>%</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>23</td>
<td>24.0</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>29</td>
<td>30.2</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>23</td>
<td>24.0</td>
</tr>
<tr>
<td>ESBLs</td>
<td>2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

ESBLs: extended spectrum beta-lactamase-producing E. coli.

a As some isolates could be resistant to more than one antibiotic, the sum of values presented in the subcolumn can exceed the total number of isolates analysed on the sampling occasion, which is presented in the main column header in parentheses.

b p value of Fisher’s exact test comparing the different sampling occasions.
Comparison of broth screening and standardised disk diffusion

To evaluate the comparability of our broth screening method with the disk diffusion methodology used for clinical isolates, 155 sewage isolates were tested for resistance against amoxicillin-clavulanic acid, cefadroxil, ciprofloxacin, nitrofurantoin, piperacillin-tazobactam, tobramycin, trimethoprim and trimethoprim-sulfamethoxazole in parallel with both methods.

Analysis of antibiotics in the sewage samples

Sewage samples were centrifuged (200 mL; 17,500 g; 20 min). The supernatants were filtered through 0.45 µm Filtropur S membranes (Sarstedt, Nürnberg, Germany). Subsequently, 150 mL of the filtered supernatant of each sample was spiked with 50 ng of internal standards and antibiotic concentrations were determined by liquid chromatography-mass spectrometry as described by Lindberg et al. [22]. Antibiotics to be analysed were chosen based on publicly available consumption statistics (provided by the Swedish eHealth Agency) for the region of Sweden where the study was conducted (Region Västra Götaland). In total 14 different antibiotics were screened and the selection included, but was not limited to, substances from antibiotic classes represented during the resistance screening of *E. coli* isolates and/or whose antibacterial spectra include *E. coli*.

Biochemical fingerprinting of sewage isolates

Diversity among sewage isolates was assessed based on substrate metabolism using the PhenePlate system for rapid screening of *E. coli* (PhPlate Microplate Techniques AB, Stockholm, Sweden) according to the manufacturer’s instructions. Isolates with similarity levels over 0.975 were considered the same biochemical phenotype. Diversity was calculated using Simpson’s index, where values close to one indicate an even distribution of multiple types and lower values indicate one or more dominant types [23]. Calculations of similarities and diversity index, as well as cluster analysis were performed using PhPWIN 7.1 software (PhPlate Microplate Techniques AB, Stockholm, Sweden).

Statistical methods

*E. coli* concentrations in different sewage samples were compared using the Welch t-test. The *E. coli* resistance rates in different samples were compared using Fisher’s exact test. Resistance rates in sewage isolates and in clinical isolates indicated strong linear relationships for the measured values, therefore linear regressions were used to model these relationships. To stabilise the variance associated with a binomial distribution, the transformation $T(x) = \sqrt{n} \times \arcsin(\sqrt{x/n})$ (where *x* is the resistance rate and *n* the number of measurements) was applied to the resistance rate before linear regression was employed and the Pearson correlation coefficient calculated [24]. Statistical analysis of the data was performed using R version 3.4.1 [25] and a significance level of 0.05 was applied.

### Table 3

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>21 Jan (95)</th>
<th>30 Mar (115)</th>
<th>3 May (95)</th>
<th>14 Jun (42)</th>
<th>23 Aug (104)</th>
<th>29 Nov (80)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>$%$</td>
<td>$n$</td>
<td>$%$</td>
<td>$n$</td>
<td>$%$</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>12</td>
<td>12.6</td>
<td>8</td>
<td>7.0</td>
<td>8</td>
<td>8.4</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>6</td>
<td>6.3</td>
<td>4</td>
<td>3.5</td>
<td>3</td>
<td>3.2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>3</td>
<td>3.2</td>
<td>2</td>
<td>1.7</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3</td>
<td>3.2</td>
<td>1</td>
<td>0.9</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4</td>
<td>4.2</td>
<td>4</td>
<td>3.5</td>
<td>3</td>
<td>3.2</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>6</td>
<td>6.3</td>
<td>4</td>
<td>3.5</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1</td>
<td>1.1</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>11</td>
<td>11.6</td>
<td>8</td>
<td>7.0</td>
<td>13</td>
<td>13.7</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>10</td>
<td>10.5</td>
<td>8</td>
<td>7.0</td>
<td>11</td>
<td>11.6</td>
</tr>
<tr>
<td>ESBLs</td>
<td>3</td>
<td>3.2</td>
<td>1</td>
<td>0.9</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

ESBLs: extended spectrum beta-lactamase-producing *E. coli*.

* As some isolates could be resistant to more than one antibiotic, the sum of values presented in the subcolumn can exceed the total number of isolates analysed on the sampling occasion, which is presented in the main column header in parentheses.

* p value of Fisher’s exact test comparing the different sampling occasions.
Results

**Escherichia coli concentrations in sewage samples**

No significant difference was observed in the viable *E. coli* concentration between hospital (mean $1.29 \times 10^4$ CFU/mL) and municipal sewage samples (mean $1.38 \times 10^4$ CFU/mL) ($p = 0.87$) (Supplement S2). Throughout the different sampling occasions, 1,252 of the 1,256 isolates (99.7%) collected were confirmed by MALDI-TOF mass spectrometry to be *E. coli*. Only four isolates (0.3%) were identified as other species (*Klebsiella oxytoca*, *Citrobacter freundii*, *Enterobacter* sp. and *Pseudomonas aeruginosa*), and these were discarded from further analysis.

**Comparison of antibiotic susceptibility testing by disk diffusion and broth screening**

For comparison, 155 sewage isolates, were tested against eight antibiotics with both disk diffusion and broth screening (generating 1,240 pairs of results). With disk diffusion as the reference method, four isolates were falsely classified as resistant against cefadroxil by broth screening. Four additional isolates were falsely classified as susceptible by broth screening. Among these, two were resistant to amoxicillin-clavulanic acid, one to piperacillin-tazobactam and one to tobramycin as determined by disk diffusion. Thus, in 99.4% (1,232/1,240) of the instances, both methods were in agreement. The broth screening method had a sensitivity of 94.4% (68/72), a specificity of 99.7% (1,164/1,168), a positive predictive value of 94.4% (68/72) and a negative predictive value of 99.7% (1,164/1,168) for detection of resistance.

**Resistance rate in sewage *Escherichia coli* isolates**

The annual mean resistance rates measured in hospital sewage were higher than in municipal sewage (Table 1) for all antibiotics tested except mecillinam (mecillinam resistance was more prevalent in municipal sewage). A higher prevalence of ESBL producers was also observed in the hospital sewage isolates. All these differences were significant, except for piperacillin-tazobactam, when cumulative data for all sampling occasions was analysed. The lowest resistance rates were measured for nitrofurantoin (0.9% of hospital sewage isolates and not detected in municipal sewage isolates) and piperacillin-tazobactam (0.9% and 0.3% for hospital and municipal sewage isolates respectively). The highest resistance rates were measured for trimethoprim (21.7% of hospital sewage isolates and 11.7% of municipal sewage isolates) followed by trimethoprim-sulfamethoxazole (19.6% and 10.8% respectively) and amoxicillin-clavulanic acid (19.4% and 9.7% respectively).

### Table 4

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>Type of clinical sample (number of <em>E. coli</em> tested)</th>
<th>p value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hospital blood (189)</td>
<td>Hospital urine (1,097)</td>
<td>Primary care urine (4,984)</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>NA</td>
<td>245</td>
<td>24.4</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>NA</td>
<td>99</td>
<td>9.0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>11</td>
<td>5.8</td>
<td>82</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>9</td>
<td>4.8</td>
<td>69</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>28</td>
<td>14.8</td>
<td>143</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>NA</td>
<td>62</td>
<td>5.7</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>NA</td>
<td>16</td>
<td>1.5</td>
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<tr>
<td>Piperacillin-tazobactam</td>
<td>5</td>
<td>2.6</td>
<td>NA</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>6</td>
<td>3.2</td>
<td>NA</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>NA</td>
<td>249</td>
<td>22.7</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>44</td>
<td>23.3</td>
<td>NA</td>
</tr>
<tr>
<td>ESBLs</td>
<td>12</td>
<td>6.3</td>
<td>81</td>
</tr>
</tbody>
</table>

ESBLs: extended spectrum beta-lactamase-producing *E. coli*; NA: not applicable.

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<tr>
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<td>Nitrofurantoin</td>
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<tr>
<td>Piperacillin-tazobactam</td>
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<td>2.6</td>
<td>NA</td>
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<tr>
<td>Tobramycin</td>
<td>6</td>
<td>3.2</td>
<td>NA</td>
</tr>
<tr>
<td>Trimethoprim</td>
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<td>ESBLs</td>
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<td>81</td>
</tr>
</tbody>
</table>

ESBLs: extended spectrum beta-lactamase-producing *E. coli*; NA: not applicable.

* Resistance rates were generally calculated for 1,097 isolates from hospital patients’ urine with the exception of amoxicillin-clavulanic acid, cefadroxil, cefotaxime, ceftazidime and ESBLs for which 1,003, 1,096, 1,096 and 1,088 isolates, respectively, were tested.

* Resistance rates were generally calculated for 4,984 isolates from primary care patients’ urine with the exception of amoxicillin-clavulanic acid and ESBLs for which 4,880 and 4,967 isolates, respectively, were tested.

* p value of Fisher’s exact tests comparing blood and urine samples from the hospital.

* p value of Fisher’s exact tests comparing urine samples from hospital and primary care.

**ESBLs**: extended spectrum beta-lactamase-producing *E. coli*; NA: not applicable.

a Resistance rates were generally calculated for 1,097 isolates from hospital patients’ urine with the exception of amoxicillin-clavulanic acid, cefadroxil, cefotaxime, ceftazidime and ESBLs for which 1,003, 1,096, 1,096 and 1,088 isolates, respectively, were tested.

b Resistance rates were generally calculated for 4,984 isolates from primary care patients’ urine with the exception of amoxicillin-clavulanic acid and ESBLs for which 4,880 and 4,967 isolates, respectively, were tested.

c p value of Fisher’s exact tests comparing blood and urine samples from the hospital.

d p value of Fisher’s exact tests comparing urine samples from hospital and primary care.
E. coli showing resistance to at least one of the investigated antibiotics were twice as prevalent in hospital sewage (264/721; 36.6%) as in municipal sewage (95/531; 17.9%) (Supplement S3). Additionally, 10 of the 11 most resistant isolates (resistant against ≥5 antibiotics) were found in hospital sewage.

The variability of the resistance rates was greater in hospital sewage than in municipal sewage (Tables 2 and 3). Indeed, resistance rates measured in hospital sewage were significantly different between sampling occasions for all antibiotics except mecillinam and piperacillin-tazobactam. In stark contrast, no significant differences were observed between the sampling occasions in municipal sewage for any antibiotic. In the hospital sewage, ciprofloxacin resistance appeared to vary the most. An exceptionally high rate of resistance (53.1%) was detected in E. coli isolated on 22 June while ciprofloxacin resistance varied between 1% and 15.6% at the other sampling occasions.

Antibiotic concentrations in sewage
Antibiotic concentrations were measured to assess if selection pressure from a particular antibiotic in the sewage could have influenced the results (Supplement S4). Ten of the 14 investigated antibiotics were detected in the sewage samples, all at concentrations below the lowest MIC reported for E. coli by EUCAST.

Biochemical phenotypes of sewage Escherichia coli
In order to assess if clonality may have influenced our results, especially on occasions with extreme resistance rates, all E. coli isolates from hospital samples collected on 21 January (presenting resistance rates close to the yearly mean), 22 June (presenting particularly high resistance rate for ciprofloxacin), and 15 November (presenting particularly low resistance rates) were subjected to biochemical phenotyping (Supplement S5). Overall, the sample presenting resistance rates close to the yearly mean (21 January) showed a diversity index of 0.97, whereas lower diversity indexes were observed for the samples from 22 June (0.85) and 15 November (0.91). In the two latter samples, there were many isolates with identical biochemical phenotypes contributing to the extreme resistance rates observed. Indeed, on 22 June, of the 51 ciprofloxacin-resistant isolates, 32 had an identical biochemical phenotype and were resistant to ciprofloxacin only. On 15 November, 28 of the 81 fully susceptible isolates had indistinguishable biochemical phenotypes.

Resistance rates in clinical Escherichia coli isolates
Measured resistance rates in E. coli urinary isolates were higher for hospital patients than for primary care patients for all antibiotics (Table 4). The differences were significant for all three cephalosporins, ciprofloxacin, and ESBL production. No significant differences were observed between resistance rates in E. coli from blood or urine samples from the hospital. Similar to what was measured in sewage isolates, the lowest resistance rates in hospital blood isolates was observed for piperacillin-tazobactam (2.6%), whereas the lowest resistance rates in urine were observed for nitrofurantoin for both primary care (0.9%) and hospital isolates (1.5%). Also in coherence with what was measured in sewage isolates, the highest resistance rates in hospital blood isolates was observed for trimethoprim-sulfamethoxazole (23.3%). The highest resistance rates in urine were observed for amoxicillin-clavulanic acid for both primary care (23.4%) and hospital isolates (24.4%).

Multiresistance was more common in E. coli isolated from hospital than primary care patients (Supplement S3), again in line with was observed for sewage samples.

Comparison of resistance rates in Escherichia coli isolates from sewage and clinical samples
Resistance rates in sewage E. coli strongly correlated with resistance rates in corresponding clinical E. coli (Figures 1 and 2). The strongest correlations were observed between resistance rates in hospital sewage and hospital clinical isolates ($r^2 = 0.95$ and 0.89 for urine and blood samples respectively). A slightly weaker correlation was observed when municipal sewage was related to primary care urine samples ($r^2 = 0.82$). The resistance rates in isolates from hospital sewage were overall close to those observed in isolates from hospital patients, whereas the resistance rates in municipal sewage isolates were in general lower than in primary care patient isolates. In the latter case, there was a twofold difference in resistance rates for the majority of antibiotics tested (five of eight). These relationships between sewage and clinical samples were also observed for the proportions of ESBL-producing isolates. The main exception to that observation was for cefadroxil for which the resistance rate was higher in E. coli from municipal sewage than from primary care patients. Noticeably, this coincided with a lower proportion of ESBL producers among the cefadroxil-resistant isolates in sewage (48/90; 53.3%) than in clinical samples (258/331; 77.9%) ($p < 0.001$).

Discussion
This study revealed stable relationships between resistance rates in E. coli from sewage and clinical samples across all tested antibiotics. The strongest correlation was observed when a well-defined and extensively sampled population (i.e. the hospital population) was investigated. Nevertheless, a strong correlation was still present when samples originating from a much larger population were analysed. These results suggest that resistance rates for other antibiotics can be estimated based on sewage analyses for these populations. Furthermore, our results show that E. coli can be isolated with high specificity from sewage samples and, by using an inexpensive broth screening, their resistance profiles can be determined in good
Concurrence with disk diffusion tests. Together, these findings provide support for the development of an inexpensive, sewage-based surveillance system for antibiotic resistance in *E. coli*.

The very good overall agreement between the broth screening method used for the sewage isolates in this study and the disk diffusion method used for the clinical isolates allows for comparison between the two types of isolates. However, the method comparison indicated an overestimation of the cefadroxil-resistant isolates by the broth screening. Such overestimation is in line with the observation of lower proportions of cefadroxil-resistant isolates that were ESBL producers in sewage compared with clinical samples. This could also explain why the measured cefadroxil resistance rate was higher in municipal sewage than in primary care patient isolates (whereas the opposite was seen for all other antibiotics).

Few studies have aimed to compare antibiotic resistance rates in sewage and clinical isolates. These studies have reported antibiotic resistance data for sewage isolates that follow general patterns seen among the clinical isolates, i.e. increased resistance rate over time and same common or rare resistance phenotypes [15,16,26]. Although these earlier reports also support the concept of surveying antibiotic-resistant bacteria in human populations via sewage monitoring, systematic comparisons between sewage and clinical isolates in order to establish their relationships were hampered for various reasons. Some of those studies had collected sewage samples after the start of sewage treatment [16,26], which inevitably alters the taxonomic composition of the samples and possibly also the proportions of resistant strains within species [27,28]. In the study by Kwak et al., similarly to the present study, *E. coli* isolates from Swedish untreated hospital and municipal sewage samples collected locally during a year were analysed. Their results showed reasonable concordance between the resistance rates in hospital sewage isolates and the clinical blood isolates for three of four antibiotics used for comparisons. However, in contrast to our study, the clinical data used for comparison by Kwak and co-workers were obtained from a much wider population than the one contributing to the sewage samples as well as being from different years (national surveillance data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) database) [15]. Furthermore, Kwak et al. did apply different resistance breakpoints for the sewage isolates than what were applied in the clinical setting (EUCAST’s clinical breakpoints). Taken together, to the best of our knowledge, the present study is the first to compare resistance rates in *E. coli* isolates from untreated sewage samples and clinical samples from

**Figure 1**

Mean resistance rates in *Escherichia coli* isolated from hospital sewage samples compared with those isolated from (A) urine and (B) blood samples from the same hospital, Gothenburg, Sweden, 2016 (n = 2,007)
the population contributing to the sewage collected during the same time period. Although we did not detect a difference in the *E. coli* concentrations between the different sewage types, the resistance rates in *E. coli* were generally higher in hospital sewage, which is in accordance with earlier studies in Sweden and other countries [15,29-33]. Still, isolates from both types of sewage samples showed resistance rates strongly correlated with those in the corresponding clinical samples. Notably, the resistance rates measured in hospital sewage isolates were very similar to the resistance rates observed in the clinical setting, both for urine and blood isolates. It should be acknowledged that the sewage monitoring by itself would not have been able to detect differences between blood and urine isolates if such existed, but would have led to different relationships between hospital sewage isolates and the different types of clinical isolates in the current study. A different relationship compared with what was seen for the hospital population was identified when municipal sewage and corresponding clinical data were compared — the resistance rates measured in municipal sewage isolates were about half of the clinical rates. This difference is however, not due to different specimen types, since urine isolates were analysed for both populations. In general, antibiotic resistance surveillance data are based on samples from a subset of the surveyed population. Even in Sweden, where surveillance from a global perspective is extensive, only a minority of the non-hospitalised patients with urinary tract infection are sampled. Hence, there is a risk for biased clinical surveillance data, not least since empiric treatment failure and recurrent urinary tract infections result in a higher degree of sampling [34]. This might partly explain the lower resistance rates in municipal sewage isolates compared with corresponding clinical isolates observed in this study. In accordance with a sentinel study conducted in Switzerland [35], it would imply that resistance rates for *E. coli* causing urinary tract infections in the non-sampled empirically treated population are lower than what is suggested by the clinical surveillance data. Consequently, there is a risk that antibiotics are discarded because of high resistance rates when they might still be of use for uncomplicated urinary tract infections. Another factor contributing to the differences in resistance rates between municipal sewage and clinical isolates is most likely that sewage isolates are predominantly originating from the gut flora of the population connected to the sewer, whereas clinical resistance rates are based exclusively on isolates causing infections. In that aspect, the observed resistance rates in the municipal sewage are in line with previous studies showing lower resistance rates in faecal *E. coli* strains compared with *E. coli* strains causing infections [36], even when the different types of strains are isolated from the same individuals [37,38]. In relation to this, our finding that resistance rates in hospital sewage isolates were generally very similar to what was observed in clinical hospital isolates is intriguing as it indicates that *E. coli* causing infections in the hospital population would, on average, have similar probabilities of being resistant as *E. coli* in their gut flora. This observation might, partly, be explained by the specificities of the hospital environment in itself. A relatively high consumption of antibiotics, which may lead to selection of resistant bacteria within patients’ intestinal flora, and transmission of resistant nosocomial strains can result in hospitalised patients carrying more resistant strains than the general population [39,40].

We observed a higher variability in the measures of resistance rates in hospital sewage than in municipal sewage, which led us to suspect that antibiotics in hospital sewage might occasionally reach concentrations capable of selecting for resistant bacteria in the sewer pipes. While all measured antibiotic concentrations were well below the lowest MICs, ciprofloxacin levels at times exceeded concentrations reported to select for resistance over many generations in pairwise competition experiments [41]. However, given the relatively short passage time from the toilets to the sampling point, and hence very limited growth opportunities,

**Figure 2**
Mean resistance rates in *Escherichia coli* isolated from influent samples collected at the municipal WWTP compared with those isolated from urine from primary care patients in the region served by the WWTP, Gothenburg, Sweden, 2016 (n = 5,515)
bactericidal or close to bactericidal concentrations would likely have been needed to manifest in detectable changes in resistance rates. Furthermore, the hospital sample for which resistance rates were particularly high for several antibiotics including ciprofloxacin, did not contain exceptionally high antibiotic concentrations. Taken together, selection by antibiotic residues in the hospital sewers were likely not an important factor behind the large variation in resistance rates between sampling dates. Another possible explanation behind the larger variation in resistance rates between dates in hospital sewage might be accidental sampling of clones due to the smaller size of the contributing population and shorter distance between the sampling point and the source compared with municipal sewage. The latter should lead to reduced suspension and mixing of the faecal material before sampling thereby increasing the risk of isolating several bacteria originating from the same individual. Biochemical fingerprinting of sewage isolates strongly supported this hypothesis by revealing a reduced diversity of the *E. coli* isolated from hospital sewage samples showing extreme resistance patterns. A similar range of *E. coli* diversity in hospital wastewater has been shown in studies by Kwak et al. and Colque Navarro et al. [15,30]. Analogous to the current study, low diversity found in a hospital wastewater sample could be attributed to the presence of highly abundant biochemical phenotypes with the same resistance pattern [30]. Limited diversity due to clonality emphasises the necessity for repeated sampling of sewage in order to obtain representative data, especially when hospital sewage is collected.

In conclusion, this study indicates that resistance data obtained from sewage samples reflects well the resistance situation in the studied populations. However, in order to use sewage monitoring to predict the clinical situation in other populations, including those for which such data are missing, further calibration is needed. Resistance rates in sewage and clinical isolates from different settings, with different levels of resistance, need to be compared in order to evaluate the stability of the relationships between different sites. Ideally, sewage monitoring should also be calibrated over time via repeated sampling at the same site while the clinical resistance situation is changing. This calibration could be extended from *E. coli* to additional important pathogens that can be present in faeces (such as *Klebsiella pneumoniae* and *Salmonella enterica*), and possibly also from the study of human populations to husbandry animals [42-44]. Given such evaluation, analyses of sewage samples have the potential to be used for population-level surveillance of antibiotic-resistant pathogens in a cost-efficient way. The approach might then complement current monitoring systems by resolving some of the problems associated with the limited sampling in clinical praxis and be applied to provide antibiotic resistance data and possibly guide empirical treatment recommendations in countries and regions where surveillance is currently very scarce or completely lacking.

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Conflict of interest

None declared.

Authors’ contributions

CFF and DGJL designed the study, which was supervised by CFF. MH did most of the laboratory work and the analysis of the data. CÅ provided the clinical data. PMCH did the biochemical fingerprinting. JF did the chemical analysis. MH wrote the manuscript. CFF, DGJL and CÅ edited the manuscript. All authors read and approved the final manuscript.

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Research

Emergence of diversity in carbapenemase-producing Escherichia coli ST131, England, January 2014 to June 2016

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Background: Escherichia coli ST131, a global, high-risk clone, comprises fluoroquinolone resistance (FQ-R) mutations and CTX-M extended-spectrum beta-lactamases associated with the fimH30-encoding clades, C1 and C2. Further carbapenem resistance development in ST131 is a public health concern.

Aim: This observational study aimed to probe the diversity of carbapenemase-producing E. coli (CP E. coli) ST131 across England.

Methods: ST131 isolates were identified using whole-genome sequencing (WGS) data generated for all non-duplicate CP E. coli from human samples submitted to the national reference laboratory from January 2014 to June 2016. Antimicrobial resistance (AMR) gene content and single nucleotide polymorphism (SNP) data were compared against a published ST131 phylogeny and analysed alongside patient metadata.

Results: Thirty-nine genetically diverse ST131 CP E. coli, from eight of nine regions, represented 10% of CP E. coli isolates sequenced. Ten and eight isolates were from the FQ-susceptible (FQ-S) clades A and B, while eight and 15 isolates belonged to the FQ-R clades C1 or C2, respectively. Seven distinct carbapenemases were identified: KPC-2 (21 isolates, 6 regions) frequently occurred among clade C2 isolates (n = 10). OXA-48-producers (10 isolates, 3 regions) were often from clade A (n = 5). NDM-1 (n = 4), NDM-5 (n = 1), VIM-1 (n = 1), VIM-4 (n = 1) and OXA-181 (n = 1) were also identified. Clade C2 isolates encoded more AMR genes than those from clades A (p = 0.02), B (p = 9.6 x 10^-3) or C1 (p = 0.03). Conclusion: When compared with its global predominance among ESBL-E. coli, ST131 represented a fraction of the CP E. coli received, belonging to diverse clades and encoding diverse carbapenemases. The greater accumulation of resistance genes in clade C2 isolates highlights the need for ongoing monitoring of this high-risk lineage.

Introduction

The increasing incidence of antimicrobial resistance worldwide and a paucity of new drugs in development presents a major threat to the treatment of bacterial infections [1,2]. Since the millennium, the successive emergences of Escherichia coli with horizontally acquired CTX-M extended-spectrum beta-lactamases (ESBLs) and, more recently, carbapenemases, have heralded the advent of resistance to last-resort treatment options for many serious infections because of E. coli and other Enterobacterales.

The pandemic of resistant E. coli has been associated particularly with the uropathogenic E. coli lineage ST131. The population structure of the ST131 lineage was shaped by the acquisition of virulence and resistance elements which has resulted in three dominant clades; A, B and C [1,2]. Around 1980, clade B diversified into clade C via subclades Bo and Co. Clade C strains were characterised by distinct alleles for the genes gyrA (coding for gyrase) and parC (coding for topoisomerase) which impart elevated fluoroquinolone resistance (FQ-R) [1,2]. This clade was further subdivided according to fimH gene variants into the C1 (fimH30-R) and C2 (fimH30-Rx) clades in 1987 [2,3]. The gene fimH encodes for the type 1 fimbrial adhesin which binds to, and facilitates colonisation of, the bladder epithelium [4,5] and has been suggested as an epidemicity factor for ST131. The C2 clade also became the principle clone associated with the spread of E. coli carrying CTX-M-15 ESBL [6] and multiple other resistance genes [7].

Recent evidence has demonstrated ongoing dynamics and diversification of clade C2, with the emergence and spread of new resistant forms. An example of further acquisition and proliferation in the ST131 clades has...
been observed with CTX-M-27 ESBL-encoding isolates being reported first in Japan, followed by India and then northern Europe [8-10]. Concerns over the continued acquisition and expansion of the ST131 resistance repertoire were manifested by early reports of the NDM carbapenemase occurring in India [11] and Vietnam [12], as well as scattered reports of the $kpc$ gene occurring in ST131 globally [13]. Other carbapenemases such as VIM, IMP and OXA-48-like enzymes have been reported to a lesser extent [14,15]. Importantly, CP $E. coli$ have also recently been recorded in Spanish rivers [16] and long-term care facilities in Italy [17]. This observation of carbapenemase-expressing CP $E. coli$ globally across many environmental niches demonstrates the breadth of reservoirs that contain CP $E. coli$.

In the United Kingdom (UK), ST131 has been widely described in $E. coli$ from community- and hospital-onset infections alike [18]. However, there has been a relative paucity of carbapenemase-producing ST131 isolates investigated to date. This observational study augments an existing ST131 phylogeny with clinical isolates encoding carbapenemases from across England, and it evaluates the genetic, geographical and temporal diversity present among the ST131 carbapenemase-producing $E. coli$ (CP $E. coli$) in England.

### Methods

**Bacterial isolates and whole genome sequencing**

CP $E. coli$ were isolated from human samples by clinical diagnostic laboratories and submitted to Public Health England’s (PHE) Antimicrobial Resistance and Health Care Associated Infections (AMRHAI) Reference Unit for carbapenemase gene detection and/or antimicrobial susceptibility testing. The referring laboratories were located in the nine English Regions (London, East of England, South East, South West, West Midlands, North West, North East, Yorkshire and the Humber and East Midlands). Between January 2014 and June 2016, AMRHAI undertook whole genome sequencing (WGS) of every initial CP $E. coli$ isolate received per patient. DNA was extracted from RNase-treated lysates via a QIAsymphony DSP DNA Midi Kit (Qiagen GmbH, Hilden, Germany). DNA libraries were prepared using the Nextera XT sample preparation method and sequenced with a standard 2 x 101 base protocol on a HiSeq 2500 (llumina, San Diego, California, United States (US)).

Data for carbapenemase-producing ST131 $E. coli$ from different patients were identified and extracted for analysis.

Ethical approval was not required as no personal identifiable information was used in this study. Short-read sequence data for the 39 CP $E. coli$ ST131 isolates were...
**Figure 2**

Evolutionary phylogeny of carbapenemase-producing *Escherichia coli* from England within the larger, global ST131 phylogeny previously validated by Stoesser et al. [2] and Ben Zakour et al. [1]

The blue box indicates a newly emerged KPC-2, CTX-M-15 negative lineage within clade C2.
Antimicrobial susceptibilities
Minimum inhibitory concentrations (MICs) of polymyxins, beta-lactams, aminoglycosides, carbapenems, tetracyclines and fluoroquinolones were determined at the time of receipt (January 2014–June 2016) by BSAC agar dilution methodology and were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines version N.0, 2018) for 26 of the CP E. coli samples.

Whole genome sequencing data analysis
WGS data were decomplexed via Casava, and nucleotides with a Phred score less than Q30 at the ends of the reads were removed with Trimmomatic [17]. Species ID was determined with Kmer-ID [19]. MLST profile was determined by the mapping tool MOST [20]. The in house tool GeneFinder [21] mapped sequenced reads to reference sequences with bowtie2 and generated an mpileup file with SAMtools version 0.1.18 [22] which enabled the rapid detection of antimicrobial resistance gene complements and plasmid replicon types [18, 20].

To provide a contextual framework for the 39 ST131 CP E. coli isolates from England, additional raw read sequences for 188 global non-carbapenemase producing ST131 isolates identified and validated previously [5] were retrieved from the National Center for Biotechnology Information (NCBI) Short Read Archive along with the EC958 assembled reference genome. Raw reads were analysed with PRINSEQ version 0.20.4 [23] and trimmed with a mean base quality score of ≥ 30 and a read length ≥ 70% of the expected read length.

Single nucleotide polymorphisms (SNPs) were determined through read mapping using the PHEnix pipeline [24]. Briefly, the quality filtered reads were mapped against reference sequence EC958 using bwa_mem, followed by variant detection via Genome Analysis Toolkit (GATK) with a minimum depth of 5, mapping quality (MQ) score of 30, allele depth ratio of 0.90, quality score of 40 and an MQ ratio of 0.1. Using methods and parameters described by Stoesser et al. [2], the variant call format (VCF) files base calls were retained if: (i) the percentage of high quality base calls was ≥ 90% and ≥ 5 high-quality bases were observed; (ii) the root of the mean square mapping quality of reads covering the putative variable site was ≥ 30; (iii) Phred scaled quality of a base call was ≥ 25; and (iv) reads spanning variable sites had high quality bases that made up ≥ 35% of variable sites [2]. Suspected recombinant regions were removed using Gubbins [25] and phylogenetic trees were constructed with RAxML version 8 using the general time-reversible Gamma model of among-site rate variation, and validated using 1,000 bootstrap repetitions to assess nodal support [26]. All trees were then viewed within the R statistics package with the ggtree library [27].

Bayesian temporal and geographical analysis
TempEST was used to assess the variance between the time of sampling and the root-to-tip divergence in maximum likelihood trees [28] using heuristic residual mean squared function (residual mean square = 6.45 × 10⁻⁶; correlation coefficient = 0.5059; R² = 0.256). BEAST 2 version 2.4.8 [29] was used to perform Bayesian temporal analysis on maximum likelihood trees to reduce variance and investigate cladal expansion for ST131 CP E. coli using the 3,779-bp non-recombinant SNPs conserved across all 227 ST131 isolates. As only SNP sites were used in the alignment a Gamma site model (Gamma Category count 0) was used, which assumes a gamma distribution for site-to-site rate heterogeneity, and the proportion of invariant sites was fixed at 0%.

The Tamura-Nei 1993 (TN93) model, which weights transitions and transversion mutations according to their likelihood, was found to be the best model to represent the phylogeny (path sampling maximum likelihood estimates are described in Supplementary Table S1A and effective sample size scores (ESS) in Supplementary Table S1B). A strict molecular clock, constant population size and uniform clock rate was found to be the most appropriate model as all isolates were from the same E. coli sequence type. The suitability of this model was reflected in the ESS scores when compared against GTR, HKY and JC69 site models under like-for-like parameters. To ensure convergence, Markov Chain Monte Carlo (MCMC) generations for each analysis were performed in triplicate for 30 million steps (totalling 90 million iterations) sampling every 1,000 steps, producing ESS scores equal to or greater than 200 (Supplementary Table S1B). Replicate analyses were then combined using the BEAST program LogCombiner with 10% burn-in.

T-test, X² and ANOVA statistical tests were all performed using standard libraries contained within the R statistics package [30].

Results
Diverse carbapenemase-producing Escherichia coli ST131 in England
Thirty-nine ST131 CP E. coli isolates were referred to and sequenced at PHE's AMRHAI reference unit between January 2014 to June 2016 (30 months) from 20 laboratories that represented eight of the nine English regions. These accounted for 10% of the total number of CP E. coli isolates submitted. Temporal analysis showed that the average number of CP E. coli ST131 isolates submitted to PHE on a monthly basis rose from 0.67 in 2014 (ST131 CP E. coli=8), to 1.67 in 2015 (ST131 CP E. coli=20), and to 1.83 in 2016 (first 6 months only, ST131 CP E. coli=11). As a percentage of the total CP E. coli isolates across England, ST131 represented 4.5% in 2014 (CP E. coli=177), 6.2% in 2015 (CP E. coli=324) and 4.9% in the first half of 2016 (CP E. coli=223). This increase in CP E. coli was linked to the number of KPC-2 producing isolates (mean/month: 2014=0.33;
2015 = 0.92; 2016 = 1.00) and OXA-48 producing isolates (mean/month: 2014 = 0.08; 2015 = 0.42; 2016 = 0.67).

Analysis of all PHE CP E. coli ST131 isolates in this study for the occurrence of regional or local clusters, irrespective of time, showed that the majority were isolated from London (n = 13) or the North West region (n = 11) (Figure 1A). Seven distinct carbapenemase alleles were detected, but only the KPC-2, OXA-48 and NDM-1 carbapenemases were encoded by isolates from multiple regions. Of these, KPC-2 was the most numerous (n = 21) and widely distributed (isolated in 6 regions), with the largest number of isolates identified in London (n = 7). The second most frequent allele, OXA-48, occurred in 10 ST131 CP E. coli and was found most often in the North West region (n = 6). The remaining carbapenemase alleles occurred sporadically across London (NDM-1, OXA-181 and VIM-4), the North East (NDM-5), West Midlands (NDM-5) and the South East (VIM-1). None of the isolates in this study were associated with known outbreaks of CP E. coli. (Figure 1A, Supplementary Table S2).

Comparison of WGS data from the CP E. coli ST131 with a previously published WGS-based population structure for ST131 identified that 23 of 39 ST131 CP E. coli from across England were from the FQ-R clades C1 (n = 8) and C2 (n = 15). Clade C2 was the most widely distributed clade of ST131 CP E. coli (isolated from 7 regions) and was the most highly represented clade in three regions (Figure 1B). The FQ-S clades, A and B, were relatively well represented (10 and 6 isolates, respectively) and widely disseminated (found in 5 and 3 regions, respectively). Opposite cladal biases were apparent in the two regions with the largest numbers of isolates: in London (n = 13), clades C1 and C2 predominated (2 and 7 isolates, respectively); whereas clades A and B predominated in the North West region (5 and 3, respectively) (Figure 1B, Supplementary Table S2).

The combined data for ST131 clades and carbapenemase alleles indicated that isolates from the largest CP E. coli clade, C2, were most frequently associated with KPC-2 (n = 13) and predominated among ST131 CP E. coli isolated in London (n = 7). In contrast, the second most frequent carbapenemase, OXA-48 (n = 10), occurred most often in clade A isolates from the North West region (n = 5), demonstrating the contrasting phylogeographical patterns in ST131 CP E. coli for the two most frequent carbapenemases.

**Emergence and expansion of clade C2**

To assess the extent of any clonality and clade specific expansion, the CP E. coli isolates were incorporated into and visualised within the context of a previously validated phylogenetic BEAST tree [5]. The CP E.
coli isolates were widely distributed across the diversity present in each of the clades with little evidence for clear expansions of sub-clades or genetic clusters of carbapenemase producers emerging, although there was a close relative cluster in clade C2 (Figure 2). This group represented KPC-2 isolates without a CTX-M-15 gene from London, two isolates from 2014 and two from 2015, and the North West region, one isolate from 2016.

MIC analyses for 26 of 39 isolates highlighted 12 isolates of ST131 CP E. coli that were phenotypically resistant to the greatest number of antimicrobials, representing at least seven drug classes (carbapenems, other beta-lactams, including third generation cephalosporins, fluoroquinolones, trimethoprim, tetracyclines, sulfonamides and aminoglycosides). Those 12 most resistant isolates remained susceptible to colistin and tigecycline. They originated from seven English regions, but were most often isolated in London (n = 6). We observed that 10 of the 12 isolates were from clade C2 and originated from six regions. Seven of the 10 isolates from clade 2 encoded KPC-2, two encoded OXA-48, and the remaining isolate encoded VIM-4 (Supplementary Table S3).

To better explore the suggestion that isolates from the C2 clade had a propensity to have a greater number of antimicrobial resistances, we examined the number of AMR genes per isolate. The number of AMR genes did not differ significantly between isolates expressing different carbapenemase genes (ANOVA: p = 0.826), but AMR gene counts were affected by the clade that an isolate belonged to (ANOVA: p = 0.0214). Clade C2 isolates were associated with significantly higher numbers of acquired AMR genes (mean = 11.1; median = 13.0) (Figure 3) (t-test: clade A, p = 0.02, mean = 8.1, median = 8.5; clade B, p = 9.6 x 10^{-1}; mean = 6.3, median = 5.0; clade C1, p = 0.03, mean = 8.25, median = 6.5). This was attributable to mobile genes that encoded resistance to aminoglycosides (X^2 = 16.657; df = 6; p = 0.01); fluoroquinolones (X^2 = 9.958; df = 3; p = 0.02) and sulfonamides (X^2 = 9.978; df = 2; p = 6.8 x 10^{-1}). Further comparisons revealed that clade C2 CP E. coli also had more AMR genes than their non-CP E. coli C2 counterparts (X^2 = 35.177; df = 19; p = 0.01), despite almost half (7/15) lacking the CTX-M-15 gene that has previously been strongly associated with the multi-resistant status of clade C2. Supplementary Table S4 describes the most common resistance genes in CP E. coli and non-CP E. coli isolates.

Plasmid replicons

The plasmid replicon sequences found in the 39 CP E. coli ST131 isolates most often indicated the presence of replicons from the FiA (n = 23), FII (n = 35) and FIB (n = 35) plasmid incompatibility groups (Supplementary Table S5). Non F-type replicons were less evenly distributed across clades as indicated by the higher numbers in isolates from clades A (mean = 2.2) and C2 (mean = 2.13) compared with isolates in clades B (mean = 0.67) and C1 (mean = 0.88) (ANOVA, p = 5.59 x 10^{-5}). For isolates in clade A, particular carbapenemase types did show unique associations with replicons, with VIM-1/4 associated with A/C replicons, KPC-2 to N-type replicons and OXA-48 association with IncI/M replicons. Isolates in the other clades (B, C1 and C2) showed no clear relationships between unique plasmid replicons and particular carbapenemase genes (Supplementary Table S5).

Discussion

The observation that ST131 CP E. coli represented only 10% of CP E. coli isolates that were submitted to the reference laboratory over the study period contrasted the current narrative that ST131 shows a global predominance among ESBL-producing E. coli. Nevertheless, the wide distribution of ST131 CP E. coli across all but one of the nine English regions indicates the ongoing success of ST131 E. coli and highlights its progression beyond being an ESBL encoding lineage in England. The diversity of clades and carbapenemase alleles also greatly differed from the emergence of a single lineage akin to the CTX-M-15 expressing C2 isolates responsible for the pandemic established in the mid-2000s. While no evidence was found for distinct newly emerged phylogenetic clusters reaching predominance, the relative success of clade C2 in particular highlights the potential to repeat the clonal success of the CTX-M-15 positive clade [1]. Specifically, the five KPC-2 encoding isolates with relative relatedness spanning 3 years and two locations indicate the potential for expansion within clade C2. Moreover, the phylogeographic diversity of isolates from C1 and C2 clades demonstrates the success of diverse isolates from within these clades as carbapenemase producers, and highlights the importance of ongoing monitoring in order to help to identify any further expansion of such isolates.

The contrast between the two most successful carbapenemases were marked. The predominance of KPC-2 among the FQ-R C2 isolates from London occurred contemporaneously to a large outbreak of KPC-2 in multiple species of the Enterobacterales in the North West region [31]. In that context, the predominance of OXA-48 among mostly FQ-S clade A isolates in the North West region did not coincide with the main clinical, healthcare-associated, circulation of CP E. coli in the region at the time. The principal mechanism of antibiotic gene acquisition in bacteria is through horizontal gene transfer, which is primarily mediated by plasmids. It is not possible to ascertain from short-read data whether these OXA-48 positive clade A isolates were part of a wider circulation of isolates and/or plasmids in the area at the time. However, nine of 10 OXA-48-positive isolates had reads mapping to the origin of replication for IncI/M-type plasmids, which have previously been identified as the main vehicles encoding OXA-48 in many Enterobacterales species [32-36]. For KPC-2, potential plasmid vectors appeared to be more diverse and highlighted the mobility that underpins the
spread of carbapenemase genes. However, accurately attributing plasmids to genes was limited by the use of short-read data in this project. Ongoing studies using additional long-read sequencing data will be required to better determine, monitor and survey the extent of mobile element and AMR flux in important, potentially public health relevant clones such as ST131.

The marked differences in the multi-resistance gene profiles, evidenced by the higher number of AMR genes noted in clade C2 when compared against their ESBL-positive counterparts, suggests that clade C2 may have been differentially affected, as compared with clades A-C1, over time by a pressure to accumulate multiple resistances. This may be because of clade C2 isolates being exposed more regularly to antimicrobial pressure in healthcare settings. We speculate that clade C2 isolates possess a mechanism(s) or are otherwise predisposed towards the increased acquisition of resistance genes; and we are investigating this further. The detection of repeated accumulation of resistance genes in this epidemic E. coli clade is concerning. The example of Klebsiella pneumoniae has shown that some high-risk clones spread in successive forms, first encoding ESBLs (CTX-M-15), followed by carbapenemases and subsequently developing resistance to colistin (an agent of last resort) [37]. This progression of resistance accumulation presents significant challenges in infection control, particularly within clinical settings. The level of concern for such a pattern in E. coli and especially ST131 would be further heightened by the emergence and wide geographic dissemination of mobile colistin resistance (mcr) genes in the species, not least as these have already been reported on IncHI2-based multi-resistance plasmids that are mobile and can replicate in E. coli and other members of the Enterobacterales [21].

The data presented highlight the ongoing diversification and success of the clade C ST131 CP E. coli within England, the emergence of multiple new resistance profiles across ST131 and a propensity for these to be newly-emerging, increasingly-resistant forms of clade C2 in particular. Detecting genomic signals associated with the repeated acquisition and evolution of resistance profiles in the most successful E. coli clades provides a platform on which to base healthcare strategies and interventions that can assist in the early detection and mitigation of high-risk lineages in the future. It also underlines the importance of continued monitoring of this high-risk lineage with known pandemic potential, given the current lack of good alternatives to carbapenems for the treatment of Gram-negative antimicrobial-resistant infections.

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Conflict of interest

None declared.

Authors’ contributions

Nicholas Ellaby conducted all bioinformatic and statistical analysis on this project, and was the primary author of the paper.

Matthew Ellington designed and managed the project, directed the analysis and aided in drafting the paper.

Michel Doumith developed bioinformatics programs and protocols used in the analysis.

Katie Hopkins was responsible for sequencing of the isolates.

Neil Woodford had oversight, design and instigation of the project, as well as editing the paper.

All authors agreed to the final version of this article.

References

9. Peiran G, Schreckenberger PC, Pitout JDD. Characteristics of NDM-1-producing Escherichia coli isolates that belong to
Q fever in Bulgaria: Laboratory and epidemiological findings on human cases and outbreaks, 2011 to 2017

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Background: Q fever is a zoonosis, included in category B of particularly dangerous infectious agents and as such merits careful surveillance and regular updating of the information about its distribution. Aim: This observational retrospective study aimed to provide an overview of Q fever incidence in Bulgaria in the period 2011 to 2017. Methods: Aggregated surveillance data from Bulgaria’s mandatory surveillance system, laboratory data on individual samples received at the National Reference Laboratory Rickettsiae and Cell Cultures and outbreak reports sent by the regional health authorities to the National Centre of Infectious and Parasitic Diseases, were used in this analysis. Cases were described by year, region, age group and most commonly identified risk behaviours. Results: A total of 139 confirmed cases were reported in the study period (average annual incidence: 0.27 cases/100,000 inhabitants). No seasonality or trend in reported cases was observed. Cases were mostly sporadic, with two small outbreaks in 2017. Identified risk behaviours among cases were occupational exposure and consumption of milk and dairy products, although exposure data were incomplete. The male/female ratio was 1.4. The identification and resolution of the two rural outbreaks in 2017 with a total of 18 cases involved good practices: active case finding and collaboration between public health and veterinary authorities. Conclusion: Between 2011 and 2017, Bulgaria retained low Q fever incidence, mostly sporadic cases and two small outbreaks. Occupational exposure and consumption of milk and dairy products were the most often reported likely exposures among cases. The outbreak investigations demonstrate the application of good control practices.

Introduction
Q fever is an endemic zoonosis spread globally, except for New Zealand [1,2]. While the majority of cases are sporadic, several outbreaks among humans have been reported in different European countries (Germany, the Netherlands, Scotland and Slovenia) [3-6]. The aetiological agent of Q fever is the obligate intracellular bacterium Coxiella burnetii, included in category B of particularly dangerous infectious agents presenting a risk to human health, and it is considered as a potential weapon for bioterrorism [7]. The infectious agent has a wide range of animal hosts. [2,8]. In animals, infection is mainly subclinical but can also cause a range of conditions in livestock such as miscarriage, infertility, retained placenta, endometritis and mastitis. Infected animals shed large numbers of bacteria in placentas, vaginal discharge, faeces and urine [9,10]. Inhalation of pathogen-contaminated aerosol particles is the main route of infection in humans [11,12]. Consumption of unpasteurised milk also poses a risk, although it is considered lower [13]. The clinical presentation of Q fever in humans varies, ranging from asymptomatic infection, self-limiting febrile reaction, atypical pneumonia and acute or chronic granulomatous hepatitis to endocarditis in patients with pre-existing valvulopathy or vascular defects and meningoencephalitis in chronic disease forms [11,14-16]. Because the clinical presentation is similar to that of other diseases, Q fever often remains underdiagnosed [14-16].

In Bulgaria, Q fever in humans was first recognised by Mitov et al. in 1949 [17]. For more than 60 years, numerous sporadic cases and small and large epidemics, involving tens to hundreds of persons, occurred in different regions [18,19]. The last two major outbreaks in the country were registered in Etropole (2002) and in Botevgrad (2003–04) [20,21].

This study aimed to provide an overview of Q fever distribution in Bulgaria in the period 2011 to 2017, with consideration given to risk factors and possible underdiagnosis and underreporting.
Methods

Study design

A retrospective descriptive analysis of cases notified and reported in our mandatory surveillance system and of samples sent to the National Reference Laboratory for Rickettsiae and Cell Cultures (NRL RCC) was carried out. Cases and positive samples were described by region, age group, sex and year of notification / laboratory test. Data from outbreak reports, as received by the National Centre of Infectious and Parasitic Diseases (NCIPD), were described.

Data sources and case definitions

In Bulgaria, Q fever is a mandatory notifiable disease and the European Union (EU) case definition and case classification have been used for surveillance purposes [22, 23]. Epidemiological surveillance of human Q fever in Bulgaria is passive and aggregated. Cases are notified by primary reporting units (general practitioners, hospitals etc) to the Regional Health Inspectors (RHI) of all 28 regions. The RHI then send aggregated reports on a weekly, monthly and annual basis to the National Center for Public Health and Analysis, which collates the data from all regions and forwards them to the NCIPD. Cases reported in monthly and annual reports are classified as probable or confirmed based on the EU case definition: A probable case is defined as any person meeting the clinical criteria (fever or pneumonia or hepatitis) with an epidemiological link, with epidemiological link defined as ‘at least one of the following two epidemiological links: (i) exposure to a common source, (ii) animal-to-human transmission’. A confirmed case is any person meeting the clinical and the laboratory criteria (C. burnetii isolation or detection of C. burnetii nucleic acid or C. burnetii-specific antibody response (IgG or IgM phase II)).

Laboratory confirmation can be carried out in different laboratories. The NRL RCC at the NCIPD is the laboratory with the highest expertise in Q fever diagnosis in the country and receives a large number of samples for testing every year. The NRL RCC collects individual information on clinical presentation, demographics and risk behaviours, identified by physicians in communication with suspected cases and added to the information sent with the samples. However, confirmation by the NRL RCC is neither mandatory nor subsidised and therefore, sample sending practices to the NRL RCC differ by region. Some physicians and hospitals send samples primarily to the NRL RCC, while others choose other labs. In addition, not all cases are laboratory-confirmed. In this study, we analysed data on samples received at the NRL RCC between 2011 and 2017.

It must be noted that samples arrive at the NRL RCC with information regarding the location of the sending physician or hospital but without information on the residency of the patient. In this way, the regional distribution that can be derived from the sample information is not the same as the regional distribution of the cases reported through surveillance, as cases reported through surveillance are assigned to regions based on the residency of the patients.

Table

Annual laboratory and surveillance data regarding samples tested for *Coxiella burnetii* at the NRL RCC and confirmed cases reported through the surveillance system, Bulgaria, 2011–2017 (n = 1,430)

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<th>Number of sera tested</th>
<th>Number of positive samples (IgM phase II)</th>
<th>Positivity rate (%)</th>
<th>Total notified cases</th>
<th>Notification rate (cases/100,000 population)</th>
<th>Confirmed cases (EU case definition)</th>
<th>Incidence rate (confirmed cases/100,000 population)</th>
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</tr>
<tr>
<td>2016</td>
<td>229</td>
<td>33</td>
<td>14.44</td>
<td>19</td>
<td>0.27</td>
<td>17</td>
<td>0.24</td>
</tr>
<tr>
<td>2017</td>
<td>373</td>
<td>38</td>
<td>10.2</td>
<td>30</td>
<td>0.42</td>
<td>28</td>
<td>0.39</td>
</tr>
<tr>
<td>Total</td>
<td>1,430</td>
<td>161</td>
<td>11.3</td>
<td>148</td>
<td>0.29a</td>
<td>139</td>
<td>0.27</td>
</tr>
</tbody>
</table>

EU: European Union; NRL RCC: National Reference Laboratory Rickettsiae and Cell Cultures.

a Average annual notification rate for the period.
Data on the regional population for each year under study was obtained from the official public database of the National Statistical Institute of Bulgaria [24].

Laboratory methods
Serum samples from ambulatory and hospitalised patients with different clinical diagnoses were collected 1–3 weeks after the onset of clinical symptoms and sent to the NRL RCC. Two diagnostic methods (serology and/or molecular detection) were used. The human serum samples were tested for IgM phase II antibodies against *C. burnetii* with a commercial indirect enzyme-linked immunosorbent assay (ELISA) (SERION ELISA classic, *Coxiella burnetii* Phase II IgG/IgM, Virion/Serion, Würzburg, Germany), known to have high sensitivity (85%) and specificity (> 99%) [20]. The assay was performed and interpreted as recommended by the manufacturer and the results were qualitatively categorised as positive, negative or equivocal. DNA was extracted from all IgM-positive human samples using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, United States (US)). The extracted DNA was subjected to a conventional PCR assay (AmpliTaq Gold 360 DNA kit, ThermoFisher Scientific, US) for the detection the *sodB C. burnetii* gene using the specific primers CB1 and CB2 [25]. All IgM-positive samples that were PCR-negative were further discussed, considering additional clinical and epidemiological information sent with the samples in the context of the timing of sample collection.

Data analysis
The numbers of cases and annual incidence per 100,000 inhabitants reported through the surveillance source were described by year at the national level and by region for the whole study period. A test for trend over time was performed with year as independent variable and the annual number of reported cases as dependent variable in negative binomial regression, through which the incidence rate ratio (IRR), 95% confidence interval (CI) and p value were obtained. The available information from surveillance reports regarding two outbreaks in 2017 was also summarised.

The individual laboratory sample database from the NRL RCC was deduplicated. The number of sera tested and the number of samples positive in serology were summarised by year. The annual positivity rate (positive samples/total number of tested samples) was calculated. A test for trend over the years with regard to the number of positive samples and positivity rate was performed at the national level, using negative binomial regression to calculate IRR and 95% CI. A test for seasonality in confirmed cases was also performed through calculating the correlation between the detrended series and sine curves in order to establish whether there was a dominant periodicity in the time series. We used the Wilcoxon rank-sum test to test the hypothesis that April to October is a period with more positive samples and the hypothesis that June to July is a peak period. Samples sent per 100,000 inhabitants and positivity rate by region were compared. The distribution of positive samples by sex, age, clinical symptoms and suspected risk factors was summarised.

Ethical statement
We present surveillance data collected routinely by the national reference laboratory and surveillance units in the country. Data are presented in aggregated and anonymous format. Publication of this analysis does not harm or influence neither cases nor institutions. Ethical committee approval was therefore not required.

Results
Overall numbers and temporal distribution of cases
In the period from 2011 to 2017, a total of 148 cases of Q fever in humans were reported through the national surveillance system, of which 139 were classified as confirmed based on the EU case definition. The cases, mostly sporadic, were reported by 18 of the 28 regions in the country. In 2017, two limited outbreaks occurred, with fewer than 10 cases each. The average annual incidence of Q fever at the national level was 0.29 per 100,000 inhabitants (ranging from 0.16 (2011) to 0.42 (2017)) (Table). There was no statistically significant trend or seasonality observed in the annual national incidence as calculated from surveillance data.

For the same period (2011–17), a total of 1,430 serum samples from patients with suspected Q fever were received at the NRL RCC from 15 regions. By means of IgM phase II ELISA, antibodies against *C. burnetii* were detected in 161 (11.3%) of the tested human sera. Sera from all IgM-positive patients were additionally tested by conventional PCR assay and the presence of *C. burnetii* DNA was confirmed in 143 samples (88.8%). After taking into account clinical and epidemiological information of the IgM-positive samples that were PCR-negative and the timing of sample collection, all IgM-positive samples were finally classified as laboratory-confirmed and are presented here (Table). A very small significant positive trend at the national level was detected for the annual number of samples received by the laboratories (IRR = 1.01; 95% CI: 1.01–1.02) and for the number of IgM-positive samples (IRR = 1.02; 95% CI: 1.01–1.03) but not for the positivity rate. There was no seasonality in the data (data not shown). Nor was there a significant difference between the periods April to October and November to March and between the periods June to July and August to May with regard to monthly number of tested samples, number of positive samples and positivity ratio.

Regional distribution of cases and positive samples
Average annual regional incidence rates of confirmed Q fever cases, as derived from surveillance data, are presented in Figure 1. Nine of 28 regions did not notify any cases during the study period. Twelve regions had an average annual incidence for the period below 0.5
per 100,000 inhabitants. Six regions stood out with higher than average incidence: Pernik, Haskovo, Stara Zagora, Kiustendil, Gabrovo and Plovdiv with average annual incidences of, respectively, 2.81, 1.12, 1.00, 0.98, 0.76 and 0.65 cases per 100,000 inhabitants (Figure 1). It must be noted that among those regions, Pernik, Gabrovo and Kiustendil differed from the other regions in their practices for sending samples to the NRL. Per 100,000 inhabitants, Pernik and Gabrovo sent the highest number of samples to the NRL RCC, with Pernik sending 10 times more than the average and Gabrovo sending four times more than the average (data not shown).

Age and sex distribution of IgM-positive samples tested at the NRL RCC

The median age of people with positive samples was 45 years (age range: 2–78). The smallest numbers of patients with positive samples were observed among children younger than 10 years and people older than 70 years. Of the 161 laboratory-confirmed samples, 94 (58.4%) were from male patients and 67 (41.6%) were from female patients (Figure 2). Overall, the number of male patients with positive samples was significantly higher than the number of female patients with positive samples for Q fever (Chi-squared p<0.05). The male to female ratio was 1.4.

Q fever-positive patients by clinical diagnosis

Within the study period, 74 of the 161 laboratory-confirmed Q fever cases were initially diagnosed with fever of unknown origin. Another 72 confirmed patients were hospitalised with atypical pneumonia and one was diagnosed as acute bronchitis. The remaining IgM phase II-positive cases were hospitalised with heart disease diagnosis: nine with endocarditis, three with pericarditis and two with myocarditis.

Possible occupational and other risk factors for Q fever

Limited epidemiological information was available in the laboratory database, where risk behaviours (i.e. suspected exposures) were identified for only 58 (36.0%) of IgM phase II-positive patients. Among the 58 for whom risk behaviour information was available, occupational hazards were identified for 25, while for 33, consumption of milk and dairy products from private stockbreeders was accepted as the possible risk for Q fever infection.

Q fever outbreak in the region of Blagoevgrad, 2017

On 13 March 2017, a resident of a village in Blagoevgrad region called the RHI and reported about an exceptionally large number of people living in the village who were suffering from pneumonia. On the next day, an investigation was initiated. The RHI began a
A retrospective search in the local hospitals for patients living in the same village and hospitalised with acute respiratory findings within the previous 2 months. The population of the affected village is about 750 people. A total of 35 were identified to have been hospitalised with pneumonia between 22 February and 10 April. Samples were taken from 32 of them. Eleven of the 32 samples were positive for C. burnetii (IgM phase II positivity). The age range of positive cases was 18–55 years and the median age was 32 years; six were women and five were men. The cases’ places of residence were scattered throughout the village. Sometimes more than one case was diagnosed in a family. Six cases gave information about consumption of milk from domestic goats while the rest shared information about exposure to the local goat herd only on the way of the animals to the pasture. The RHI contacted the regional veterinary centre and asked for investigation of private ruminants (sheep and goats) in the village. Q fever-positive goats were identified by the veterinarians and it was concluded that the source of this outbreak had been domestic goats. The possible spread was through infected aerosol and/or infected milk (in some households).

Q fever outbreak in the region of Gabrovo, 2017
A routine serological screening among sheep and goats, carried out in the Gabrovo region, established C. burnetii positivity in sheep and goats from one village. After the signal from the veterinarians, an epidemiological investigation was conducted in mid-November 2017 among workers in sheep breeding facilities in the area. A total of 39 samples were collected of which seven were positive for C. burnetii (IgM and PCR positivity). The age range of positive workers was 40–68 years and the median age was 50 years; five were women and two were men. Five reported having had fever of unknown origin and two had atypical pneumonia, but none had been hospitalised.

Control measures
Q fever control measures in Bulgaria according to standard guidelines for veterinary [26] and public health [27,28] authorities include: (i) hospitalisation and appropriate treatment of patients with antibiotics (tetracyclines, quinolones or macrolides in effective doses for at least 2–3 weeks), (ii) follow up of contacts for 30 days, including two serological tests for C. burnetii per contact within this period, (iii) disinfection of the environment and work place, (iv) manure composting for at least 6 months under nylon or treatment of manure with lime, (v) temporary halting of milk collection from animals positive for Q fever, lasting until the animals had completed antibiotic treatment (vi) pasteurisation of milk, (vii) measures related to animals, as implemented by veterinary authorities, including establishing a separate birthing zone, removal of placenta and disinfection of the birthing zone after birthing and active animal case finding within the affected herd, (viii) enhanced surveillance during the outbreak (contact tracing and active case finding), (ix) health promotion activities, including the distribution of information, reinforcing messages about good preventive practices in livestock farming (separate birthing areas, regular disinfection, personal protective equipment such as gloves, gumboots, protective clothing, eye protection and respiratory protection, cleaning, handwashing, etc.)

In the case of the two outbreaks, the RHI reported having applied all appropriate measures. Active case finding and collaboration with the veterinary authorities in both cases contributed to better characterisation of the outbreaks. It must be noted that because of the complexity of the diagnosis, both outbreaks were detected at a late stage and therefore, not all measures could be implemented in the most timely manner. Nevertheless, information about the findings was immediately shared by the regional health authorities with various stakeholders (veterinary authorities, representatives of the respective municipalities etc.), and field work related to enhancing control measures was carried out in order to raise awareness about prevention and control measures and prevent future outbreaks.

Discussion
Most Q fever cases registered in Bulgaria between 2011 and 2017 were sporadic, similar to other EU countries [29]. Notification rates were low for the whole period, with the average notification rate comparable to the average EU levels observed in 2014, 2015 and 2016 (average notification rate for EU for this period was 0.2 cases per 100,000 population) [29]. Q fever cases are registered during the entire year in Bulgaria without significant seasonality in reporting. Overall in the countries in the EU and European Economic Area (EEA), cases are also reported throughout the year. However, combining the data from the all EU/EEA countries leads to an observable seasonality, with peaks in June and July [30]. Interestingly, European surveillance data from 2016, unlike data from other years, show no
clear seasonality in the EU/EEA, because France and Germany reported consistently higher case numbers from January to August 2016 [29]. The lack of statistically significant seasonality in Bulgaria may be due to the overall small number of notified cases, to differences in farming practice or to other reasons. The data available to us do not allow us to draw definitive conclusions with regard to the determinants behind the observed lack of seasonality. Some regions are affected more than others, which may be due to the specific economic activities in these regions, but could also be attributable to varied detection of cases, considering that the diagnosis is complex and symptomatic cases may remain undiagnosed because of non-specific symptoms, as reported elsewhere [31]. It is indeed possible that the differences in notification rates among regions are due to a combination between variation in risk factors and variation in surveillance and testing practices. Information on risk behaviours among cases was incomplete. Nevertheless, it helped identify the most important suspected risk behaviours such as occupational hazard and consumption of milk and dairy products. While this information is indicative of likely exposure, lack of information on the prevalence of the same behaviours among a control group limits our ability to establish which exposures are truly risk factors. There were more cases among men, which may be linked to their higher likelihood of occupational exposure – a hypothesis that needs to be confirmed further through dedicated studies.

The non-specific clinical symptoms make it difficult to diagnose Q fever. Therefore, laboratory diagnostic capacities should allow for quick identification, but also for the differentiation between acute and past Q fever infections. This is why the NRL RCC employs both serological and PCR testing in order to improve the diagnostic capacities with regard to this disease.

Over the studied period, we observed a small significant positive trend for number of tested samples and number of positive samples, but not for the positivity rate. This may indicate improvement in diagnostic practices (i.e. sample sending to the NRL RCC) rather than an increase in incidence. There are indications of underreporting based on our data, especially for the last years, as there are more positive samples from the NRL RCC than reported cases through surveillance. Unfortunately, there is no means for us to link the individual dataset from the NRL RCC and the aggregated surveillance data in order to pinpoint the reasons for the difference between the two systems. This issue demonstrates the importance of introducing case-based surveillance integrating laboratory and surveillance data in order to monitor and improve surveillance performance. An additional limitation of our study is that the ELISA test used has a sensitivity of 85%, which can lead to false negative results and an underestimation of positivity rates. For diagnostic purposes, the NRL RCC tested by PCR some IgM-negative samples for which laboratory material was available and samples that were considered equivocal (data not shown). However, resource limitations did not allow this to be done in a systematic manner, and in order to present and analyse systematically collected data, we have summarised here only the data for IgM-positive samples, for which the laboratory results had been aligned with the clinical manifestation of Q fever in patients.

In 2017, in addition to sporadic reports of Q fever cases, two limited outbreaks were reported by the local hospitals and the RHI in two regions in Bulgaria. All cases were among rural populations; one was suspected to be linked to consumption of milk and dairy products and the other to occupational exposure. Both outbreaks involved active case finding and demonstrated how this approach leads to identification of more cases, which underlines the importance of active case finding during outbreaks. In addition, the outbreaks were good examples of collaboration between public health and veterinary units in the control of zoonotic diseases.

Conclusions

During the period from 2011 to 2017, Bulgaria retained a low Q fever incidence with mostly sporadic cases and two small outbreaks. Occupational exposure, and consumption of milk and dairy products were the main risk factors, and men were more affected by the disease. The NRL RCC employs methods that allow quick diagnosis of cases and differentiation between current and past infections and is best placed to provide accurate and experienced diagnosis of suspected cases. The practices of sending samples to the NRL RCC have improved during the studied period – a positive development that should continue in the future. At the same time, our data indicate underreporting, which could be addressed by introducing case-based surveillance, although this is strongly dependent on available resources. The outbreak investigations carried out in 2017 involved active case finding and collaboration with veterinary units and demonstrate the application of good practices to limit the spread of Q fever.

Conflict of interest

None declared.

Authors’ contributions

PG-K and SK performed serological and PCR screening of human samples. NV, SS and AK prepared analysis of epidemiological and laboratory surveillance data and wrote the manuscript. PG-K, NV, SS, AK and TK edited the manuscript.

References

