Surveillance and outbreak report

Active surveillance scheme in three Romanian hospitals reveals a high prevalence and variety of carbapenemase-producing Gram-negative bacteria: a pilot study, December 2014 to May 2015

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We report the findings of an active surveillance scheme for detection of asymptomatic carriers with carbapenemase-producing Gram-negative bacteria (CP-GNB) in Romanian hospitals. During a pilot study from December 2014 to May 2015, faecal cultures were screened in three hospitals (two large, one medium-size) for patients newly admitted to selected wards or inpatients transferred from other wards to an intensive-care unit. The study revealed a high prevalence of CP-GNB detected in 22/27 and 28/38 of the carbapenem non-susceptible isolates from Hospitals 1 and 3, respectively. CP-GNB identified through faecal screening included NDM-1-producing *Serratia marcescens* and *Klebsiella pneumoniae*, OXA-48-producing *K. pneumoniae* and OXA-23-producing *Acinetobacter baumannii*. The distribution of the CP-GNB varied between the hospitals, with NDM-1-producing *S. marcescens* and *K. pneumoniae* being prevalent in the north-central part of the country and OXA-23/24-producing *A. baumannii*, OXA-48-producing *K. pneumoniae*, *Morganella morganii* and VIM-2-producing *Escherichia coli/Pseudomonas aeruginosa* detected in the northeast of the country. Conjugation studies showed that carbapenem resistance was transferable and PCR-based replicon typing identified blaNDM-1 on IncFIIIs in *S. marcescens* and *K. pneumoniae* from Hospital 1 and blaOXA-48 on IncL plasmids in all *Klebsiella* spp. isolates from Hospitals 1 and 3. Our findings underline the importance of active surveillance for detection of CP-GNB asymptomatic faecal carriers and suggest a likely endemic spread of CP-GNB in Romania.

Introduction

The worldwide emergence of carbapenemase-producing Gram-negative bacteria (CP-GNB) is widely accepted as a major public health threat that has caused international concern. The rapid spread of CP-GNB provides many challenges for healthcare systems, with implications that span from laboratory detection and infection control, to identifying suitable treatment options for organisms that are now starting to exhibit co-resistance to multiple last-resort antimicrobials [1].

The epidemiology of CP-GNB in Europe varies between northern and southern countries. A European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE) among national experts from 39 European countries in 2013 showed that carbapenemase-producing *Enterobacteriaceae* (CPE) were continuing to spread in Europe, with *Klebsiella pneumoniae* carbapenemase (KPC)-producing isolates having the widest distribution and the number of OXA-48-producing isolates continuing to rise [2]. In contrast, NDM-1-producing isolates are sporadically reported, mainly from the United Kingdom (UK) [2]. The same report also found that 30 of the 39 European countries had a dedicated surveillance system for CPE and 22 of the countries had national recommendations or specific guidelines for infection control [2]; however, Romania does not have such a surveillance system or CPE infection control guidelines.

Romania contributes to the European Antimicrobial Resistance Surveillance Network (EARS-Net) although only via a small number of laboratories that report only carbapenem-resistant invasive *K. pneumoniae* and
**Methods**

Microbiology laboratories from three Romanian hospitals participated in the study. The hospitals were from two different geographical regions, with two hospitals (Hospitals 1 and 2) from the north-central part of the country, while the third (Hospital 3) was from the north-east of Romania. Hospitals 1 and 2 were large, with 1,299 and 1,892 beds, respectively, while Hospital 3 had a smaller capacity, with 232 beds and about 1,900 patients treated each year (the annual number of patients treated in Hospitals 1 and 2 could not obtained). Ethical approval for the study was obtained from the Hospital Research Ethics Committee at each hospital. Written consent was obtained from all patients for biological sample collection for routine investigation according to the hospitals’ protocols.

During the study period (December 2014 to May 2015), surveillance of faecal cultures was introduced in the routine admission procedures as part of infection prevention and control measures. Each hospital analysed faecal cultures within the first 48 hours of hospitalisation for patients newly admitted to selected wards (such as haematology units with transplant recipients, ICUs or cardiac ICUs, nephrology and neonatology, gastroenterology, infectious diseases units and several surgical specialities) and also for inpatients transferred from other wards to an ICU. Screening criteria for newly admitted patients, if applied by the hospital, included previous hospitalisation, previous infections with multidrug-resistant bacteria (if known) or previous antimicrobial treatment.

Carbapenem non-susceptible clinical isolates (from blood, urine, tracheal secretions) obtained from patients from the selected wards, were also included in the surveillance and when detected, these patients and their contacts were also checked for faecal colonisation. Suspicion of an ongoing outbreak with a CP-GNB during the study period in Hospital 1 lead to environmental samples being collected, which were also included in the study.

**Escherichia coli** from blood and cerebrospinal fluid. As a result, most European epidemiological reports show that data on CP-GNB from Romania are unknown or uncertain [3]. To get a snap-shot of the epidemiology of CP-GNB in hospitals that do not report to EARS-Net and that were not included in the 2013 EuSCAPE, a pilot CP-GNB surveillance project – introducing faecal screening for carbapenemase-producing bacteria on admission of patients to hospital and following transfer of inpatients from other wards to an intensive-care unit (ICU), as well as screening of clinical isolates – was implemented in three Romanian hospitals during December 2014 to May 2015. Few studies have investigated intestinal colonisation with carbapenemase-producing bacteria at the point of hospital admission: here we report the findings of an active surveillance programme for carbapenemase-producing bacteria in three hospitals in Romania.

**Carbapenem-hydrolysing class D beta-lactamases (CHDLs)**

Carbapenemase screening protocols for *Enterobacteriaceae* and non-fermenting Gram-negative organisms were considered and a single protocol was used in the participating hospitals. For screening of faecal samples, a small sample inoculum was placed in 5 mL trypticase soy broth (TSB) containing a meropenem (10 µg) disc and incubated at 37°C overnight. The following day, 100 µL of TSB was transferred onto a MacConkey agar plate (all discs and media were from Oxoid, Basingstoke, UK) and one disc each of ertapenem (10 µg) and meropenem (10 µg) were placed on different sectors of the inoculum. Clinical breakpoints and screening cut-off values for CPE were according to the European Committee on Antimicrobial Susceptibility (EUCAST) methodology [4]. All carbapenem non-susceptible isolates were identified (VITEK 2 system, Biomerieux, France) and stored at –80°C for further testing.

Molecular characterisation of isolates was performed at Liverpool University, UK, which consisted of screening for resistance markers by multiplex PCR followed by sequencing. The PCR included screening for extended spectrum beta-lactamases (ESBLs) including *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>OXA</sub> and carbapenem-resistance genes (*bla*<sub>NDM</sub>, *bla*<sub>KPC</sub>, *bla*<sub>GES</sub>, *bla*<sub>IMI</sub>, *bla*<sub>ZIM</sub>, *bla*<sub>SPM</sub>) as previously described [5-7]. Carbapenem-hydrolysing class D beta-lactamases (CHDLs) corresponding to OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58 and OXA-148 groups, as well as OXA-10, previously described as narrow-spectrum and recently shown to have CHDL activity in *Acinetobacter* spp. [8,9], were also included. Positive and negative controls were included in all PCR reactions. Minimum inhibitory concentration (MIC) testing for isolates with a unique genotype was performed by

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**Figure 1**

Genes associated with carbapenem resistance (here including *bla*<sub>OXA-48</sub>) identified in two hospitals, north-central (Hospital 1, 22 isolates) and north-east Romania (Hospital 3, 28 isolates), December 2014–May 2015.
broth microdilution with Sensititre GNX3F plates (TREK Diagnostic Systems, West Sussex, UK). The results were interpreted according to EUCAST breakpoints for all antimicrobials except for polymyxin B, where Clinical and Laboratory Standard Institute (CLSI) criteria were applied [10,11].

To determine whether the carbapenem-resistance genes were transferable, conjugation was performed by broth mating as previously described [12] and was attempted for all CPE identified in this study. Donor and recipient cells (plasmid-free streptomycin-resistant E. coli HB101) were grown to logarithmic phase, mixed in nutrient broth and incubated at 37 °C for 18 hours. Transconjugants were selected on nutrient agar (Oxoid, UK) supplemented with streptomycin (50 µg/mL) and cefotaxime (1 µg/mL). To ensure that selected isolates were the recipient E. coli and that they contained the transferred resistance gene, PCR was performed for the uidA gene [13] as well as carbapenem-resistance genes. In addition, PCR was performed to identify if ESBL resistance determinants were co-transferred with the carbapenem-resistance. Plasmid DNA was extracted from clinical isolates and transconjugants using a QIAprep spin miniprep kit (QIAGEN, Germany). Plasmid identification using a PCR-based replicon typing (PBRT) scheme [14] was carried out on total DNA from all CPE clinical isolates carrying carbapenem-resistance genes, as well as on plasmid DNA extracted from the clinical isolates and their transconjugants. It was previously observed that PBRT can be inefficient at identifying IncL/M plasmid type, therefore an up-dated method designed to identify and distinguish between IncL and IncM plasmids was used [14,15].

Results
A total of 820 clinical, faecal and environmental specimens were analysed: 528 samples were from Hospital 1 (7 clinical, 336 faecal, 185 environmental), 105 from Hospital 2 (all faecal) and 187 from Hospital 3 (23 clinical, 164 faecal). Carbapenem non-susceptible Gram-negative isolates were identified in 27/528 (5%), 12/105 (12%) and 38/187 (20%) of the samples from Hospitals 1, 2 and 3, respectively. Molecular testing identified carriage of at least one gene associated with carbapenem resistance in 22/27 of the carbapenem non-susceptible isolates from Hospital 1, none from Hospital 2 and 28/38 isolates from Hospital 3 (Figure 1).

A total of 19 isolates from Hospital 1 carried blaNDM-1 (17 Serratia marcescens and two K. pneumoniae) while three isolates (all K. pneumoniae) carried blaOXA-48. The NDM-1 positive isolates were from clinical specimens (seven isolates from pharyngeal secretions), faecal screening (nine isolates) and hospital environmental samples (three isolates, from feeding tube, bedside and food jar) while the OXA-48-producing K. pneumoniae isolates were all from faecal screening. Of the 19 NDM-1-positive isolates, 15 co-harboured blaCTX-M-15 while two produced LEN-25 beta-lactamases. Moreover, an unexpected variety of species carrying one or sometimes two genes associated with carbapenem resistance were identified in Hospital 3 (Figure 2).

A total of 11 A. baumannii isolates from Hospital 3 (nine from clinical samples (blood, tracheal secretions, urine) and two faecal isolates) were found to harbour blaoXA-23 while another clinical isolate carried blaoXA-24/27. The second most prevalent gene associated with carbapenem resistance in this hospital was bloaOXA-48 detected in clinical isolates of K. pneumoniae (n = 4) and Morganella morganii (n = 1). In addition, two Pseudomonas aeruginosa clinical isolates carried blavim-2 while eight P. aeruginosa isolates (six faecal, two clinical) co-harbourd blaoXA-10 and blaveb-1a beta-lactamase. Furthermore, one patient was found to have faecal colonisation with E. coli-producing VIM-2. KPC
Carbapenemases were not identified in any of the hospitals investigated.

Previously unreported species/gene combinations were also identified in Hospital 3. For instance, *A. baumannii* isolates co-harboured *bla*_{OXA-23} as well as *bla*_{SHV-12/18}, while one *E. coli* isolate obtained from faecal screening co-harboured *bla*_{VIM-1} and *bla*_{SHV-18}. All except two isolates from Hospital 3 that carried a gene associated with carbapenem resistance also co-produced CTX-M-15 and/or SHV-12 beta-lactamases.

Overall, of the 50 CP-GNB identified in this study, 27 were from clinical specimens, 23 were obtained through faecal screening and three from environmental samples. Two patients with *A. baumannii* OXA-23-producing infections (pneumonia, bacteraemia) had faecal colonisation with the same CP-GNB. Another patient was found to be colonised with multiple CP-GNBs, where OXA-23-producing *A. baumannii* and OXA-48-producing *K. pneumoniae* were isolated from a wound infection while OXA-10-producing *P. aeruginosa* was detected through faecal screening.
Susceptibility testing showed variable levels of resistance to carbapenems, spanning from NDM-1-producing *S. marcescens* and OXA-48-producing *K. pneumoniae* exhibiting susceptibility to doripenem, imipenem and meropenem through to *P. aeruginosa* co-producing OXA-10, VEB-1a and SHV-12 beta-lactamases, which manifested resistance to all antimicrobials tested except for polymyxin B and colistin (Table). Taken together, these results highlight the difficulties faced by laboratories that only rely on phenotypic methods for detection of carbapenemases.

Due to the multidrug-resistant profiles of many clinical isolates, a suitable recipient for conjugation experiments was identified for only five isolates (one NDM-1-producing *S. marcescens* and two NDM-1-producing *K. pneumoniae* from Hospital 1 and two OXA-48-producing *K. pneumoniae* from Hospital 3); conjugative plasmids were successfully transferred in all five isolates. PBRT showed that *bla*₅₉₃₇₄ was associated with IncFIIs plasmids in both *K. pneumoniae* and *S. marcescens* from Hospital 1. However, PBRT performed on clinical *Serratia* NDM-1-positive isolates also identified IncM plasmids in five isolates and IncL in three isolates, while no typable plasmids could be found in six clinical isolates carrying *bla*₅₉₃₇₄. IncL plasmids were found to be associated with *bla*₅₉₃₇₄ in *K. pneumoniae* clinical isolates and transconjugants from resistance-transfer studies. In addition, IncL was the only plasmid found in all *K. pneumoniae* clinical isolates that carried *bla*₅₉₃₇₄ from both hospitals (Hospitals 1 and 3). PCR also showed that no ESBL-resistance determinants had co-transferred with the carbapenem-resistance genes.

**Discussion**

This pilot study demonstrated a high prevalence of CP-GNB in two of three Romanian hospitals over a six-month period. Furthermore, a variety of carbapenemase-producing species and genes associated with carbapenem resistance were also identified in this study. The distribution of the CP-GNB varied between the two hospitals in which these bacteria were detected: NDM-1-producing *S. marcescens* and *K. pneumoniae* were prevalent in Hospital 1, in the north-central part of the country, but were not identified in Hospital 3, in the north-east. No history of travel abroad could be identified in the patients and the origin of the NDM-1 isolates needs further investigation to understand the epidemiology of NDM-1-producing *Enterobacteriaceae* in eastern Europe [16].

In our study, *bla*₅₉₃₇₄ was identified in 17 isolates of *S. marcescens* across clinical, environmental and faecal isolates, all of which were obtained from the same neonatal unit, suggesting the likelihood of an outbreak, which will be further investigated. This is the largest number of NDM-1-producing *S. marcescens* detected in one hospital in Europe, with only one previous report of a clinical isolate in Germany [17], highlighting the spread of *bla*₅₉₃₇₄ into another *Enterobacteriaceae* species. Although an opportunistic pathogen, *S. marcescens* has emerged in the past decades as an important source of hospital-acquired-infections and outbreaks in both adult and paediatric intensive-care patients [18,19]. Hospital 1, where the NDM-1-producing *S. marcescens* and *K. pneumoniae* were isolated, had previous *Serratia* spp. outbreaks during 2010–12 in the same neonatal unit with *Serratia* spp. isolates that did not produce carbapenemase (data not shown). Our conjugation studies identified *bla*₅₉₃₇₄, to be associated with conjugative IncFIIs plasmids in both *K. pneumoniae* and *S. marcescens*, which could suggest interspecies transfer of *bla*₅₉₃₇₄ via mobile genetic elements. Whole genome sequencing will be carried out of the NDM-1-negative and NDM-1-producing *Serratia* and *Klebsiella* isolates obtained in this study, as well isolates from previous outbreaks in this neonatal unit, to understand the molecular epidemiology of NDM-1-producing *S. marcescens* in this hospital and in surrounding areas in eastern Europe.

In contrast, NDM-1 was not found in Hospital 3, whereas OXA-23- and OXA-24-producing *A. baumannii*, OXA-48-producing *K. pneumoniae* and *M. morganii*, as well as OXA-10-producing *P. aeruginosa*, were the most prevalent in this hospital. Interestingly, the *bla*₅₉₃₇₄-carrying *P. aeruginosa* isolates exhibited resistance to all tested carbapenems, which could imply that OXA-10 beta-lactamases in *Pseudomonas* spp. may have catalytic activity against carbapenems, as has been demonstrated for *Acinetobacter* spp. [9]. Further research is necessary, however, to establish the ability of OXA-10 beta-lactamases to compromise therapeutic effectiveness of carbapenems in *Pseudomonas* spp. OXA-48-producing *K. pneumoniae* was the most common CPE in Hospital 3 and conjugation studies identified *bla*₅₉₃₇₄ to be associated with IncL plasmids. Previous studies have shown that plasmids carrying *bla*₅₉₃₇₄ share similar features, in that they have an IncL/M backbone and are very similar in size (about 60–70 kb) suggesting their wide dissemination in different countries through an epidemic plasmid [20]. A recent study specifically assigned *bla*₅₉₃₇₄-carrying plasmids to the IncL group [15]; in our study, we demonstrated the association of *bla*₅₉₃₇₄ with IncL plasmids in all carbapenemase-producing *K. pneumoniae* isolates from two hospitals in different geographical regions in Romania.

Previously unreported species/gene combinations, or bacteria co-harbouring more than one carbapenemase-encoding gene, were also identified in Hospital 3. Detection of a patient colonised with *E. coli*-producing VIM-2 is the first known report of *E. coli* harbouring *bla*₅₉₃₇₄ in a hospital patient in Romania, following previous detection of this genotype in patients from Germany between September 2009 and May 2013 [21]. Our study also detected *M. morganii*-producing OXA-48, a CP-GNB that has not been previously reported in Europe, but has been described in clinical isolates from Lebanon and Kuwait [22,23].
A. baumannii is known to be an agent of nosocomial infections associated with ICUs [24]. It is widely recognised that this bacterium is very well adapted to withstand dry environmental conditions and disinfectants, being able to survive for a long time (several months) on hospital surfaces [24]. Previous molecular analyses of A. baumannii isolates from Hospital 3’s cardiac ICU obtained in 2011–12, identified widespread infections with OXA-23-producing A. baumannii belonging to European clones I and II (data not shown). Such findings should trigger appropriate infection control measures, however, previous studies have shown that very often routine infection control measures are insufficient to control A. baumannii and enhanced measures, such as using closed tracheal suction systems, patient isolation or sometimes ward closure, are necessary to control this resilient opportunistic pathogen [24].

There are only a few previous reports on the isolation and/or characterisation of CP-GNB from Romanian hospitals, which described NDM-1-producing E. cloacae, OXA-48-producing K. pneumoniae, OXA-23-producing A. baumannii and VIM-2-producing P. aeruginosa clinical isolates [25-29]. In one study, Deshpande et al. analysed clinical isolates from three Romanian hospitals in 2011 and found 3% of isolates from two hospitals with reduced susceptibility to carbapenems, two of which were NDM-1-producing E. cloacae, the other being OXA-48-producing K. pneumoniae [25]. Only a few years later, our study demonstrates a high prevalence of carbapenem non-susceptible Gram-negative isolates, ranging from 5% to 20%, as well as a great diversity of bacterial species and genes associated with carbapenem resistance. Detection of CP-GNB through the implementation of a patient screening programme demonstrated the presence of these bacteria in clinical and faecal samples of newly admitted hospital patients, providing a snapshot of what is likely to be an endemic spread of CP-GNB in this country. Larger, nationwide screening studies are necessary to determine the true prevalence of CP-GNB in Romanian hospitals.

Our study has some limitations. Firstly, the criteria used to select newly admitted patients for screening (previous hospitalisation, previous infection with multidrug-resistant bacteria or previous antimicrobial treatment) were applied in Hospital 3 but could not be applied in Hospitals 1 and 2, where all patients newly admitted to selected wards were screened. Therefore, risk factors for CP-GNB colonisation of newly admitted patients could not be determined. Secondly, clinical data were not available from Hospitals 1 and 2, therefore risk factors for CP-GNB infection could also not be determined.

Despite these limitations, data from Hospital 3, where screening criteria were used for newly admitted patients, showed that two patients selected for faecal screening had multiple hospitalisations in the previous year, one whom was found to have concurrent OXA-23-producing A. baumannii faecal and tracheal exudate colonisation. Also, hospital records showed that six patients from whom surveillance cultures yielded faecal carbapenem non-susceptible bacteria had been previously admitted to the same hospital during the past year, during which time they had been treated with imipenem. Of these, one patient had a wound infection with an OXA-48-producing K. pneumoniae and also faecal carriage of carbapenem non-susceptible P. aeruginosa. Another patient had concurrent faecal and tracheal exudate colonisation, as well as blood cultures containing OXA-23-producing A. baumannii. Finally, faecal carriage of VIM-2-producing E. coli was found in another patient who had been previously admitted to Hospital 3. These findings highlight the importance of active surveillance of asymptomatic carriers and identification of patients who may have a higher risk of CP-GNB carriage, such as those with previous multiple hospital admissions.

The high prevalence and variety of CP-GNB identified in two of the three hospitals investigated in this study are of concern and suggest the need for a review of the local surveillance and infection control measures. Romania is one of the few European countries that does not currently have a national surveillance system or specific guidelines for the management of CP-GNB infections, nor are hospitals obliged to report such infections to the health authorities [30]. As a consequence, the importance of these infections may be underestimated by hospital authorities. In the hospitals investigated, the ICUs had no facilities for patient isolation and at best, mobile curtains are used to separate beds. Although financial constraints may not allow infrastructure changes that would ensure strict patient isolation, more could be done to educate staff and implement feasible measures to prevent the introduction and spread of CP-GNB. Therefore, reinforcement of measures such as staff education on hand hygiene and contact precautions combined with active faecal culture surveillance are likely to have a direct impact on preventing the spread of CP-GNB and thus reducing the risk of such infections. Introduction of mandatory screening and reporting of CP-GNB by hospitals, as part of a coordinated surveillance programme, should provide an effective framework for the early detection and control of CP-GNB.

In conclusion, our findings emphasise the need for early detection of patients with CP-GNB colonisation or infection, which should lead to the implementation of appropriate infection control measures to prevent the silent spread of these isolates in hospitals. Improvement of surveillance of carbapenemase-producing bacteria in Romania – including introducing guidelines for laboratory detection of intestinal asymptomatic carriers and for infection control and outbreak management – will not only benefit the local patients but will also have an impact at European level, as it is recognised that the spread of carbapenemase-producing organisms is often linked to travel of patients between various countries [16].
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Conflict of interest

None declared.

Authors’ contributions

DT coordinated the study, analysed data, wrote the manuscript; CVP and MD assisted in designing the study; organised implementation of surveillance of carbapenemase-producing bacteria in one of the hospitals; collected clinical data, read and revised the manuscript; IEM assisted in designing the study, performed molecular testing, analysed data, read and revised manuscript; ADM, AM and FT assisted in designing the study, organised implementation of surveillance of carbapenemase-producing bacteria in two of the hospitals; collected clinical data, read and revised the manuscript.

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