ECDC SURVEILLANCE

Rapid investigation of New Delhi metallo-beta-lactamase (NDM)-1-producing *Providencia stuartii* in hospitals in Romania

November 2024

Summary

This report describes the results of an epidemiological and genomic study carried out in six hospitals in Romania to determine the extent of the spread of *Providencia stuartii* carrying bla_{NDM-1}. Most of the 74 submitted *P. stuartii* isolates were resistant to penicillins, cephalosporins, carbapenems, amikacin and ciprofloxacin. Almost all the isolates were associated with infections (in decreasing order of frequency) lower respiratory tract infection, urinary tract infection, multiple sites/systemic infection, bloodstream infection, skin and soft tissue infection, thus showing the clinical relevance of the detected multidrug-resistant *P. stuartii* isolates for hospitalised patients.

We identified four multi-hospital clusters including isolates from December 2021 to September 2023, which were detected over more than a year pointing to ongoing and sustained spread of *P. stuartii* carrying bla_{NDM-1} within the healthcare system in Romania. Clusters included isolates from up to four hospitals and three different regions indicating interregional spread. The phylogenetic tree revealed that several clusters were part of distinct clades. This indicates that *P. stuartii* carrying bla_{NDM-1} had already been circulating and diversifying in hospitals in Romania over a longer period which is in line with previous reports.

Three of the multi-hospital clusters were caused by a specific *P. stuartii* sequence type (ST)46 lineage carrying metallo-beta-lactamase (MBL) genes. Investigating this lineage in an international context showed that it had already been detected in nine countries, i.e. Bulgaria, France, Germany, Ireland, the Netherlands, Romania, Switzerland, the United Kingdom and the United States. Isolates belonging to this lineage carry a large set of resistance determinants and show sustained healthcare-associated and interregional transmission in Romania for more than a year. This suggests that this lineage can colonise and effectively transmit between hosts and, in this study, most isolates were associated with infections (although this can partially be attributed to the selection criteria which gave preference to isolates from clinical samples). All these features are part of the criteria proposed for the definition of international high-risk clones.

Although, in European Union (EU)/European Economic Area (EEA) countries, *P. stuartii* carrying bla_{NDM-1} initially received attention when detected in relation with patient transfers from Ukraine, there is evidence of wider dissemination in Eastern Europe and the Balkan region including Bulgaria (published genomic analysis), Greece (published study), Hungary (reported epidemiological links), North Macedonia (reported epidemiological link), Romania (this report) and Serbia (described epidemiological link). Nevertheless, evidence is still limited at this stage and further studies are required to determine the extent of spread of *P. stuartii* in Eastern Europe and the Balkan region, and the emergence and persistence of *P. stuartii* high-risk clones. From a public health perspective, however, current evidence regarding the spread of *P. stuartii*, including sustained transmission in hospitals in Romania, is sufficient for a warning regarding the potential of *P. stuartii* carrying MBL genes to further disseminate throughout healthcare systems in the EU/EEA and the related adverse consequences for patients.

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Background

Molecular surveillance of carbapenemase-producing Enterobacterales (CPE) and related outbreak investigations in the European Union (EU)/European Economic Area (EEA) have so far mainly focused on *Klebsiella pneumoniae* and *Escherichia coli* as the most frequent species of CPE [1-3]. However, national studies [4] as well as unpublished results of an ECDC questionnaire on the epidemiological situation of CPE in the EU/EEA in 2023 show that carbapenemase-producing isolates from several other genera and species of Enterobacterales, including *Citrobacter* spp., *Enterobacter* spp.*, Klebsiella oxytoca, Proteus* spp., *Providencia* spp. and *Serratia* spp. were reported with increasing frequency, which requires further investigation.

In January 2023, the National Reference Laboratory (NRL) of the Netherlands reported an increase of New Delhi metallo-beta-lactamase (NDM)-producing *Providencia stuartii* isolates via ECDC's EpiPulse platform. Among these, a genomic cluster of multidrug-resistant *P. stuartii* isolates from patients with a link to Ukraine was identified using whole-genome sequencing (WGS). In reply, reports were received from other EU/EEA countries about cases of NDM-producing *P. stuartii* with and without a link to Ukraine. This led to a joint investigation with the sharing of epidemiological and WGS data between countries to determine the extent of the cross-border spread of carbapenemase-producing *P. stuartii* in Europe. Analysis of *P. stuartii* isolates from ten EU/EEA countries showed clustering of isolates with a link to Ukraine [5].

Data received by ECDC in reply to the above mentioned questionnaire on the epidemiological situation of CPE in the EU/EEA showed that Romania reported 355 cases of NDM-producing *P. stuartii* isolates from 2018 to 2022, the highest frequency of detection of participating EU/EEA countries. These cases originated from the same seven hospitals initially invited for this study due to their capacity to detect NDM (unpublished data). Dissemination of NDM-1-producing *P. stuartii* in five Romanian hospitals had also been reported in a previous study based on 77 isolates collected from January 2016 to September 2017 [6]. However, more timely national surveillance and control information could be generated by WGS of recent isolates in combination with basic epidemiological data. Furthermore, comparison of recent *P. stuartii* isolates detected in Romanian hospitals to isolates collected for the above-mentioned European investigation in 10 EU/EEA countries [5] would give valuable insights into the spread of carbapenemase-producing *P. stuartii* at the European level.

The objectives of this study were:

- to determine the epidemiological and genomic characteristics of isolates of carbapenemase-producing *P. stuartii* in Romanian hospitals;
- to compare these characteristics with carbapenemase-producing *P. stuartii* isolates detected in other EU/EEA countries.

Methods

Study design

This study protocol was based on the protocol for the European survey of carbapenem- and/or colistin-resistant Enterobacterales (CCRE survey) conducted in 2019 [1], with a few modifications to reduce the time between isolate collection and WGS results. Hospitals that detected carbapenem-resistant and/or carbapenemase-producing *P. stuartii* isolates within the last two years were invited to participate in the study. The study included carbapenem-resistant and/or carbapenemase-producing isolates of *P. stuartii* from infected and/or colonised individual patients. *P. stuartii* isolates with resistance to any carbapenem (ertapenem, imipenem, meropenem) according to the current EUCAST breakpoints (v14.0) valid for 2024 [7] were eligible for inclusion if identified during clinical routine testing in hospital laboratories, as well as *P. stuartii* isolates confirmed as carrying carbapenemase genes by molecular testing.

Sampling

Each participating hospital was invited to submit up to 20 isolates of carbapenem-resistant and/or carbapenemase-producing *P. stuartii* with preference given to recent isolates from 2022 and 2023. Non-duplicate bacterial isolates, preferably from clinical specimens collected for diagnostic purposes (e.g., blood, urine, sputum, wound secretion, etc.), originating from non-duplicate patients and meeting the above definition were also included. Each isolate was accompanied by microbiological, clinical, and epidemiological data as outlined below.

Data collection

Microbiological and epidemiological data

The collected microbiological data included the isolate's unique identifier, the date of sample collection, the code of the healthcare institution submitting the sample and its National Territorial Unit for Statistics level 2 (NUTS 2) location, the type of sample and routine antimicrobial susceptibility testing (AST) results for a panel of 16 antibiotics including ertapenem, imipenem, meropenem, aztreonam, cefotaxime, cefepime, ceftazidime, amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftazidime-avibactam, amikacin, gentamicin, tobramycin, ciprofloxacin, trimethoprim-sulfamethoxazole and fosfomycin. Isolates were tested at hospital laboratories with established routine methods. Disk diffusion zone diameters and minimum inhibitory concentrations were interpreted using EUCAST breakpoints [7].

Demographic, epidemiological and clinical data included:

- age;
- sex;
- type of patient (e.g. inpatient or outpatient);
- infection or colonisation/carriage status or unknown clinical significance, and in case of infection, site of infection;
- most likely mode of acquisition (e.g. community-associated and healthcare-associated differentiated by a hospital stay of more or less than 48 hours at the time of sample collection);
- an epidemiological link to another patient with *P. stuartii* infection or colonisation/carriage;
- previous travel within the past 12 months;
- previous hospitalisation within the past 12 months;
- direct hospital transfer from another country.

Whole-genome sequencing and bioinformatic analysis

Eurofins Genomics (Germany), contracted by ECDC, performed WGS. The final dataset included:

- 74 *P. stuartii* genomes from hospitals in Romania collected for this study;
- 68 published and unpublished *P. stuartii* genomes from the *P. stuartii* outbreak in Rome [8] as well as the investigation linked to patient transfers from Ukraine (originating from Denmark, Finland, France, Germany, Ireland, the Netherlands, Norway, Poland, Spain [5]) and Latvia;
- 236 *P. stuartii* genomes from the public domain.

Metadata for these isolates were gathered and standardised where possible. Assemblies were produced using SPAdes v3.15.5 and alleles were called with ChewBBACA v3.2.0 [9]. Clustering was performed using the in-house whole genome multilocus sequence typing (wgMLST) scheme developed by the National Institute for Public Health and the Environment (RIVM) comprising 3 079 core genes and 665 accessory genes. The cluster cut-off was increased from 15 ADs [5] to 25 ADs due to the implementation of ChewBBACA in the ECDC WGS analysis pipeline which tended to produce slightly higher pairwise distances. Sequence types (STs) were assigned using the Institute Pasteur-hosted *Providencia* spp. MLST scheme [10,11]. Antimicrobial resistance genes were identified using ResFinder v4.1.11 (database downloaded on 29 September 2022) with standard settings. [12]. An interactive phylogenetic tree based on a neighbour-joining algorithm including relevant metadata was visualised in Microreact [13].

Results

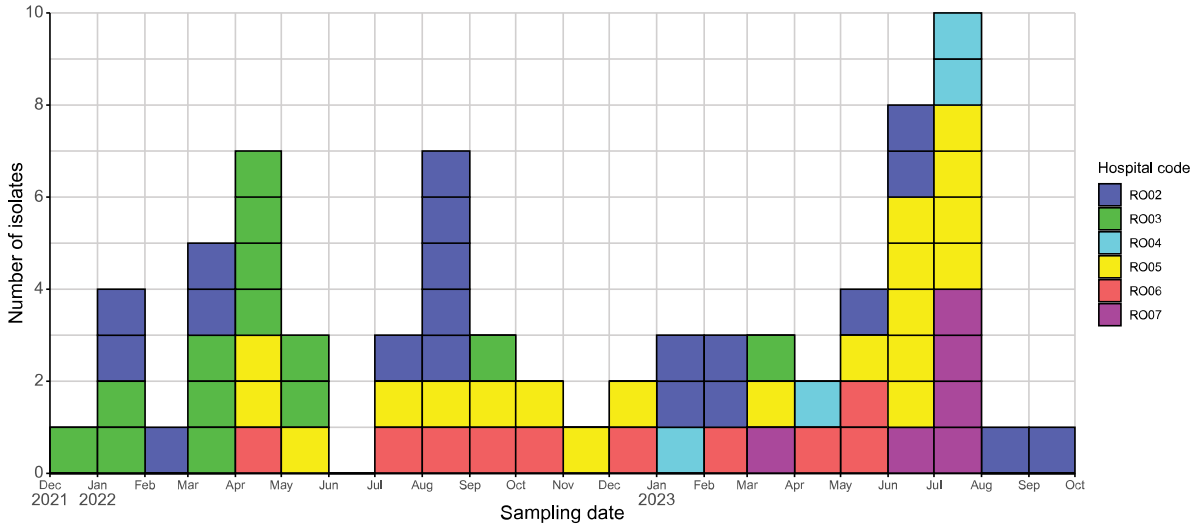
Geographic and time distribution

Six of the seven initially registered hospitals submitted carbapenem-resistant and/or carbapenemase-producing *P. stuartii* isolates for WGS, with the number of submitted isolates ranging from four to the maximum number of 20 isolates permitted per protocol (Table 1). The six hospitals were from the north, the centre and the capital of the country, and covered four of the eight NUTS-2 regions in Romania. Three hospitals (RO05, RO06 and RO07) were located in Bucharest (Table 1). A total of 74 isolates were included in the dataset with the earliest isolate collected on 29 December 2021 and the last isolate on 5 September 2023 (Table 1, Figure 1). All isolates except one were confirmed by WGS to carry *bla*$_{\text{NDM-1}}$.

Table 1. Number of *Providencia stuartii* isolates (n=74) by geographic region, hospital and period covered (first to last isolate), Romania, December 2021–September 2023

NUTS 2 region	Hospital code	Number of isolates	Date of first isolate	Date of last isolate
Bucharest	RO05	20	08/04/2022	31/07/2023
	RO06	10	19/04/2022	27/05/2023
	RO07	6	30/03/2023	30/07/2023
Centre	RO03	14	29/12/2021	30/03/2023
North-East	RO04	4	17/01/2023	10/07/2023
North-West	RO02	20	02/01/2022	05/09/2023
Total	**6**	**74**	**29/12/2021**	**05/09/2023**

Figure 1. Time distribution of *Providencia stuartii* isolates (n=74) by hospital, Romania, December 2021–September 2023



The time distribution illustrated above should not be interpreted as an epidemic curve as the variation over time may be affected by retrospective inclusion of stored P. stuartii *isolates with preference given to more recent isolates and the maximum number of 20 isolates included by hospital. The figure therefore does not reflect true temporal trends in incidence.*

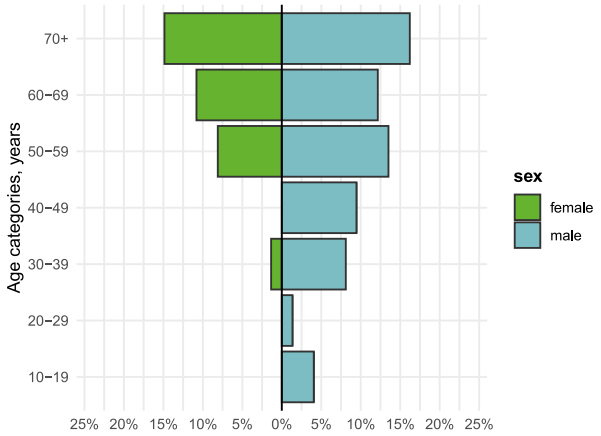
Epidemiological characteristics

Most (n=72, 97.3%) of the isolates were associated with an infection, while only two were reported as colonisation/carriage. The recorded sites of infection are listed in Table 2. Fifty-eight (78.4%) isolates were categorised as healthcare-associated, while the mode of acquisition was unknown for the remaining isolates (n=16, 21.6 %). More patients were male (n=48, 64.9%) and the median age was 64.5 years (range 19-91). The age and sex distribution of cases are displayed in Figure 2.

Table 2. Sites of infection for *Providencia stuartii* isolates (n=72), Romania, December 2021–September 2023

Site of infection	Number of isolates	Percentage
Lower respiratory tract infection	24	33.3
Urinary tract infection	18	25.0
Multiple sites/systemic infection	12	16.7
Bloodstream infection	10	13.9
Skin and soft tissue infection	5	6.9
Surgical site infection	1	1.4
Unknown	2	2.8
Total	**72**	**100**

Figure 2. Age and sex distribution of patients with *Providencia stuartii* (n=74), Romania, December 2021–September 2023



Microbiological characteristics

Seventy-three (98.6%) of 74 included isolates were from samples collected for clinical diagnostic purposes, most frequently urine (n=22, 30.1%), blood (n=15, 20.5%) and lower respiratory tract samples (n=15, 20.5%). One sample was collected for screening purposes. The majority (>90%) of tested *P. stuartii* isolates were resistant to penicillins, cephalosporins, amikacin, ciprofloxacin and trimethoprim-sulfamethoxazole (Table 3). Of the tested isolates carrying *bla*$_{\text{NDM-1}}$, 97.0% were resistant to ertapenem, 97.1% to imipenem and 79.2% to meropenem according to EUCAST clinical breakpoints (Table 3). None of the *P. stuartii* isolates carrying *bla*$_{\text{NDM-1}}$ were fully susceptible to carbapenems. Even though AST for aztreonam was performed for only 44% of isolates carrying *bla*$_{\text{NDM-1}}$, 43.8% of them were resistant to this antimicrobial. Aztreonam-avibactam and cefiderocol AST were not performed and fosfomycin results were not reported due to the absence of valid clinical breakpoints.

Table 3. Antimicrobial susceptibility testing results for *Providencia stuartii* isolates carrying *bla*$_{\text{NDM-1}}$ **(n=72), Romania, December 2021–September 2023**

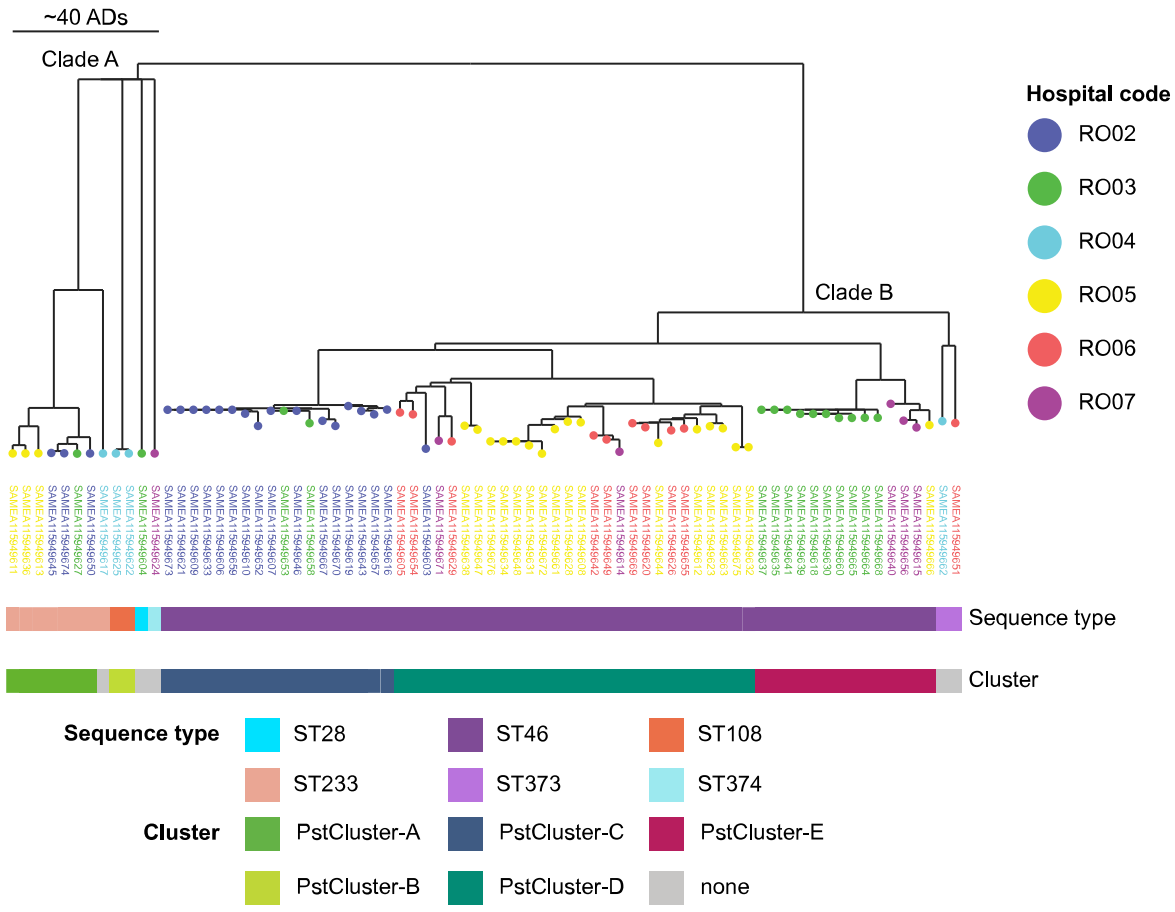
Antimicrobial	Number of isolates tested	Percentage resistant*
Piperacillin-tazobactam	71	98.6
Ceftazidime	72	100
Cefepime	72	93.1
Ceftazidime-avibactam	59	96.6
Aztreonam	32	43.8
Ertapenem	67	97.0
Imipenem	70	97.1
Meropenem	72	79.2
Amikacin	72	94.4
Ciprofloxacin	72	95.8
Trimethoprim-sulfamethoxazole	72	90.3

Two isolates in the dataset did not carry bla$_{NDM-1}$ *and are therefore excluded from this table. Results were interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints v14.0.*

Genomic clusters

The phylogenetic tree of the *P. stuartii* isolates from Romania indicated a separation into two main clades: a smaller clade A with 12 isolates and a larger clade B with 62 isolates. Using a cut-off of 25 allelic differences (ADs), five clusters of potential recent transmission were identified with a size ranging from two to 28 isolates (Figure 3, Table 4). Isolates of PstCluster-A and PstCluster-B belonged to the smaller clade A while PstCluster-C, PstCluster-D and PstCluster-E were part of the larger clade B. All clusters except PstCluster-B were multi-hospital clusters involving up to four hospitals and three NUTS 2 regions. The three clusters with more than ten isolates each (PstCluster-C, PstCluster-D and PstCluster-E) were located on the same branch within 44 ADs, indicating recent rapid expansion, while the two smaller clusters (PstCluster-A and PstCluster-B) were much more distant from each other and from PstCluster-C, PstCluster-D and PstCluster-E (Figure 3).

Figure 3. Sequence types and clusters of *Providencia stuartii* isolates (n=74) by hospital, Romania, December 2021–September 2023



AD – allelic difference; ST – sequence type.

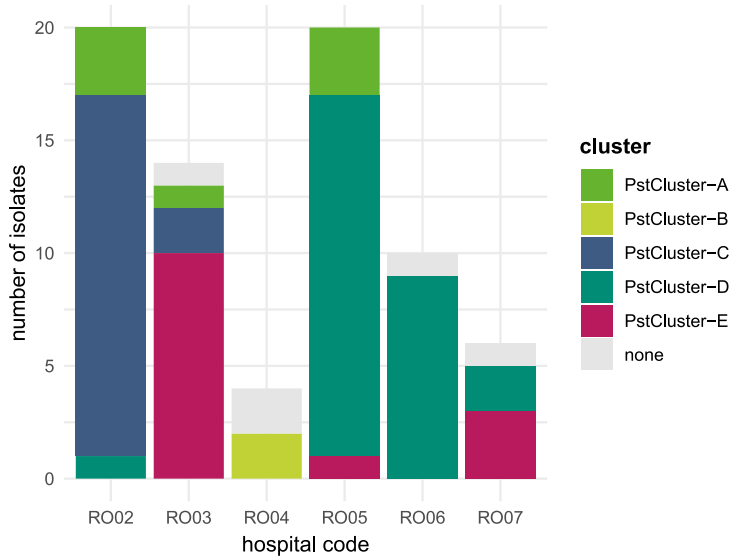
All four multi-hospital clusters included isolates detected over more than a year which confirming sustained transmission within the healthcare system (Table 4). In addition, *P. stuartii* isolates being part of up to three different clusters were detected within the same hospitals indicating co-existence of various transmission chains in these hospitals (Figure 4).

Table 4. Clusters (n=5) of *Providencia stuartii* isolates, Romania, December 2021–September 2023

Clade	ST	PstCluster (25 ADs)	Number of involved hospitals	Hospital code	Number of isolates	Date of first isolate	Date of last isolate	NUTS 2 region(s)
A	233	A	3	RO02, RO03, RO05	7	22/03/2022	24/06/2023	Bucharest, Centre, North-West
	108	B	1	RO04	2	05/07/2023	10/07/2023	North-East
B	46	C	2	RO02, RO03	18	02/01/2022	05/09/2023	Centre, North-West
		D	4	RO02, RO05, RO06, RO07	28	19/04/2022	31/07/2023	Bucharest, North-West
		E	3	RO03, RO05, RO07	14	29/12/2021	30/07/2023	Bucharest, Centre

AD – allelic difference; ST – sequence type.

Figure 4. Presence of different clusters among *Providencia stuartii* isolates (n=74) from the six participating hospitals, Romania, December 2021–September 2023



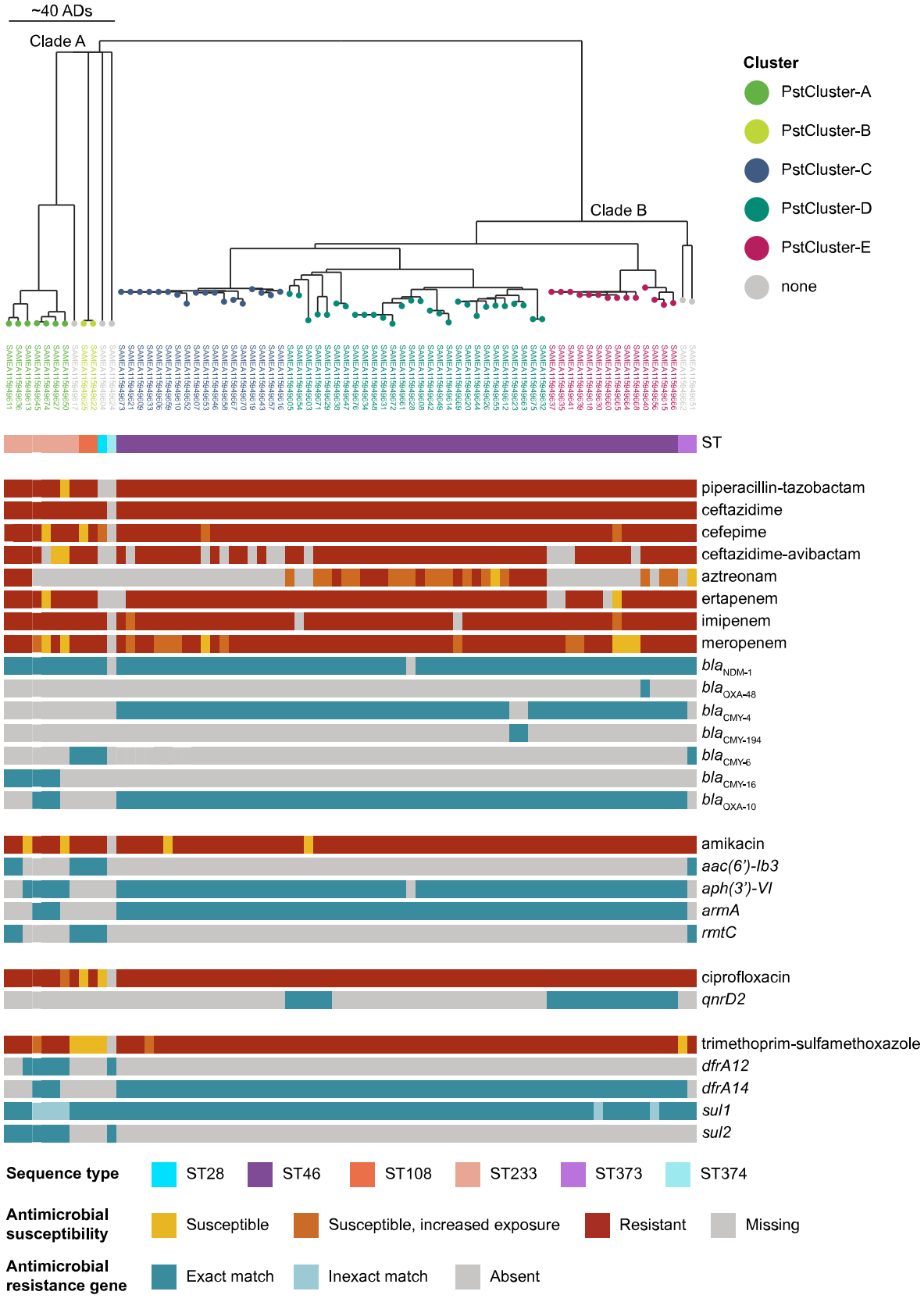
Nearly all within-cluster isolates, i.e. 68 (98.6%) of 69 isolates, carried the metallo-beta-lactamase (MBL) gene *bla*$_{NDM-1}$, while the remainder of the resistome differed between the clades despite a similar phenotypic AST profile (Figure 5). Of all isolates within clusters, the majority (n=62) co-carried two additional beta-lactamases. These included *bla*$_{OXA-10}$ and one of the detected *bla*$_{CMY}$ variants, i.e. *bla*$_{CMY-4}$ (two isolates carried inexact match of *bla*$_{CMY-4}$ with the same nucleotide mutation T757A resulting in W253R amino acid change; these variants were designated as *bla*$_{CMY-194}$) or *bla*$_{CMY-16}$. Five isolates had only one additional beta-lactamase, either *bla*$_{CMY-6}$ or *bla*$_{CMY-16}$, and one isolate did not carry any additional beta-lactamase gene. The plasmid-mediated AmpC beta-lactamase gene variants differed between clades and clusters, with *bla*$_{CMY-16}$ detected in six out of seven isolates of PstCluster-A, *bla*$_{CMY-6}$ found in both isolates of PstCluster-B, and *bla*$_{CMY-4}$ found in isolates from PstCluster-C, PstCluster-D and PstCluster-E. Two PstCluster-D isolates carried *bla*$_{CMY-194}$. In addition to *bla*$_{NDM-1}$, *bla*$_{CMY-4}$ and *bla*$_{OXA-10}$, one isolate in PstCluster-E also carried the *bla*$_{OXA-48}$ carbapenemase gene (Figure 5).

Detection of *bla*$_{NDM-1}$ alone is predicted to cause resistance to most of the beta-lactam antibiotics included in Figure 5, except for aztreonam. However, nine within-cluster isolates remained susceptible to various beta-lactams (Figure 5). Eleven out of 14 aztreonam-resistant within-cluster isolates carried a combination of three beta-lactamase genes, i.e. *bla*$_{NDM-1}$, *bla*$_{CMY-4}$/*bla*$_{CMY-194}$ and *bla*$_{OXA-10}$, while the remaining three isolates carried *bla*$_{NDM-1}$ and *bla*$_{CMY-16}$. It is worth mentioning that, within clusters, 17 aztreonam-'susceptible, increased exposure' (I) isolates and one aztreonam-susceptible isolate all carried the same above-mentioned triple combination of beta-lactamase genes.

For amikacin resistance, all within-cluster isolates were predicted to be resistant by carrying the combination of two aminoglycoside resistance genes, either *armA* and *aph(3')-VI* (n=62) or *aac(6')-Ib3* and *rmtC* (n=4), or single genes, i.e. *aph(3')-VI* (n=2) or *armA* (n=1). Despite the presence of these genes, four isolates (two in each clade) were susceptible to amikacin. All isolates in PstCluster-E (n=14) and five PstCluster-D isolates carried the *qnrD2* gene encoding resistance to ciprofloxacin. The remaining 50 within-cluster isolates did not carry any acquired fluoroquinolone resistance genes, which would indicate that chromosomal mutations may be the main explanation for the high proportion (97.1%, 67 out of 69 within-cluster isolates) of ciprofloxacin resistance.

Trimethoprim resistance was predicted by carriage of the *dfrA14* gene by all isolates within PstCluster-C, PstCluster-D and PstCluster-E (n=60) and three isolates of PstCluster-A, as well as the *dfrA12* gene only detected in five isolates from PstCluster-A. Isolates in PstCluster-B did not carry any detected trimethoprim resistance genes. The sulfamethoxazole resistance gene *sul1* was found in all within-cluster isolates (including six inexact matches) followed by the *sul2* gene only detected in isolates from PstCluster-A. Phenotypic trimethoprim-sulfamethoxazole resistance was confirmed for most (94.2%, 65 out of 69 isolates) within-cluster isolates.

Figure 5. Phenotypic antimicrobial susceptibility testing results and relevant genotypic antimicrobial resistance results of *Providencia stuartii* isolates (n=74), by cluster, Romania, December 2021–September 2023



Results were interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints v14.0. AD – allelic difference; ST – sequence type. Inexact match: <100% coverage and/or <100% identity.

International context

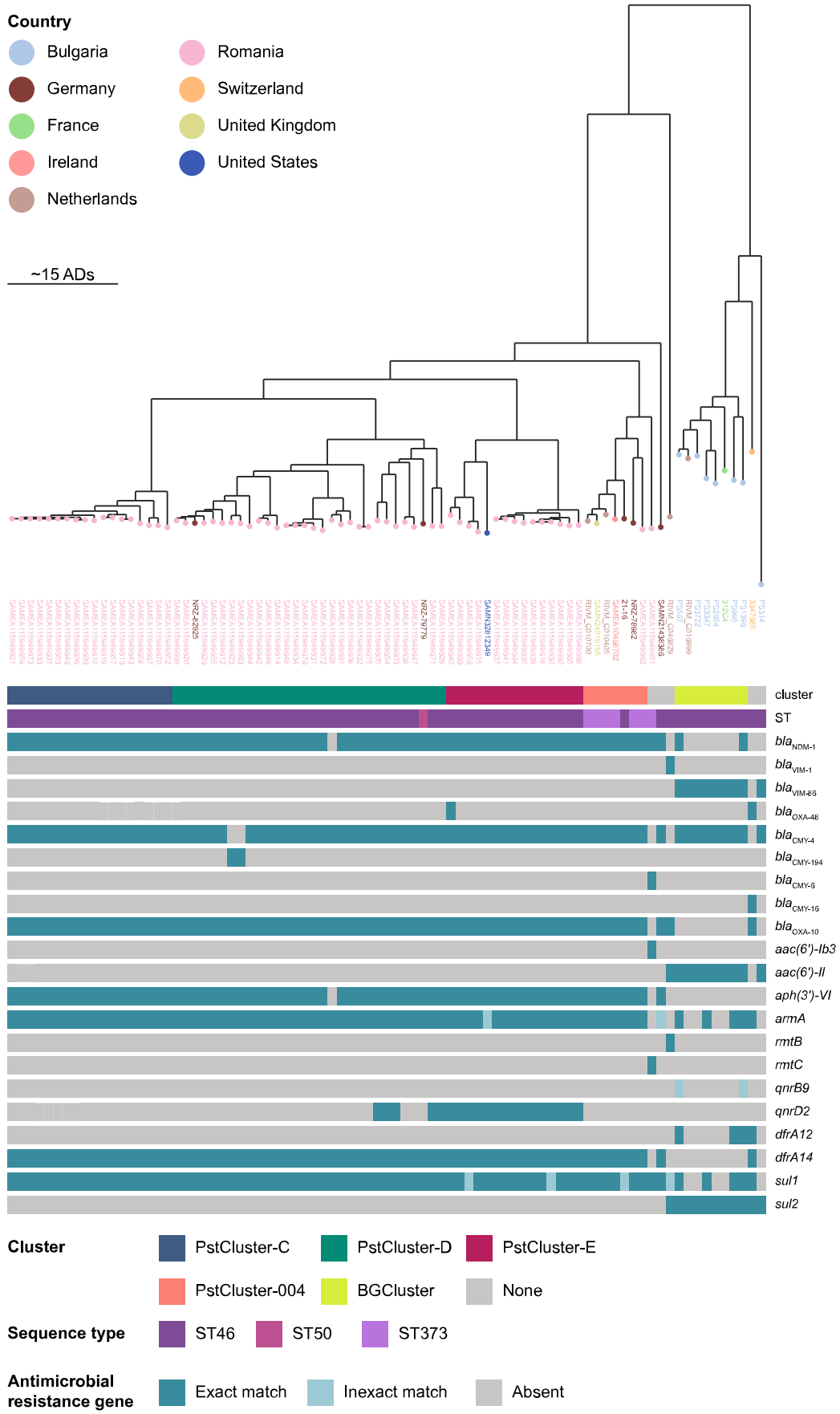
After the addition of data from *P. stuartii* isolates available in the public domain, three of the clusters detected in Romania and described above (PstCluster-A, PstCluster-D and PstCluster-E) also included isolates from other countries. PstCluster-A was related to an outbreak in Rome [8] with three Italian isolates (41, 65 and 883) clustering within PstCluster-A and carrying *bla*$_{NDM-1}$, with otherwise varied resistome. PstCluster-D included two isolates from Germany (NRZ-79779 and NRZ-82925) collected for the European investigation into the spread of *P. stuartii* related to medical transfers from Ukraine [5] and PstCluster-E contained an isolate from the United States (SAMN32812349). Both isolates from Germany, as well as the isolate from the United States, had a resistome similar to the respective clusters, and harboured *bla*$_{NDM-1}$, *bla*$_{CMY-4}$, *bla*$_{OXA-10}$, *aph(3')-VI*, *armA*, *dfrA14* and *sul1* genes, known to confer resistance to clinically relevant antibiotics (Figure 6). The isolate from the United States also carried ciprofloxacin resistance *qnrD2* gene.

PstCluster-C, PstCluster-D and PstCluster-E had a distance of 27, 21 and 21 ADs, respectively, from PstCluster-004, from the European investigation into spread of *P. stuartii* related to medical transfers from Ukraine [5], which involved four isolates (two from Germany and two from the Netherlands) (Figure 6). Additionally, two public domain isolates (one from Ireland, SAMEA10468702, and one from the United Kingdom, SAMN24019195) and one isolate from Romania (SAMEA115949662) clustered within PstCluster-004. For one German and one Dutch isolate from this cluster, there was related information on hospitalisation of the respective patients in Hungary. All these isolates carried *bla*$_{NDM-1}$, *bla*$_{CMY-4}$, *bla*$_{OXA-10}$, *aph(3')-VI*, *armA*, *dfrA14* and *sul1* genes (Figure 6).

In the immediate surroundings of the clade with PstCluster-C, PstCluster-D and PstCluster-E as well as PstCluster-004, we identified a clade containing seven isolates from Bulgaria and one isolate each from France (312C4), the Netherlands (RIVM_C016999) and Switzerland (3347685), the latter from a patient transferred from a hospital in North Macedonia [14]), with a distance of approximately 80 ADs in the phylogenetic tree (Figure 6). Within this clade, six isolates originating from three different hospitals in Bulgaria and isolates from France and the Netherlands formed a multi-country multi-hospital cluster (BGCluster) of VIM-86-producing *P. stuartii* [15,16] also harbouring *bla*$_{CMY-4}$, *aac(6')-Il* and *sul2* (Figure 6). Four isolates from Bulgaria within this cluster also carried the *armA* gene. Of those, two were isolated from the same hospital and carried *bla*$_{NDM-1}$. Other genes detected among some isolates of this cluster were *qnrB9*, *dfrA12*, and *sul1*.

The main difference in resistome between the *bla*$_{NDM-1}$ clusters involving isolates from Romanian hospitals (PstCluster-C, PstCluster-D and PstCluster-E) and international clusters with isolates linked to Ukraine (PstCluster-001 and PstCluster-002) were redundancy of RNA methylase genes *armA* and *rmtC* leading to resistance to all aminoglycosides. Most of the isolates in the Ukraine-related cluster (PstCluster-003), characterised by the detection of the chromosomally encoded *bla*$_{NDM-5}$ gene, also carried the *armA* gene.

Figure 6. Phylogenetic tree of *Providencia stuartii* isolates belonging to the ST46 lineage and carrying carbapenemase genes, Romania and other countries (n=83 isolates), 2015–2023



AD – allelic difference; ST – sequence type. Inexact match: <100% coverage and/or <100% identity.

Discussion

This report describes the results of an epidemiological and genomic study in six hospitals in Romania to determine the extent of the spread of *P. stuartii* carrying bla_{NDM-1}. Most of the 74 submitted *P. stuartii* isolates were resistant to penicillins, cephalosporins, carbapenems, amikacin and ciprofloxacin and therefore multidrug-resistant [17]. In addition, *P. stuartii* isolates are intrinsically resistant to gentamicin, tobramycin, colistin and the activity of tigecycline is considered insufficient [7]. A treatment option unaffected by NDM production would be aztreonam. However, most of the isolates in this study co-carried bla_{CMY} genes that can confer resistance to aztreonam [18], even though phenotypic resistance to aztreonam was only present in 42.4% of tested isolates. Other remaining treatment options would be cefiderocol and aztreonam-avibactam, however, AST for these antimicrobials was not performed in this study. The high number of isolates associated with infections shows the clinical relevance of the detected multidrug-resistant *P. stuartii* isolates.

Among 74 submitted isolates for this study, we found four multi-hospital clusters including recent isolates that were detected over the duration of more than a year, pointing to ongoing and sustained spread of *P. stuartii* within the healthcare system in Romania. Clusters included isolates from up to four hospitals and three regions indicating interregional spread. The shape of the phylogenetic tree, with several clusters being part of distinct clades, indicates that *P. stuartii* carrying bla_{NDM-1} has already been circulating and diversifying over a longer time in the healthcare system in Romania. This is confirmed by the reports of a previous study of NDM-1-producing *P. stuartii* including 77 isolates collected in Romania from January 2016 to September 2017 [6] and the detection of a NDM-1-producing *P. stuartii* isolate in a burn patient transferred to the Netherlands following the fire in the Colectiv nightclub in Bucharest in 2015 [19], thus indicating that *P. stuartii* carrying bla_{NDM-1} was already circulating.

P. stuartii isolates carrying bla_{NDM-1} have been reported from countries on other continents, including Afghanistan [20] Bangladesh [21], Brazil [22], Canada [23], Egypt [24], India [25,26], Peru [27], and the US [28] indicating global distribution. While outbreaks of NDM-1-producing *P. stuartii* have previously been described [8], there had not been solid evidence of sustained healthcare-associated transmission before this study. We also found that the behaviour of the MBL gene-carrying *P. stuartii* ST46 lineage, including its single locus variants (ST50 and ST373), involving various multi-hospital clusters described above, appears to be similar to the behaviour of carbapenemase-producing *K. pneumoniae* high-risk clones.

The following criteria have been proposed to determine international multidrug-resistant high-risk clones:

- a global distribution;
- an association with various antimicrobial resistance determinants;
- the ability to colonise and persist in hosts for long intervals (>6 months);
- the ability for effective transmission among hosts;
- enhanced pathogenicity and fitness;
- the ability to cause severe and/or recurrent infections [29],

Although the exact definition for some of these features remains unclear [30].

Reviewing the current evidence related to these criteria for the *P. stuartii* ST46 lineage (including ST50 and ST373) carrying MBL genes, and for clade A, shows that they fulfil many of these criteria as described in the next paragraph.

The *P. stuartii* ST46 lineage carrying MBL genes was detected in nine countries (Bulgaria, France, Germany, Ireland, the Netherlands, Romania, Switzerland, the United Kingdom and the United States). Isolates belonging to this lineage carry a large set of resistance determinants as described above. The lineage shows sustained healthcare-associated and interregional transmission in Romania for more than a year which implies that *P. stuartii* were able to colonise the hosts and transmit between them (from person to person). A high number of isolates in this study were also associated with infections. Clade A had similar features albeit with less evidence related to the criteria for high-risk clones outlined above. Clade A was detected in two countries (Italy and Romania), involving three hospitals in Romania with sustained spread over a year and the documented outbreak in Italy [8]. Isolates in clade A are also highly drug-resistant due to carriage of various resistance determinants and include isolates associated with infections.

Although *P. stuartii* carrying bla_{NDM-1} initially received attention in EU/EEA countries after its detection related to patient transfers from Ukraine [5], there is evidence for wider dissemination in countries in Eastern Europe and the Balkan region including Bulgaria [15], Greece [31], Hungary (described epidemiological links) [5], North Macedonia (described epidemiological link) [14], Romania (this study) and Serbia (described epidemiological link) [32]. Although there is limited data at this stage, the *P. stuartii* ST46 lineage (including ST50 and ST373) carrying MBL genes, and to a lesser extent clade A, seem to have many characteristics of international multidrug-resistant high-risk clones. However, further assessment is currently hampered by the limited availability of genomic, microbiological and epidemiological data on *P. stuartii*. In addition, our results and those of other investigations may be biased due to their focus on carbapenem- or multidrug-resistant isolates.

Systematic surveillance on the extent of spread of carbapenem-resistant *P. stuartii* in the EU/EEA and adjacent countries is urgently needed. Further studies of *P. stuartii*, for example, related to duration of carriage, pathogenicity and population structure would also be required to determine its capacity to develop and sustain high-risk clones. However, from a public health perspective, current evidence regarding the spread of *P. stuartii*, especially including the sustained transmission in the healthcare system in Romania, is sufficient for being alert to the potential of *P. stuartii* to further disseminate throughout healthcare systems in the EU/EEA and about possible adverse consequences for patients. In the hospitals participating in this study, the number of *P. stuartii* isolates carrying $bla_{\text{NDM-1}}$ declined in 2024 compared with previous years. It remains unclear if this decline can be attributed to enhanced infection prevention and control (IPC) measures. Still, the evidence for sustained healthcare-associated spread of *P. stuartii* isolates carrying $bla_{\text{NDM-1}}$ outlined in this report argues for instituting enhanced IPC measures in line with international guidance [33,34] as soon as cases are detected in healthcare facilities.

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