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Outbreak of NDM-1-producing *Acinetobacter baumannii* in France, January to May 2013

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We report the first outbreak of carbapenem-resistant NDM-1-producing *Acinetobacter baumannii* in Europe, in a French intensive-care unit in January to May 2013. The index patient was transferred from Algeria and led to the infection/colonisation of five additional patients. Concurrently, another imported case from Algeria was identified. The seven isolates were genetically indistinguishable, belonging to ST85. The bla_{NDM-1} carbapenemase gene was part of the chromosomally located composite transposon Tn*125*. This report underscores the growing concern about the spread of NDM-1-producing *A. baumannii* in Europe.

Background

The emergence and spread of New-Delhi metallo-beta-lactamase (NDM)-producing Gram negative isolates constitutes a new wave of multidrug-resistant (MDR) bacteria [1]. First identified from *Enterobacteriaceae*, the bla_{NDM} gene has since been identified in non-fermenting bacterial species such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [2,3]. Considering its ability to be the source of nosocomial outbreaks, carbapenem-resistant *A. baumannii* (AB) represents a threat for critically ill hospitalised patients [4]. We report here the first outbreak of NDM-1-producing AB in Europe, which occurred in a French surgical intensive-care unit in January to May 2013.

Outbreak description

The index case (Patient 1) was a female patient in her early 80s suffering from end-stage cirrhosis. She originated from Algeria but lived mostly in France. During a stay in Algeria in December 2012, she was admitted into a private hospital in the city of Tizi Ouzou following renal failure, which required dialysis. After one month in hospital, she was repatriated to France due to liver decompensation. On 18 January 2013, she was admitted to a 15-bed surgical intensive-care unit of a tertiary care university hospital in a Paris suburb. In accordance with local and national policy, she was screened on admission for carriage of MDR bacteria. Rectal screening revealed MDR-*A. baumannii* (MDR-AB) (Isolate 1) that was susceptible only to amikacin, netilmicin and colistin (Table). The same day, she was intubated for respiratory failure. Protected distal bronchial brushing yielded a culture of MDR-AB with the same antibiotic resistance profile. A combination of intravenous tigecycline and amikacin was given. On 24 January, she developed multivisceral failure and died four days later. During the following days, three additional patients with MDR-AB infection and/or colonisation were identified in the same unit. A cirrhotic male patient in his mid-60s (Patient 2) – who had been hospitalised since 3 January 2013 and confirmed free of MDR bacteria on admission – developed a ventilator-associated pneumonia on 26 January. Culture of a distal protected specimen yielded MDR-AB (Isolate 2). This patient was successfully treated by a combination of tigecycline and amikacin and was extubated two days later. Patient 3 was a male liver-transplant patient in his mid-60s who was not colonised on admission but developed a dialysis catheter-related bloodstream infection due to a MDR-AB on 28 January (Isolate 3). Imipenem and amikacin combination was prescribed but the patient died of haemorrhagic shock before antibacterial susceptibility results could be obtained. Patient 4 was a dual renal- and liver-transplant female patient in her late 40s from whom an abdominal drain yielded an MDR-AB culture on 2 February (Isolate 4). This patient recovered without receiving any antibiotic therapy and was discharged from the hospital on 11 February.

Two weeks after the admission of the index case, a woman in her early 80s (Patient 5) suffered from a cerebrovascular accident and was repatriated from the

Table

Antimicrobial susceptibility of carbapenem-resistant NDM-1-producing *Acinetobacter baumannii* isolates, France, January–May 2013 (n=7)

Antibiotic	Isolates MIC [µg/mL] (S/I/R[a])[b]						
	1	2	3	4	5	6	7
Ampicillin-sulbactam	16	24	16	24	24	32	192
Ticarcillin-clavulanic acid	›256	›256	›256	›256	›256	›256	›256
Piperacillin	›256	›256	›256	›256	›256	›256	›256
Piperacillin-tazobactam	›256	›256	›256	›256	›256	›256	›256
Aztreonam	›256	›256	›256	›256	›256	›256	192
Ceftazidime	›256	›256	›256	›256	›256	›256	›256
Cefepime	›256	›256	›256	›256	›256	›256	›256
Meropenem	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)
Imipenem	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)
Imipenem/imipenem + EDTA ratio[b]	96	96	64	128	96	64	128
Doripenem	›32 (R)	›32 (R)	›32 (R	›32 (R)	›32 (R)	›32 (R)	›32 (R)
Ciprofloxacin	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)
Gentamicin	32 (R)	24 (R)	24 (R)	32 (R)	24 (R)	32 (R)	64 (R)
Amikacin	8 (S)	12 (I)	8 (S)	8 (S)	8 (S)	8 (S)	64 (R)
Tobramycin	24 (R)	32 (R)	24 (R)	24 (R)	24 (R)	32 (R)	64 (R)
Netilmicin	0.75 (S)	1.5 (S)	0.5 (S)	0.75 (S)	0.75 (S)	0.75 (S)	1 (S)
Tetracycline	4	2	2	2	2	2	2
Tigecycline	0.75	1	1	1	1	0.25	0.38
Colistin	0.125 (S)	0.19 (S)	0.38 (S)	0.25 (S)	0.38 (S)	0.25 (S)	0.38 (S)
Trimethoprim-sulfamethoxazole	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)	›32 (R)
Fosfomycin	256	384	384	192	256	256	192
Rifampicin	6	6	6	6	6	32	8

MIC: mimimum inhibitory concentration; NDM: New-Delhi metallo-beta-lactamase.

[a] Susceptible/Intermediary Resistant/Resistant categories from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [5], if determined.

[b] Except for the imipenem/imipenem + EDTA ratio. The ratio was considered significant if ›4.

same Algerian county that Patient 1 was repatriated from (but from a different healthcare facility) to the emergency unit of our hospital. A screening test performed on admission identified MDR-AB (Isolate 5).

Two months after this first cluster of five patients with MDR-AB, two additional patients free of MDR bacteria on admission to the surgical intensive-care unit described acquired a MDR-AB during their stay in this unit. A woman in her late 50s (Patient 6) was admitted to the surgical intensive-care unit on 6 April and placed in the room where the index case had stayed. This patient was found positive for MDR-AB on 15 April in specimens from a catheter and the respiratory tract (Isolate 6). She was treated with intravenous tigecycline and aerosolised colistin. She underwent successful liver transplantation on 22 April and recovered well. The last patient (Patient 7) was a man in his late 50s admitted to the surgical intensive-care unit on 3 April for a liver transplant and from whom a rectal swab yielded MDR-AB a month later (Isolate 7). The patients' duration of hospital stay, time of infection and/or colonisation and location in the hospital are reported in Figure 1.

Laboratory analysis

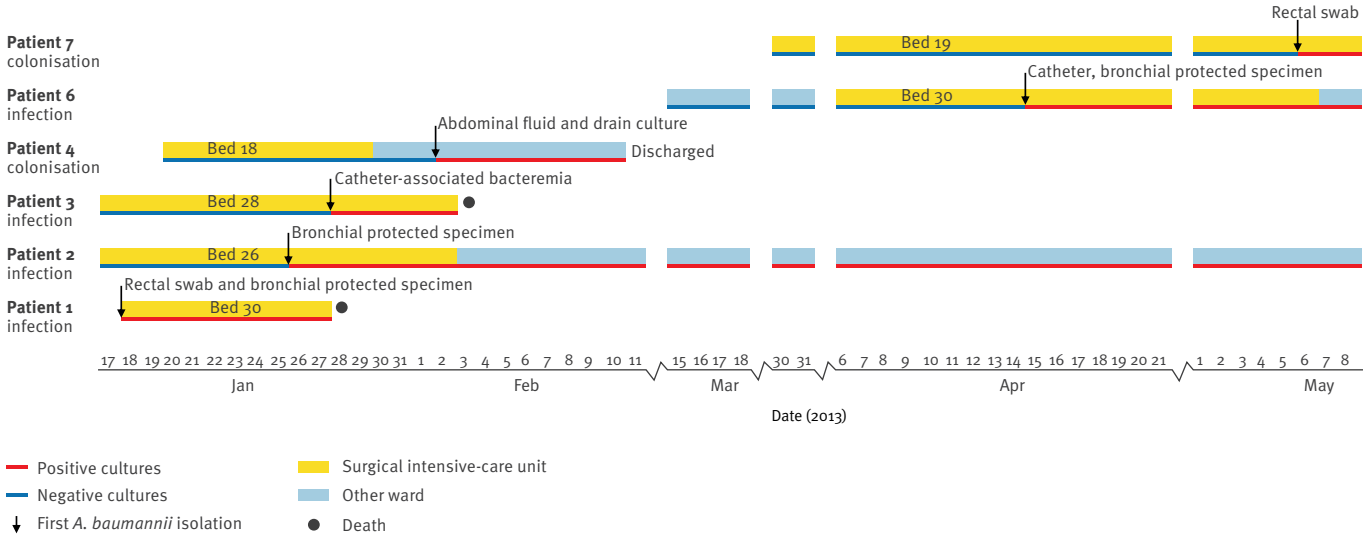
Identification of the seven MDR-AB strains at the species level was confirmed by 16S RNA sequencing (data not shown). Their antimicrobial susceptibilities were tested by minimum inhibitory concentration (MIC) determination (Etest, bioMérieux, France) (Table) and interpreted according to EUCAST guidelines [5]. All isolates exhibited a high level of resistance to penicillins, broad-spectrum cephalosporins, carbapenems, fluoroquinolones and trimethoprim-sulfamethoxazole. Those isolates remained susceptible only to netilmicin, colistin and amikacin. The production of a class B carbapenemase was suspected by the positive results of the imipenem/imipenem plus EDTA test using MIC double strips (Etest, bioMérieux, France) (Table 1) and confirmed by UV spectrophotometry [6]. Carbapenemase genes were screened by PCR as described and the bla_{NDM-1} gene was amplified in the seven isolates [7]. Genotypic comparison by pulsed-field gel electrophoresis using restriction enzyme *Sma*I revealed an indistinguishable profile (data not shown). Diversilab (bioMérieux, France) analysis and multilocus sequence typing (MLST) typing confirmed that these isolates were clonally related and belonged to the same sequence type, ST85 (Figure 2) [8]. The genetic environment of bla_{NDM-1} was investigated as previously described [8] and showed that it was located in the composite transposon Tn*125* made of two copies of insertion sequence (IS) ISAba*125*.

Discussion

Carbapenem-resistant *A. baumannii* are a source of deep concern due to their multidrug resistance pattern and the ability of this bacterial species to persist in the environment [4,9,10]. Intensive-care units are particularly susceptible to outbreaks associated with

Figure 1

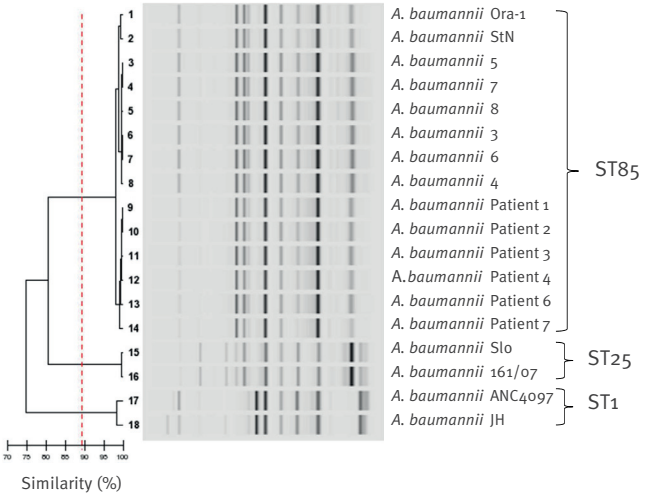
Timeline of patients infected/colonised with carbapenem-resistant NDM-1-producing *Acinetobacter baumannii* hospitalised in a surgical intensive-care unit, Créteil, France, January–May 2013 (n=6)



NDM: New-Delhi metallo-beta-lactamase.

Figure 2

Results of Diversilab and multilocus sequence typing analysis of isolates from patients infected/colonised with carbapenem-resistant NDM-1-producing *Acinetobacter baumannii* hospitalised in a surgical intensive-care unit, Créteil, France, January–May 2013 (n=6)



NDM: New-Delhi metallo-beta-lactamase.

The isolates from the hospitalised patients were compared with a collection of characterised strains [8,17]. A similarity line (89.4%) shows the cut-off to separate different clones.

MDR-AB: it is sometimes difficult for them to adhere strictly to infection control measures when patients require a high and persistent care-load. Four years ago, the same hospital faced a hospital-wide outbreak of MDR-AB colonisations and infections due to the importation of an index case from Tahiti [11]. Despite this experience and the implementation in 2010 at the national and local level of strict measures on hospital admission to detect, screen and place under contact-isolation precautions repatriated patients, another outbreak linked to the admission of a patient previously hospitalised abroad again occurred [12].

Since 2010, NDM-producing MDR-AB has been identified in various parts of the world, in particular in North Africa and the Middle East [8,13-17]. A series of imported cases have been identified recently in Europe, such as in the Czech Republic, Germany, Slovenia, Switzerland and Belgium [8,13,14,17]. In France, the emergence of an NDM-1-producing MDR-AB strain originating from North Africa was recently highlighted [15,17]. We describe here the first outbreak associated with the importation of this NDM-1-producing *A. baumannii* clone ST85 in Europe. This report underlines the need for dedicated measures for patients previously treated in a hospital located in a 'high risk' geographical area. Such measures (e.g. screening for colonisation/infection with MDR organisms and isolation nursing) should be maintained until the screening for colonisation/infection (e.g. using rectal, throat and wound swabs) has shown that these patients are free of MDR organisms. Because of intermittent carriage or lack of sensitivity of the current culture-based screening methods, repeated specimen collection and

molecular-based methods of detection may help to control such outbreaks.

Taking in account the relationship between North African countries and many European countries, it is possible that the spread of NDM-1 carbapenemase may occur rapidly, mostly through *A. baumannii* rather than Enterobacteriacae, since *A. baumannii* may become much more difficult to eradicate.

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Conflict of interest

None declared.

Authors' contributions

Jean-Winoc Decousser: laboratory work, manuscript preparation. Chloé Jansen: infection control, manuscript preparation. Aurélie Emirian: laboratory and clinical work. Rémy Bonnin: laboratory work. Leslie Anais: laboratory work. Jean-Claude Merle: clinical work. Patrice Nordmann: manuscript preparation, analysis of data. Laurent Poirel: manuscript preparation, analysis of data.

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First report of IMI-1-producing colistin-resistant *Enterobacter* clinical isolate in Ireland, March 2013

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We report the first case in Ireland of an IMI-1 carbapenemase-producing *Enterobacter asburiae*, which was resistant to both colistin and fosfomycin. The circumstances under which this isolate was acquired were unclear. Several reports of IMI-producing *Enterobacter* spp. have emerged in recent years, and colistin resistance in *Enterobacteriaceae* is also increasingly reported. Laboratories should be aware of the unusual antibiograms of IMI-producing isolates.

In late March 2013, a patient was admitted to the Mid-Western Regional Hospital, Limerick, Ireland with fractured ribs. She had not been hospitalised in the previous 24 months; her last hospital stay had been in December 2010.During the admission in 2013, she received a five-day course of amoxicillin-clavulanate for an *Escherichia coli* urinary tract infection, and routine rectal screening for gastrointestinal carriage of carbapenemase-producing *Enterobacteriaceae* (CPE) was performed in accordance with the surveillance and infection control policies of the hospital. Carbapenem-resistant *Enterobacteriaceae* were isolated from the culture of the rectal swab. The isolate was identified as *Enterobacter asburiae* using matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (BrukerDaltonics, Bremen, Germany) and was designated ME52 in this report.

Antimicrobial susceptibility testing using disc diffusion and gradient minimal inhibitory concentration (MIC)(Etest, BioMerieux, Basingstoke, United Kingdom) methods showed the isolate ME52 to be resistant to amoxicillin-clavulanate, cefoxitin, ertapenem and meropenem, but susceptible to cefotaxime, ceftazidime, and piperacillin-tazobactam. Among the non-beta-lactam agents, it was susceptible to ciprofloxacin, aminoglycosides and tigecycline. The isolate was resistant to colistin and fosfomycin according to interpretive criteria from the European Committee on Antimicrobial Susceptibility Testing (EUCAST), with MICs of 96 mg/L and 64mg/L, respectively [1]. Synergy testing of meropenem with the beta-lactamase inhibitorsboronic acid, dipicolinic acid, and cloxacillin was performed (RoscoDiagnostica, Taastrup, Denmark). Significant potentiation of the meropenem inhibitory zone was observed in the presence of boronic acid, but not with dipicolinic acid or cloxacillin, implying the presence of an Ambler class Acarbapenemase. Real-time PCR for various carbapenemase genes was performed in the Department of Medical Microbiology in Galway University Hospitals, and *bla* genes for KPC, GES, NDM, VIM, IMP, and OXA-48-like carbapenemases

Table

Antimicrobial susceptibility results of the IMI-producing *Enterobacter asburiae* ME52 isolated in the Mid-Western Regional Hospital, Limerick, Ireland, March 2013

Antimicrobial agent	MIC (mg/L)	Susceptibility[a]
Ampicillin	› 64	R
Amoxicillin-clavulanate	64	R
Piperacillin-tazobactam	2	S
Cefotaxime	1	S
Ceftazidime	1	S
Cefoxitin	› 64	R
Aztreonam	0.25	S
Ertapenem	16	R
Imipenem	64	R
Meropenem	16	R
Ciprofloxacin	≤ 0.125	S
Gentamicin	1	S
Tobramycin	1	S
Amikacin	2	S
Tigecycline	0.5	S
Fosfomycin	64	R
Colistin	› 32	R

MIC: minimum inhibitory concentration; R, resistant; S, susceptible.

[a] Based on EUCAST interpretive criteria [1].

were not detected. The isolate was subsequently referred to Public Health England (PHE) Colindale, London, United Kingdom (UK), for further investigation of the mechanism of carbapenem resistance. PCR identified the presence of bla_{IMI} in ME52. Nucleotide sequencing confirmed the carbapenemase to be IMI-1. MICs by agar dilution also confirmed susceptibility to third-generation cephalosporins and piperacillin-tazobactam, as well as resistance to carbapenems and colistin. The Table shows the antimicrobial susceptibility profile (MICs) of the isolate.

On further review, the patient had never received either colistin or fosfomycin therapy in the past. She had travelled in Europe during the past 15 years including France and Italy, but not to the American continent where the first isolates had been reported [2,3]. The only aquatic exposure of note was a visit to the River Jordan in Israel 10 years ago. In the current hospitalisation, the patient made an uneventful recovery and was discharged home.

Discussion

This is the first report in Ireland of an IMI carbapenemase-producing *Enterobacter* clinical isolate, coupled with the phenotype of colistin and fosfomycin resistance. It seems that the isolation of ME52 was a chance finding and the period of rectal colonisation by the patient was unknown. The clinical significance of the patient's travel history and aquatic exposure with respect to the acquisition of the IMI-producing *E. asburiae* is unclear.

IMI enzymes, together with another closely related beta-lactamase NMC-A, are found in *Enterobacter* spp. and form a relatively uncommon group within the Ambler class A carbapenemases [4]. The chromosomally located IMI-1 enzyme was first reported in 1996 in two *Enterobacter cloacae* isolates in the United States (US) [2]. Subsequently, plasmid-mediated IMI-2 carbapenemase was detected in clonally related environmental *E. asburiae* isolates recovered from seven of 16 rivers in the mid-western regions of the US [3], as well as in an *E. cloacae* clinical isolate in China [5]. While IMI enzymes are relatively uncommon carbapenemases, their presence in *Enterobacter* clinical isolates have been reported in recent years in France, Finland and Singapore [6-9]. They consist mainly of *E. cloacae* isolates producing either the IMI-1 or IMI-2 enzyme. Apart from our current report, IMI-producing *E. asburiae* clinical isolates have also been found in three patients from different cities in France between 2007 and 2011 [9].

To date, the common feature with IMI-producing isolates of the *E. cloacae* complex is the retention of susceptibility to third-generation cephalosporins such as cefotaxime and ceftazidime, while being resistant to the carbapenems, particularly imipenem. Additionally, IMI-producing *E. asburiae* isolates also retain susceptibility to piperacillin-tazobactam, as shown in the antibiograms of our isolate as well as of those isolated from US rivers from 1999 to 2001 [3].

The finding of a colistin-resistant *Enterobacter* isolate in a patient without a history of polymyxin therapy is unusual and unexpected. Unlike certain *Enterobacteriaceae* such as Proteaeor Serratia spp., *Enterobacter* spp. do not possess intrinsic resistance to colistin [10]. Acquired colistin resistance in *Enterobacteriaceae* has mainly been reported in Klebsiella pneumoniae, particularly multidrug-resistant clones producing carbapenemases such as KPC enzymes [11-13]. Prior colistin therapy has been documented in some patients, but acquisition of such colistin- and carbapenem-resistant strains in other patients is likely to be the result of cross-transmission in healthcare settings [11-13]. However, a recent study has found unexpectedly high rates of colistin resistance amongst non-multidrug-resistant *E. cloacae* complex isolates from the UK and Ireland [14]. Colistin resistance rates of 6% and 10% were found in blood and respiratory isolates, respectively [14]. Fosfomycin is another useful agent for the treatment of multidrug-resistant (MDR) *Enterobacteriaceae* [15]. However, fosfomycin susceptibility rates of *Enterobacter* spp. were lower than those of *E. coli* or *K. pneumoniae* [15,16]. Based on EUCAST interpretive criteria, fosfomycin susceptibility rates ranged from 47% to 72% in *E. cloacae* [16,17]; while one third of E. *asburiae* isolates (seven of 21) were resistant to fosfomycin in one European study [17]. Notably, our patient had not received colistin or fosfomycin therapy in the past.

Conclusion

This is the first report in Ireland of IMI-producing *E.asburiae* with co-resistance to colistin and fosfomycin. For the accurate detection of IMI-producing *Enterobacteriaceae*, laboratories should be awareof the unusual antimicrobial resistance profiles of such isolates, particularly if synergy test results with beta-lactamaseinhibitors suggest the presence of a class A carbapenemase. In the era of mounting antimicrobial resistance and diminishing therapeutic options, laboratories should monitor trends in colistin and fosfomycin resistance amongst *Enterobacteriaceae* isolates, particularly in *Enterobacter* spp.

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Conflict of interest

None declared.

Authors' contributions

Teck-Wee Boo prepared the first and subsequent drafts of the manuscript and collated the clinical and laboratory data. Nuala O'Connell, Margaret O'Connor and Lorraine Power provided the clinical and epidemiological data; while Nuala O'Connell, Joanne King, Elaine McGrath, Robert Hill, Katie Hopkins and Neil Woodford provided relevant sections of laboratory data. All authors read and critically revised the first, subsequent and final drafts of the manuscript.

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Intercontinental spread of OXA-48 beta-lactamase-producing *Enterobacteriaceae* over a 11-year period, 2001 to 2011

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OXA-48 beta-lactamase producers are emerging as an important threat mostly in the Mediterranean area. We report here the molecular epidemiology of a collection of OXA-48 beta-lactamase-positive enterobacterial isolates (n=107) recovered from European and north-African countries between January 2001 and December 2011. This collection included 67 *Klebsiella pneumoniae*, 24 *Escherichia coli* and 10 *Enterobacter cloacae*. Using the EUCAST breakpoints, ninety-eight isolates (91.6%) were of intermediate susceptibility or resistant to ertapenem, whereas 66% remained susceptible to imipenem. Seventy-five per cent of the isolates co-produced an extended-spectrum beta-lactamase, most frequently CTX-M-15 (77.5%). Susceptibility testing to non-beta-lactam antibiotics showed that colistin, tigecycline, amikacin, and fosfomycin remain active against most of the isolates. Multilocus sequence typing indicated that the most common sequence types (ST) were ST101 and ST38 for *K. pneumoniae* and *E. coli*, respectively. The bla_{OXA-48} gene was located on a 62 kb IncL/M plasmid in 92.5% of the isolates, indicating that a single plasmid was mainly responsible for the spread of that gene. In addition, this study identified multiple cases of importation of OXA-48 beta-lactamase producers at least in Europe, and spread of OXA-48 beta-lactamase producers giving rise to an endemic situation, at least in France.

Introduction

Currently, an emergence of carbapenem resistance in *Enterobacteriaceae* is reported, mostly related to the spread of carbapenemases [1]. Those carbapenem-hydrolysing beta-lactamases belong to the Ambler class A (e.g. KPC), class B (e.g. IMP, VIM and NDM) [1], and class D (e.g. OXA-48 and its variants possessing weaker but significant carbapenemase activity) [2]. OXA-48 had first been identified from a clinical *Klebsiella pneumoniae* isolate recovered in Istanbul, Turkey, in 2001 [3]. The corresponding gene, namely bla_{OXA-48}, was then also identified in *Escherichia coli* and *Citrobacter freundii*, still in Turkey [4]. For several years, OXA-48 was identified only in Turkey, and almost all OXA-48 beta-lactamase producers were reported from patients hospitalised in Turkey or with a link to that country [4,5]. Since 2008, this gene has been identified in many other countries, most often in *K. pneumoniae* isolates [2,5-10]. OXA-48 is now identified in the Middle East and in North African countries, and those countries are considered as reservoirs of OXA-48 beta-lactamase producers [2]. In addition to sporadic cases, an increasing number of outbreaks due to OXA-48-producing *K. pneumoniae* are currently observed, not only in Turkey but also in Belgium, France, Greece, the Netherlands and Spain [2,11-13]. *K. pneumoniae* strains belonging to specific sequence types (ST), such as ST395 and ST101, have been involved in those outbreaks [12,14].

In order to gain further understanding of that phenomenon, our study aimed at comparing the genetic features of OXA-48 beta-lactamase-producing strains recovered from various countries by analysing an existing collection of 107 bla_{OXA-48}-positive enterobacterial isolates. The genetic context and the location of the bla_{OXA-48} gene were investigated, as well as resistance to broad-spectrum beta-lactams and non-beta-lactam antibiotics.

Methods

Bacterial isolates

A total of 107 OXA-48 beta-lactamase-producing enterobacterial isolates were investigated retrospectively. Enterobacteriacae producing OXA-48-like beta-lactamases were not included in this study. All isolates had been recovered from clinical specimens except a single isolate (one *Serratia marcescens* strain from an environmental water sample in Morocco), and had been received between January 2001 and December 2011 in our National Reference Laboratory which is also used as an International Reference Laboratory by many colleagues worldwide who send us their isolates for

further characterisation. Of identical strains in an outbreak, only one was included in this work. The distribution of clinical samples was as follows: rectal swabs (n=33), urine samples (n=24), blood samples (n=12), wound samples (n=7), respiratory specimens (n=4), catheters (n=4), bone specimens (n=2), peritoneal fluids (n=2), and placenta specimen (n=1). One sample per patient was included. Detailed information could not be obtained for 18 clinical samples. The isolates were identified to species level using the API 20E system (bioMérieux, La Balme-les-Grottes, France).

Susceptibility testing

Routine antibiograms were determined by disk diffusion method on Mueller-Hinton (MH) agar (Bio-Rad, Marnes-la-Coquette, France) and interpreted using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (updated 2012) and of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for tigecycline and colistin [15,16]. In addition, MICs were determined for imipenem, meropenem, ertapenem, cefotaxime, and ceftazidime using E-test (bioMérieux, La Balme-les-Grottes, France). The production of extended-spectrum beta-lactamases (ESBL) was evidenced by a double-disk synergy test performed with cefepime, ceftazidime, and ticarcillin/clavulanic acid disks [17] and more recently by using the rapid ESBL NDP test [18].

PCR and sequencing of beta-lactamase-encoding genes

Whole-cell DNA was extracted using the QiaAmp DNA minikit and following the manufacturer's recommendations (Qiagen, Courtaboeuf, France). All isolates were screened by PCR for the Ambler class A and B carbapenemase-encoding genes bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM} [19-20]. For each isolate, the bla_{OXA-48} gene was amplified using primers preOXA-48A and preOXA-48B, and subsequently sequenced [21]. Detection of other beta-lactamase genes such as bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{AmpC}-like, and bla_{OXA-1} was performed with internal primers, as described previously [19,22]. PCR products were analysed on agarose gel and sequenced by using the amplification primers with an automated sequencer (ABI PRISM 3100; Applied Biosystems). The nucleotide and deduced protein sequences were analysed using software from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Strain typing

Multilocus sequence typing (MLST) with seven housekeeping genes (*rpoB, gapA, mdh, pgi, phoE, infB* and *tonB*) was performed for *K. pneumoniae* isolates according to Diancourt et al. [23]. Allele sequences and STs were verified at http://pubmlst.org/Kpneumoniae. Fragments of seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) were amplified and sequenced for *E. coli* isolates as described on the following website http://mlst.ucc.ie/mlst/dbs/Ecoli. A different allele number was given to each distinct sequence within a locus, and a distinct ST number was attributed to each distinct combination of alleles. *E. coli* isolates were assigned to the major *E. coli* phylogenetic groups (A, B1, B2, and D) by multiplex PCR, as described [24]. The genetic relationship between the *Enterobacter cloacae* isolates was studied using Diversilab, a semi-automated typing system based on repetitive sequence-based PCR (rep-PCR) following the manufacturer's instructions (bioMérieux).

Plasmid DNA analysis, transformation and mating-out assays

Plasmid DNA was extracted from the isolates using the Kieser technique [25]. *E. coli* NCTC50192, harbouring four plasmids of 154, 66, 48 and 7 kb, was used as plasmid size marker. Plasmid DNAs were analysed by agarose gel electrophoresis. Direct transfer of the carbapenem resistance markers was attempted by liquid mating-out assays at 37°C, using *E. coli* J53 as recipient, or by electrotransformation of plasmid DNA, using *E. coli* TOP10 as recipient as reported [3,4]. Selection was performed on agar plates supplemented with ertapenem (0.5 µg/ml) and azide (100 µg/ml) for mating-out assays. In order to search for a possible chromosomal location of the bla_{OXA-48} gene in *E. coli* isolates 19 to 24, restriction with endonuclease I-*CeuI* followed by pulsed-field gel electrophoresis (PFGE) analysis was performed as described [26].

Replicon and transposon typing

PCR-based replicon typing (PBRT) of the main plasmid incompatibility groups reported in *Enterobacteriaceae* was performed as described [27] and using the specific primers designed from plasmid pOXA-48a [28]. Genetic structures surrounding the bla_{OXA-48} gene were determined according to the Tn*1999*-like PCR-mapping scheme as described [29].

Results

Bacterial isolates

A total of 107 isolates were studied, including *K. pneumoniae* (n=67), *E. coli* (n=24), and *E. cloacae* (n=10) (Table 1). Other enterobacterial species were identified: *Citrobacter koseri* (n=2), *C. freundii* (n=1), *Klebsiella oxytoca* (n=1), *Providencia rettgeri* (n=1), and *S. marcescens* (n=1). They had been isolated in France (n=61), Morocco (n=22), Turkey (n=11), Egypt (n=3), Lebanon (n=2), Tunisia (n=2), Switzerland (n=2), South Africa (n=2), Belgium (n=1), and the Netherlands (n=1), respectively (Table 1). Among the 61 strains collected in France, 30 had a history of international travel to the following countries: Morocco (n=14), Tunisia (n=2), Libya (n=5), Algeria (n=4), Egypt (n=3), Senegal (n=1), and Kuwait (n=1). In 12 cases, no travel history from a foreign country was identified. For the remaining 19 cases, no precise travel information could be obtained (Table 1).

Table 1A

Genetic features associated with OXA-48 beta-lactamase producers, 2001–11 (n=107)

Species		Country of isolation	Travel history	beta-lactams MICs (µg/mL)					Sequence type	Genetic location of bla_{OXA-48}	Incompatibility group of bla_{OXA-48}-positive plasmid	Non-beta-lactam-associated resistances	Associated beta-lactam resistance determinants[a]	Phylogenetic group	Transposon bearing bla_{OXA-48}
				ERT	IMP	MER	CAZ	CTX							
Enterobacter cloacae	1	France	Morocco	8	1	1	64	>256	ND	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
E. cloacae	2	France	?	>32	1	1.5	32	>256	ND	Plasmidic	IncL/M	Q, Tm, Ak, TET, TGC, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
E. cloacae	3	France	None	4	0.5	0.5	>256	>256	ND	Plasmidic	IncL/M	Q, Ami, TET, TGC, Cm, Fos, SXT, FT	CTX-M-15, TEM-1, OXA-1 DHA-7, SHV-12	ND	Tn*1999.2*
E. cloacae	4	France	Algeria	3	0.75	0.38	48	>256	ND	Plasmidic	IncL/M	FOS	CTX-M-15	ND	Tn*1999.4*
E. cloacae	5	France	Morocco	16	1	0.75	32	>256	ND	Plasmidic	IncL/M	Q, Gm, Tm, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
E. cloacae	6	France	Morocco	1.5	0.5	0.25	16	>256	ND	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.1*
E. cloacae	7	Morocco	None	1	0.5	0.38	0.5	8	ND	Plasmidic	IncL/M	Ami, TET, SXT, FT	CTX-M-9, TEM-1	ND	Tn*1999.2*
E. cloacae	8	Morocco	None	1	0.5	0.38	48	16	ND	Plasmidic	IncL/M	OFX, Ami, TET, SXT, FT	CTX-M-9, TEM-1, SHV-12	ND	Tn*1999.2*
E. cloacae	9	Morocco	None	0.75	0.38	0.19	8	128	ND	Plasmidic	IncL/M	Gm, Tm, TET, Cm, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
E. cloacae	10	Morocco	None	6	1	1	48	16	ND	Plasmidic	IncL/M	OFX, Gm, Tm, Cm, SXT, FT	TEM-1, DHA-7, SHV-12	ND	Tn*1999.2*
C. koseri	11	France	?	2	0.38	0.38	0.38	2	ND	Plasmidic	IncL/M	None	None	ND	Tn*1999.2*
C. koseri	12	France	?	2	0.75	0.38	2	2	ND	Plasmidic	IncL/M	None	None	ND	Tn*1999.2*
Klebsiella oxytoca	13	Morocco	None	4	1	0.5	256	256	ND	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT	CTX-M-15, TEM-1	ND	Tn*1999.2*
Citrobacter freundii	14	France	None	4	0.75	0.38	64	12	ND	Plasmidic	IncL/M	Q, Gm, Tm, TET, TGC, Cm, SXT	SHV-12, TEM-1	ND	Tn*1999.2*
Providencia rettgeri	15	Turkey	None	>32	>32	>32	32	16	ND	Plasmidic	Inc A/C	Q, Gm, Tm, TET, Cm, SXT, FT	TEM-101	ND	Tn*1999.1*
Serratia marcescens	16	Morocco	None	>32	8	4	1	3	ND	Plasmidic	IncL/M	Q, TET, Cm, SXT, FT	OXA-1	ND	Tn*1999.1*
E. coli	17	France	Algeria	0.5	0.38	0.12	16	>256	10	Plasmidic	IncL/M	Q, SXT	CTX-M-15, TEM-1	A	Tn*1999.4*

Ak: amikacin; Ami: aminoglycosides; Cm: chloramphenicol; Cs: colistin; △Tn*1999*: truncated transposon Tn*1999*; FOS: fosfomycin; FT: nitrofurantoin; Gm gentamicin; MIC: minimum inhibitory concentration; ND: not determinable; OFX: ofloxacin; Q: fluoroquinolones; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; TGC: tigecycline; Tm: tobramycin.

[a] Resistance markers being co-harboured by the bla_{OXA-48}-carrying plasmid are underlined.

Table 1B

Genetic features associated with OXA-48 beta-lactamase producers, 2001–11 (n=107)

Species		Country of isolation	Travel history	beta-lactams MICs (µg/mL)					Sequence type	Genetic location of bla_{OXA-48}	Incompatibility group of bla_{OXA-48}-positive plasmid	Non-beta-lactam-associated resistances	Associated beta-lactam resistance determinants[a]	Phylogenetic group	Transposon bearing bla_{OXA-48}
				ERT	IMP	MER	CAZ	CTX							
E. coli	18	France	Libya	1	0.5	0.19	›256	32	10	Plasmidic	IncL/M	Ak, Tm, TET, Cm, SXT	CMY-2, VEB-8, TEM-1	A	Tn*1999.2*
E. coli	19	France	Egypt	3	0.5	0.19	0.5	24	38	Chromosomic	ND	OFX, Gm, Tm, SXT	CTX-M-24, TEM-1	D	△Tn*1999.2*
E. coli	20	France	Turkey	3	0.75	0.38	0.75	24	38	Chromosomic	ND	OFX, Gm, Tm, SXT	CTX-M-24, TEM-1	D	△Tn*1999.2*
E. coli	21	France	Egypt	0.5	0.25	0.19	0.5	24	38	Chromosomic	ND	OFX, Gm, Tm, SXT	CTX-M-24, TEM-1	D	△Tn*1999.2*
E. coli	22	France	None	1	0.38	0.25	0.5	24	38	Chromosomic	ND	OFX, Gm, Tm, SXT	CTX-M-24, TEM-1	D	△Tn*1999.2*
E. coli	23	Switzerland	?	8	0.75	1	1.5	48	38	Chromosomic	ND	OFX, Gm, Tm, SXT	CTX-M-24, TEM-1	D	△Tn*1999.2*
E. coli	24	Egypt	None	2	0.5	0.25	0.5	24	38	Chromosomic	ND	OFX, Gm, Tm, SXT	CTX-M-24, TEM-1	D	△Tn*1999.2*
E. coli	25	Lebanon	None	2	0.75	0.25	1.5	48	38	Plasmidic	IncL/M	SXT	CTX-M-14, TEM-1	D	Tn*1999.2*
E. coli	26	France	?	0.5	0.38	0.12	0.12	0.09	46	Plasmidic	IncL/M	Q, TET, SXT	TEM-1	A	Tn*1999.2*
E. coli	27	France	Egypt	0.5	0.5	0.19	8	›256	69	Plasmidic	IncL/M	TET, SXT	CTX-M-15, TEM-1	D	Tn*1999.2*
E. coli	28	France	None	0.75	0.5	0.25	0.12	0.38	95	Plasmidic	IncL/M	None	TEM-1	B2	Tn*1999.2*
E. coli	29	Egypt	None	›32	32	›32	›256	256	101	Plasmidic	IncL/M	Q, Ami, TET, Cm, SXT	TEM-1, VIM-1, CMY-4	B1	Tn*1999.1*
E. coli	30	France	?	3	1.5	0.5	0.19	1	362	Plasmidic	IncL/M	TET, SXT	TEM-1	D	Tn*1999.2*
E. coli	31	France	Morocco	2	0.38	0.25	0.5	0.5	410	Plasmidic	IncL/M	Q, TET	None	A	Tn*1999.2*
E. coli	32	France	?	0.75	0.38	0.19	16	128	617	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT	CTX-M-15, OXA-1	A	Tn*1999.2*
E. coli	33	France	?	0.75	0.38	0.19	8	64	617	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT	CTX-M-15, OXA-1	A	Tn*1999.2*
E. coli	34	Turkey	None	24	1.5	12	16	192	648	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT	CTX-M-15, TEM-1, OXA-1	D	Tn*1999.1*
E. coli	35	Turkey	None	›32	1.5	12	16	256	648	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT	CTX-M-15, TEM-1, OXA-1	D	Tn*1999.1*
E. coli	36	France	Morocco	1.5	0.38	0.25	24	›256	746	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT	CTX-M-15, TEM-1, OXA-1	A	Tn*1999.1*
E. coli	37	France	?	1.5	0.5	0.19	24	256	963	Plasmidic	Inc F	None	CTX-M-15, TEM-1	D	△Tn*1999.1*
E. coli	38	France	?	1	0.5	0.19	0.12	0.75	1092	Plasmidic	IncL/M	None	None	B2	Tn*1999.2*
E. coli	39	France	None	1	0.5	0.19	1.5	24	2969	Plasmidic	IncL/M	TET	CTX-M-15	D	Tn*1999.2*

Ak: amikacin; Ami: aminoglycosides; Cm: chloramphenicol; Cs: colistin; △Tn*1999*: truncated transposon Tn*1999*; FOS: fosfomycin; FT: nitrofurantoin; Gm gentamicin; MIC: minimum inhibitory concentration; ND: not determinable; OFX: ofloxacin; Q: fluoroquinolones; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; TGC: tigecycline; Tm: tobramycin.

[a] Resistance markers being co-harboured by the bla_{OXA-48}-carrying plasmid are underlined.

Table 1c

Genetic features associated with OXA-48 beta-lactamase producers, 2001–11 (n=107)

Species		Country of isolation	Travel history	beta-lactams MICs (µg/mL)					Sequence type	Genetic location of bla_{OXA-48}	Incompatibility group of bla_{OXA-48}-positive plasmid	Non-beta-lactam-associated resistances	Associated beta-lactam resistance determinants[a]	Phylogenetic group	Transposon bearing bla_{OXA-48}
				ERT	IMP	MER	CAZ	CTX							
E. coli	40	France	Morocco	0.75	0.5	0.19	1	16	2969	Plasmidic	IncL/M	TET	CTX-M-15	D	Tn*1999.2*
K. pneumoniae	41	Morocco	None	6	0.75	0.75	192	256	11	Plasmidic	IncL/M	Q, Ami, TET, Cm, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	42	Turkey	None	›32	›32	›32	›256	64	14	Plasmidic	IncL/M	Q, Ami, Cm, SXT	SHV-2a , TEM-1, OXA-47	ND	Tn*1999.1*
K. pneumoniae	43	Turkey	None	›32	2	4	256	48	14	Plasmidic	IncL/M	Q, Ak, Tm, FT	SHV-12, TEM-1, OXA-1	ND	Tn*1999.1*
K. pneumoniae	44	Turkey	None	›32	›32	›32	›256	48	14	Plasmidic	IncL/M	Q, Ami, Cm, FOS	OXA-1, TEM-1, SHV-12	ND	Tn*1999.1*
K. pneumoniae	45	Egypt	None	1.5	2	0.75	0.19	1.5	14	Plasmidic	IncL/M	FT	OXA-1	ND	Tn*1999.2*
K. pneumoniae	46	France	?	›32	8	6	1	3	15	Plasmidic	IncL/M	Q, Tm, TET, TGC, Cm, FOS, SXT, FT	TEM-1	ND	Tn*1999.2*
K. pneumoniae	47	France	Morocco	2	0.5	0.5	192	›256	15	Plasmidic	IncL/M	Q, Gm, Tm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	48	France	Morocco	2	1	0.5	48	128	15	Plasmidic	IncL/M	Q, Gm, Tm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	49	Morocco	None	12	1	2	8	6	15	Plasmidic	IncL/M	Q, Ami, TET, TGC, Cm, FOS, SXT, FT	DHA-1, TEM-1	ND	Tn*1999.2*
K. pneumoniae	50	Morocoo	None	›32	›32	›32	256	›256	15	Plasmidic	IncL/M	Q, Gm, Tm, TET, FOS, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.1*
K. pneumoniae	51	Morocco	None	12	1	2	8	6	15	Plasmidic	IncL/M	Q, Ami, TET, TGC, Cm, FOS, SXT, FT	DHA-1, TEM-1	ND	Tn*1999.2*
K. pneumoniae	52	Morocco	None	2	0.38	0.5	24	›256	15	Plasmidic	IncL/M	Q, Gm, Tm, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	53	Turkey	None	2	0.5	0.5	64	›256	16	Plasmidic	IncL/M	Q, Tm, Ak, TET, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	54	Morocco	None	0.38	0.38	0.5	12	96	25	Plasmidic	IncL/M	Q, Gm, Tm, TET, TGC, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	55	Morocco	None	0.38	0.38	0.5	12	96	25	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.1*
K. pneumoniae	56	France	Koweit	›32	32	24	1.5	2	29	Plasmidic	IncL/M	OFX, TET, TGC, Cm, FOS, SXT, FT	None	ND	Tn*1999.2*

Ak: amikacin; Ami: aminoglycosides; Cm: chloramphenicol; Cs: colistin; △Tn*1999*: truncated transposon Tn*1999*; FOS: fosfomycin; FT: nitrofurantoin; Gm gentamicin; MIC: minimum inhibitory concentration; ND: not determinable; OFX: ofloxacin; Q: fluoroquinolones; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; TGC: tigecycline; Tm: tobramycin.

[a] Resistance markers being co-harboured by the bla_{OXA-48}-carrying plasmid are underlined.

Table 1D

Genetic features associated with OXA-48 beta-lactamase producers, 2001–11 (n=107)

Species		Country of isolation	Travel history	beta-lactams MICs (µg/mL)					Sequence type	Genetic location of bla_{OXA-48}	Incompatibility group of bla_{OXA-48}-positive plasmid	Non-beta-lactam-associated resistances	Associated beta-lactam resistance determinants[a]	Phylogenetic group	Transposon bearing bla_{OXA-48}
				ERT	IMP	MER	CAZ	CTX							
K. pneumoniae	57	France	Morocco	0.5	0.38	0.25	0.19	0.38	35	Plasmidic	IncL/M	None	None	ND	Tn*1999.2*
K. pneumoniae	58	France	None	0.75	0.5	0.25	0.09	0.38	37	Plasmidic	IncL/M	TET, FT	None	ND	Tn*1999.2*
K. pneumoniae	59	France	?	1	0.5	0.25	0.12	0.5	45	Plasmidic	IncL/M	None	TEM-1	ND	Tn*1999.2*
K. pneumoniae	60	France	?	1	0.5	0.25	0.12	0.25	45	Plasmidic	IncL/M	FOS	TEM-1	ND	Tn*1999.2*
K. pneumoniae	61	France	?	0.75	0.5	0.25	0.19	0.25	45	Plasmidic	IncL/M	None	TEM-1	ND	Tn*1999.2*
K. pneumoniae	62	France	None	4	4	1	0.5	0.5	45	Plasmidic	IncL/M	TET, FOS	None	ND	Tn*1999.2*
K. pneumoniae	63	Tunisia	None	>32	1.5	12	48	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	64	France	?	>32	24	16	32	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	67	Tunisia	None	>32	2	8	>256	>256	101	Plasmidic	IncL/M	Ami, TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	68	France	None	3	0.5	0.38	48	96	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	69	Switzerland	?	6	0.5	0.75	48	>256	101	Plasmidic	IncL/M	Q, Tm, Ak, TET, SXT, FT, TGC	CTX-M-15, OXA-1	ND	Tn*1999.2*
K. pneumoniae	70	France	Morocco	>32	3	16	192	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	71	France	None	4	0.5	0.5	48	128	101	Plasmidic	IncL/M	Q, Tm, SXT, FT	CTX-M-15, OXA-1	ND	Tn*1999.2*
K. pneumoniae	72	France	Libya	>32	3	8	>256	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	73	France	Libya	>32	>32	>32	>256	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	74	South Africa	?	>32	3	8	>256	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, TGC, Cm, SXT, FT	CTX-M-15, OXA-1	ND	Tn*1999.2*
K. pneumoniae	75	South Africa	?	>32	3	8	>256	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cs, SXT, FT	CTX-M-15, OXA-1	ND	Tn*1999.2*
K. pneumoniae	76	Morocco	None	>32	>32	16	192	>256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, FOS, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	77	Morocco	None	>32	>32	>32	256	>256	101	Plasmidic	IncL/M	Q, Tm, Ak, TET, FOS, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*

Ak: amikacin; Ami: aminoglycosides; Cm: chloramphenicol; Cs: colistin; △Tn*1999*: truncated transposon Tn*1999*; FOS: fosfomycin; FT: nitrofurantoin; Gm gentamicin; MIC: minimum inhibitory concentration; ND: not determinable; OFX: ofloxacin; Q: fluoroquinolones; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; TGC: tigecycline; Tm: tobramycin.

[a] Resistance markers being co-harboured by the bla_{OXA-48}-carrying plasmid are underlined.

Table 1E

Genetic features associated with OXA-48 beta-lactamase producers, 2001–11 (n=107)

Species		Country of isolation	Travel history	beta-lactams MICs (µg/mL)					Sequence type	Genetic location of bla_{OXA-48}	Incompatibility group of bla_{OXA-48}-positive plasmid	Non-beta-lactam-associated resistances	Associated beta-lactam resistance determinants[a]	Phylogenetic group	Transposon bearing bla_{OXA-48}
				ERT	IMP	MER	CAZ	CTX							
K. pneumoniae	78	Morocco	None	2	0.38	0.5	192	256	101	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	79	Morocco	None	2	0.38	0.5	>256	>256	101	Plasmidic	IncL/M	Q, Tm, Ak, TET, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	80	Belgium	None	4	1	1	1	0.5	147	Plasmidic	IncL/M	Q, TET, TGC, Cm, SXT	None	ND	Tn*1999.2*
K. pneumoniae	81	Turkey	None	>32	32	8	96	>256	147	Plasmidic	IncL/M	Q, Tm, Ak, TET, TGC, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	82	France	Tunisia	3	0.38	0.38	64	>256	147	Plasmidic	IncL/M	Q, Gm, Tm, TET, TGC, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	83	France	?	2	0.38	0.38	96	192	147	Plasmidic	IncL/M	Q, Tm, Ak, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	84	France	Libya	3	1.5	3	>256	>256	147	Plasmidic	IncL/M	Q, Gm, Tm, Ak, TET, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1, CMY-2	ND	Tn*1999.2*
K. pneumoniae	85	France	Libya	>32	3	8	>256	>256	147	Plasmidic	IncL/M	Q, Ami, TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1, CMY-2	ND	Tn*1999.2*
K. pneumoniae	86	France	?	1	0.25	0.25	48	>256	307	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	87	France	Algeria	0.5	0.38	0.19	32	192	336	Plasmidic	IncL/M	Q, Tm, Ak, SXT	CTX-M-15, OXA-1	ND	Tn*1999.2*
K. pneumoniae	88	Morocoo	None	1	0.5	0.5	8	48	392	Plasmidic	IncL/M	Q, Gm, Tm, TET, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	89	Morocco	None	12	6	3	24	>256	392	Plasmidic	IncL/M	Q, Gm, Tm TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	90	France	None	3	0.75	0.75	96	>256	395	plasmidic	IncL/M	Q, Tm, Gm, TET, FOS, SXT, FT, Cm	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	91	France	None	2	0.5	0.38	1	1	395	Plasmidic	IncL/M	Q, Tm, Ak, TET, TGC, Cm, FOS, SXT, FT	OXA-1	ND	Tn*1999.2*
K. pneumoniae	92	Netherlands	None	2	0.5	0.38	32	192	395	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	93	France	Morocco	2	0.38	0.38	256	256	395	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*

Ak: amikacin; Ami: aminoglycosides; Cm: chloramphenicol; Cs: colistin; △Tn*1999*: truncated transposon Tn*1999*; FOS: fosfomycin; FT: nitrofurantoin; Gm gentamicin; MIC: minimum inhibitory concentration; ND: not determinable; OFX: ofloxacin; Q: fluoroquinolones; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; TGC: tigecycline; Tm: tobramycin.

[a] Resistance markers being co-harboured by the bla_{OXA-48}-carrying plasmid are underlined.

Table 1f

Genetic features associated with OXA-48 beta-lactamase producers, 2001–11 (n=107)

Species		Country of isolation	Travel history	beta-lactams MICs (µg/mL)					Sequence type	Genetic location of bla_{OXA-48}	Incompatibility group of bla_{OXA-48}-positive plasmid	Non-beta-lactam-associated resistances	Associated beta-lactam resistance determinants[a]	Phylogenetic group	Transposon bearing bla_{OXA-48}
				ERT	IMP	MER	CAZ	CTX							
K. pneumoniae	94	France	Morocco	>32	24	32	128	>256	395	Plasmidic	IncL/M	Q, Gm, Tm, TET, TGC, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	95	Morocco	None	3	0.5	0.5	>256	>256	395	Plasmidic	IncL/M	Q, Gm, Tm, TET, TGC, Cm, FOS, SXT, FT	CTX-M-15, TEM-1	ND	Tn*1999.2*
K. pneumoniae	96	Morocoo	None	3	0.5	0.5	>256	>256	395	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	97	France	Morocco	1.5	3	0.25	32	96	405	Plasmidic	IncL/M	Q, Gm, Tm, TET, FOS, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.1*
K. pneumoniae	98	Lebanon	None	>32	>32	>32	0.38	3	496	Plasmidic	IncL/M	FOS, SXT, FT	None	ND	Tn*1999.2*
K. pneumoniae	99	France	Algeria	1	0.38	0.19	0.25	0.25	530	Plasmidic	IncL/M	FT	TEM-1	ND	Tn*1999.2*
K. pneumoniae	100	France	?	4	0.75	0.5	0.5	0.75	685	Plasmidic	IncL/M	FT	None	ND	Tn*1999.2*
K. pneumoniae	101	Turkey	?	0.38	0.38	0.12	0.09	0.12	981	Plasmidic	IncL/M	OFX	None	ND	Tn*1999.2*
K. pneumoniae	102	Turkey	None	>32	>32	32	32	>256	982	Plasmidic	IncL/M	OFX, Tm, TET, TGC, FOS, SXT, FT	CTX-M-15, OXA-1	ND	Tn*1999.2*
K. pneumoniae	103	Turkey	None	>32	2	6	>256	48	984	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT, FT	SHV-12, TEM-1	ND	Tn*1999.1*
K. pneumoniae	104	France	Senegal	1	0.5	0.25	12	48	985	Plasmidic	IncL/M	OFX, Tm, Gm, TET, TGC, Cm, FOS, SXT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	105	France	Morocco	3	0.5	0.38	96	256	986	Plasmidic	IncL/M	Q, Gm, Tm, TET, Cm, SXT, FT	CTX-M-15, TEM-1, OXA-1	ND	Tn*1999.2*
K. pneumoniae	106	France	?	3	0.5	0.38	1.5	0.75	987	Plasmidic	IncL/M	OFX, TET, TGC, Cm, FT	None	ND	Tn*1999.2*
K. pneumoniae	107	Morocco	None	1	0.38	0.5	12	3	1027	Plasmidic	IncL/M	Q, Ami, TET, Cm, FOS, SXT, FT	TEM-1, SHV-27, DHA-1	ND	Tn*1999.2*

Ak: amikacin; Ami: aminoglycosides; Cm: chloramphenicol; Cs: colistin; △Tn*1999*: truncated transposon Tn*1999*; FOS: fosfomycin; FT: nitrofurantoin; Gm gentamicin; MIC: minimum inhibitory concentration; ND: not determinable; OFX: ofloxacin; Q: fluoroquinolones; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; TGC: tigecycline; Tm: tobramycin.

[a] Resistance markers being co-harboured by the bla_{OXA-48}-carrying plasmid are underlined.

Table 2

Susceptibility to carbapenems of *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter cloacae* isolates, 2001–11 (n=107)

Species	Antimicrobial drug	Susceptibility Number (%) of isolates		
		Susceptible	Intermediate	Resistant
K. pneumoniae (n=67)	Imipenem	40 (60)	13 (19)	14 (21)
	Ertapenem	5 (7)	9 (13)	53 (79)
	Meropenem	40 (60)	6 (9)	21 (31)
E. coli (n=24)	Imipenem	20 (84)	3 (12)	1 (4)
	Ertapenem	4 (17)	8 (33)	12 (50)
	Meropenem	21 (88)	0 (0)	3 (12)
E. cloacae (n=10)	Imipenem	10 (100)	0 (0)	0 (0)
	Ertapenem	0 (0)	3 (30)	7 (70)
	Meropenem	9 (90)	1 (10)	0 (0)

The percentages are rounded so as to add up to 100%.

The results in this Table are from E-tests.

Susceptibility to carbapenems and broad-spectrum cephalosporins

Results of susceptibility testing are shown in Tables 2 and 3. According to the CLSI guidelines, 40 (60%) *K. pneumoniae* isolates, 20 (83%) *E. coli* isolates, and 10 (100%) *E. cloacae* isolates were susceptible to imipenem (Table 2). In addition, 40 (60%) *K. pneumoniae*, 21 (88%) *E. coli*, and 9 (90%) *E. cloacae* isolates were susceptible to meropenem. By contrast, 62 (92%) *K. pneumoniae*, 20 (83%) *E. coli*, and 10 (100%) *E. cloacae* isolates were found of intermediate susceptibility or resistant to ertapenem (Table 2). Regarding the broad-spectrum cephalosporins, 73 (68%) and 90 (84%) isolates were resistant or of intermediate susceptibility to ceftazidime and cefotaxime, respectively (Table 1 and 3).

Beta-lactamase genes

Among the 107 OXA-48-producing isolates, 80 (75%) co-produced an ESBL. A $bla_{\text{CTX-M}}$-like gene was detected in 71 (66%) of the isolates (89% of the ESBL-producing isolates). Among the different CTX-M variants identified, those belonging to the CTX-M-1 and CTX-M-9 groups accounted for 87.5% (n=62) and 12.5% (n=9), respectively. CTX-M-15 was the only representative of the CTX-M-1 group. In the CTX-M-9 group, the $bla_{\text{CTX-M-14}}$ was identified in a single *E. coli* (Table 1). Two *E. cloacae* isolates harboured a $bla_{\text{CTX-M-9}}$ gene and 6 *E. coli* isolates harboured a $bla_{\text{CTX-M-24}}$ gene. The other ESBL determinants were SHV-2a (one *K. pneumoniae*), SHV-12 (three *K. pneumoniae*, two *E. cloacae*, and one *C. freundii*), SHV-27 (one *K. pneumoniae*) and TEM-101 (*P. rettgeri* isolate no. 15). Among the SHV-12-producing isolates, one *E. cloacae* co-produced CTX-M-9 (Table 1). In addition, a novel VEB variant, namely VEB-8, was identified in a single *E. coli* isolate from Libya that co-produced CMY-2 (*E. coli* 18). VEB-8 differed from VEB-5 by a single amino acid substitution (GenBank accession number JX679208) [30,31]. It is interesting to note that ESBLs were not related to date or geographic area of isolation.

Furthermore, nine isolates (8.5%) co-produced a plasmid-mediated AmpC-type beta-lactamase. Four isolates (3.8%) produced a CMY-type beta-lactamase, namely CMY-4 in a single *E. coli* isolate from Egypt and CMY-2 in three isolates (a single *E. coli* and two *K. pneumoniae* isolates from Libya). Five isolates (5%) produced a DHA-like AmpC, namely DHA-1 in three *K. pneumoniae* isolates and DHA-7 in two *E. cloacae* isolates. All the DHA-producing isolates originated from Morocco. A single isolate (*E. coli* 29 from Egypt) co-produced OXA-48 and another carbapenemase, namely VIM-1, in addition to CMY-4. The non-ESBL beta-lactamases TEM-1 and OXA-1 were detected in 79 (74%) and 47 (44%) isolates, respectively.

Susceptibility to non-beta-lactam antibiotics

Results of susceptibility testing for non-beta-lactam antibiotics are shown in Table 3. Four antibiotics were active against the majority of the isolates; 104 (99%) of the 107 isolates were susceptible to colistin, 90 (84.1%) to tigecycline, 83 (77.6%) to amikacin, and 77 (72%) to fosfomycin. Conversely, 84 (78.5%) of the 107 isolates were resistant to sulfamethoxazole-trimethoprim, 72 (67.3%) to tetracycline, 64 (59.8%) to ciprofloxacin, and 61 (57%) to gentamicin. Resistant isolates that produced an ESBL were mostly resistant also to non-beta-lactam antibiotics (Table 3).

Table 3

Susceptibility of the study isolates determined by disk diffusion method, 2001–11 (n=107)

Antimicrobial drug	Susceptibility Number (%) of isolates								
	Susceptible			Intermediate			Resistant		
	Total	ESBL	Non-ESBL	Total	ESBL	Non-ESBL	Total	ESBL	Non-ESBL
Ceftazidime	34 (31.8)	10 (9.4)	24 (22.4)	9 (8.4)	7 (6.5)	2 (1.9)	64 (59.8)	63 (58.9)	1 (0.9)
Cefotaxime	17 (15.9)	0 (0)	17 (15.9)	8 (7.5)	1 (1.0)	7 (6.5)	82 (76.6)	79 (73.8)	3 (2.8)
Tetracycline	34 (31.8)	21 (19.6)	13 (12.2)	1 (0.9)	1 (0.9)	0 (0)	72 (67.3)	58 (54.2)	14 (13.1)
Tigecycline	90 (84.1)	69 (64.5)	21 (19.6)	5 (4.7)	4 (3.7)	1 (1.0)	12 (11.2)	7 (6.5)	5 (4.7)
Fosfomycin	77 (72.0)	58 (54.2)	19 (17.8)	2 (1.8)	2 (1.8)	0 (0)	28 (26.2)	20 (18.7)	8 (7.5)
Sulfamethoxazol/ trimethoprim	23 (21.5)	7 (6.5)	16 (15.0)	0 (0)	0 (0)	0 (0)	84 (78.5)	73 (68.2)	11 (10.3)
Colistin	104[a] (99.0)	78 (74.3)	26 (24.7)	0 (0)	0 (0)	0 (0)	1 (1.0)	1 (1.0)	0 (0)
Ciprofloxacin	39 (36.4)	21 (19.6)	18 (16.8)	4 (3.8)	4 (3.8)	0 (0)	64 (59.8)	55 (51.4)	9 (8.4)
Amikacin	83 (77.6)	60 (56.1)	23 (21.5)	15 (14.0)	14 (13.1)	1 (0.9)	9 (8.4)	6 (5.6)	3 (2.8)
Gentamicin	43 (40.2)	19 (17.8)	24 (22.4)	3 (2.8)	3 (2.8)	0 (0)	61 (57.0)	58 (54.2)	3 (2.8)

ESBL: extended-spectrum beta-lactamases.

[a] The *Providencia rettgeri* and the *Serratia marcescens* isolates were excluded because of their natural resistance to colistin.

The percentages are rounded so as to add up to 100%.

Phylogenetic groups of the Escherichia coli isolates

More than half of *E. coli* isolates belonged to the phylogenetic group D (14 of the 24 *E. coli* isolates), seven *E. coli* isolates belonged to the phylogenetic group A, two belonged to the phylogenetic group B2, and one isolate belonged to the phylogenetic group B1 (Table 1).

Mulilocus sequence typing

The distribution of the sequence types among the *K. pneumoniae* and *E. coli* isolates is shown in Figures 1 and 2, respectively. ST101 was the most commonly observed ST for the *K. pneumoniae* isolates, accounting for 17 out of 67 isolates (25.4%), followed by ST395 and ST15 (7 isolates, 10.5%) (Figure 1). Six isolates (9%) belonged to ST147 (9%) and the other isolates to diverse STs, namely ST14 (n=4), ST45 (n=4), ST25 (n=2), ST392 (n=2), and one to other STs (Figure 1). Among the 24 OXA-48-positive *E. coli* isolates, seven belonged to ST38 (29.2%). The remaining 17 isolates belonged to STs 10, 617, 648 and 2969 (two isolates each) and to STs 46, 69, 95, 101, 362, 410, 746, 963 and 1092 (one isolate each) (Figure 2).

Since no MLST system has been developed for typing the *E. cloacae* species, these isolates were genotyped using the DiversiLab method. *E. cloacae* 7 and 9 recovered from Morocco were closely related, and *E. cloacae* 1, 2 and 5 (also from Morocco) belonged to the same cluster. The other *E. cloacae* isolates were distinct (data not shown).

Genetic location the bla_{OXA-48} gene

Using the specific primers designed from the reference plasmid pOXA-48a of *K. pneumoniae* 11978 [27] to amplify its replicase gene, 99 of the 107 isolates (92.5%) carried an IncL/M-pOXA-48a-like backbone. For the eight other isolates (*P. rettgeri* isolate no. 15, *E. coli* isolates no. 19 to 24, and *E. coli* isolate no. 37), mating-out assays were performed and transconjugants harbouring the bla_{OXA-48} gene were obtained for *P. rettgeri* isolate no. 15 and *E. coli* isolate no. 37. Plasmid DNA analysis of the two *E. coli* transconjugants revealed a single plasmid. Those two bla_{OXA-48}-positive plasmids corresponded to a ca. 150 kb IncA/C-type plasmid identified from *P. rettgeri* isolate no. 15 from Turkey and a ca. 160-kb IncF-type plasmid from an *E. coli* isolate from France. Despite repeated attempts, transconjugants or transformants were not obtained for six of the seven *E. coli* isolates belonging to ST38. Interestingly, I-*Ceu*I analysis confirmed the chromosomal location of the bla_{OXA-48} gene in those six isolates (data not shown). Furthermore, one out of the seven bla_{OXA-48}-positive ST38 *E. coli* harboured the epidemic OXA-48 IncL/M-type plasmid.

Genetic environment of the bla_{OXA-48} gene

The bla_{OXA-48} gene was flanked by two copies of IS*1999*. In 21 isolates (19.6%), the upstream copy remained intact. This structure corresponded to transposon Tn*1999*, whereas 84 isolates (78.5%) had a Tn*1999.2* transposon structure in which the IS*1999* is disrupted by insertion of an IS1R element [4]. In two isolates

Figure 1

Sequence types represented among OXA-48-producing *Klebsiella pneumoniae* isolates, 2001–11 (n=67)

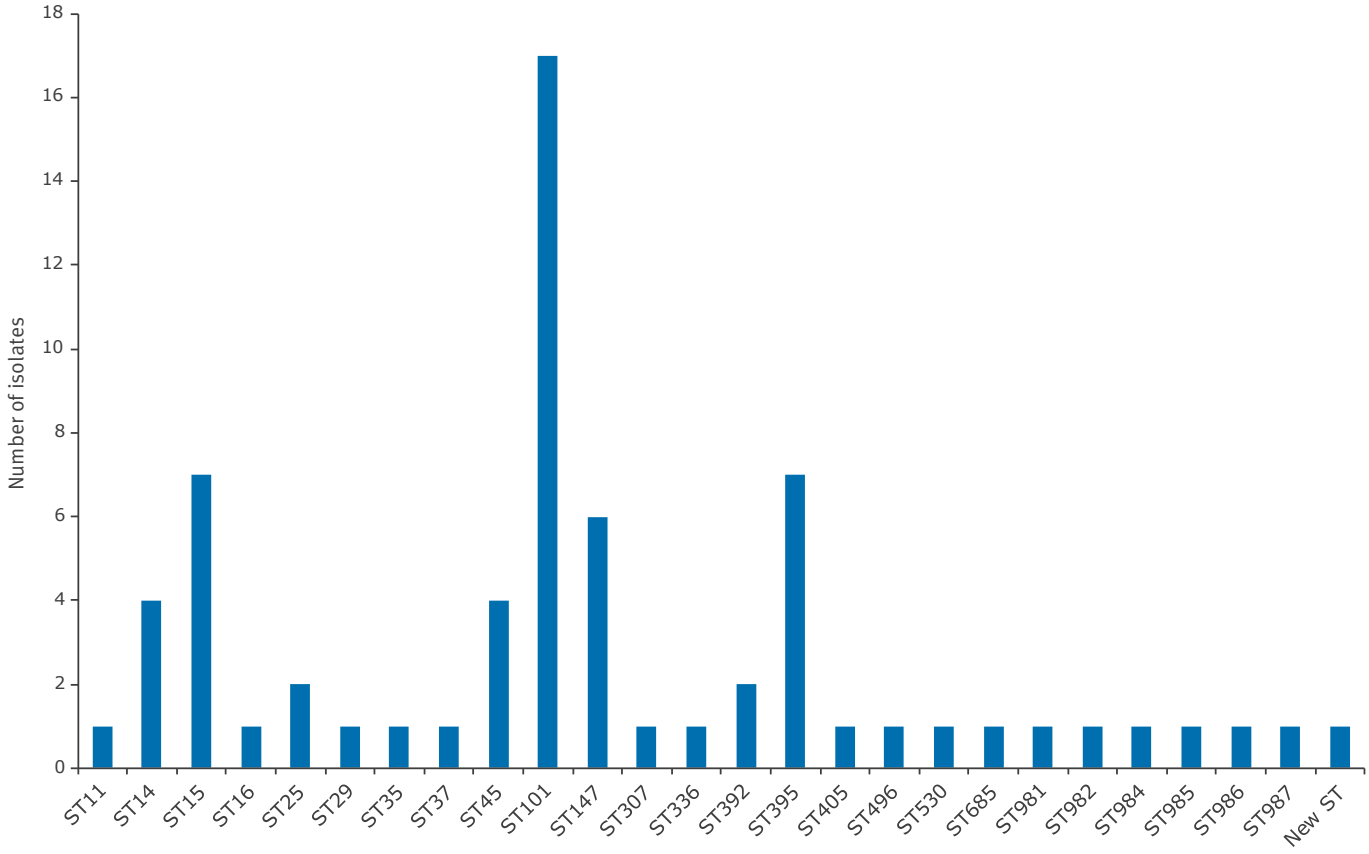
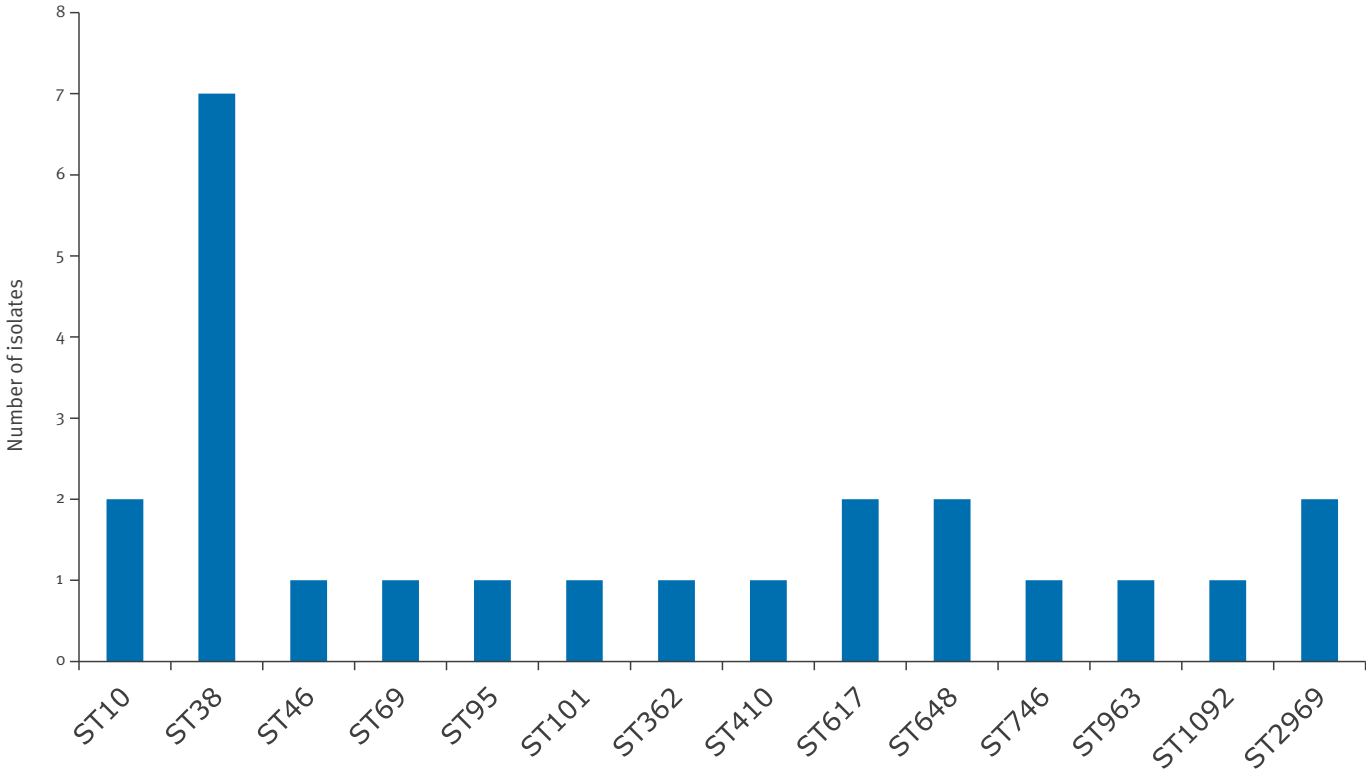


Figure 2

Sequence types represented among OXA-48-producing *Escherichia coli* isolates, 2001–11 (n=24)



(*E. cloacae* isolate no. 4 and *E. coli* isolate no. 17, recovered from the same patient), a new Tn*1999* derivative was identified. This new transposon Tn*1999.4* was composed of Tn*1999.2* disrupted by another transposon, Tn*2015* which, in turn, was composed of IS*Ecp1*, $bla_{CTX\text{-}M\text{-}15}$ and a truncated Tn*2* transposase [32].

Discussion

We have analysed here many different features of 107 known OXA-48-positive enterobacterial isolates which are widely distributed at least in several European and North African countries, and also in Turkey. Noticeably, 25% of the OXA-48 beta-lactamase producers remained susceptible to broad-spectrum cephalosporins, which therefore present possible therapeutic options. At least positive therapeutic outcomes have been obtained using an animal model of infection and broad-spectrum cephalosporins [33]. Those ESBL-negative isolates were most often susceptible to the other classes of antibiotics, which is in line with the fact that the epidemic plasmid encoding the $bla_{OXA\text{-}48}$ gene does not carry additional resistance determinants [28].

However, 75% of the OXA-48-positive isolates in our study harboured an additional ESBL-encoding gene that confers resistance to broad-spectrum cephalosporins. We have recently reported the genetic association of the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}48}$ genes on the same transposon, indicating that this combination of multidrug-resistance genes may spread further in the future [32]. In addition, most of those ESBL-producing isolates were resistant to non-beta-lactam antibiotics, due to other resistance mechanisms. It is worth mentioning that 70 isolates (65%) were susceptible to imipenem and meropenem according to CLSI guidelines, further complicating the detection of OXA-48-producing isolates in laboratories. Conversely, most isolates showed intermediate susceptibility or resistance to ertapenem. Ertapenem may thus be the most appropriate carbapenem molecule for detecting OXA-48 producers. Therefore, a selective medium containing ertapenem has recently been developed for the detection of all types of carbapenemase producers including the OXA-48 beta-lactamase producers [34]. Taking into account the fact that 75% of the OXA-48 isolates were ESBL producers and the level of resistance to non-beta-lactam molecules, treatment options for infections caused by OXA-48 beta-lactamase producers may be limited. The efficacy of carbapenems in treating infections due to OXA-48 beta-lactamase producers with susceptibility or low-level resistance to several carbapenems remains debatable, because carbapenems have been shown to be an inefficient therapy for treating mice with induced peritonitis caused by an OXA-48-producing *K. pneumoniae* [33]. Also, imipenem-containing therapy failed to treat several OXA-48 infections in humans [4,11]. A single report described imipenem as efficient treatment against bacteraemia due to an OXA-48 *K. pneumoniae* isolate [35]. Controlled trials are needed to evaluate the real clinical efficacy of carbapenems in treating infections due to OXA-48 beta-lactamase producers.

The clonal distribution of OXA-48 beta-lactamase-positive isolates is interesting because a quarter of the *K. pneumoniae* isolates belonged to ST101. OXA-48-positive *K. pneumoniae* isolates belonging to ST101 have recently been implicated in an outbreak in Spain, and have also been detected in Tunisia [11,12]. We report here that the ST101 isolates were recovered from Tunisia, Morocco, and from South Africa and France from patients who did not travel abroad, suggesting that this ST has now widely spread in European countries and in Africa. Seven *K. pneumoniae* isolates belonged to ST395, a ST implicated in clonal outbreaks in Europe [11,14]. Interestingly, we detected seven ST15 among *K. pneumoniae* isolates recovered from patients who had a link with Morocco. That sequence type corresponds to an internationally occurring clone and has been associated with different ESBL genes, but also with the metallo-beta-lactamase genes coding for NDM and VIM [36,37]. The occurrence of OXA-48 beta-lactamase in a ST15 *K. pneumoniae* isolate had been reported only once, in 2012, in an isolate from Finland [38]. Those data are likely to indicate that a novel OXA-48 *K. pneumoniae* clone belonging to ST15 may emerge in Morocco. *K. pneumoniae* isolates belonging either to ST392 or ST147 (differing at a single locus) were identified in a total of eight isolates, with the two ST392 collected in Morocco and the six ST147 collected in Belgium, Turkey and France, and also from patients originating from Tunisia or Libya. This result highlights the dissemination of another OXA-48-producing clone, mainly in the Mediterranean area. The other *K. pneumoniae* isolates belonged to diverse ST, supporting the hypothesis of the widespread dissemination of a single $bla_{OXA\text{-}48}$-positive IncL/M plasmid among various genetic backgrounds. Overall, there is no association between ST type and ESBL type among OXA-48 producers.

Among the 24 *E. coli* isolates, seven were of ST38, showing that this clone is widely disseminated, as previously suggested [7,39]. Interestingly, the $bla_{OXA\text{-}48}$ gene was chromosomally located in six of those isolates, as was speculated for the ST38 *E. coli* isolates recovered in the United Kingdom [7]. Such chromosomal location of the $bla_{OXA\text{-}48}$ gene in *E. coli* may be associated to a lower level of resistance (a single gene copy). The other 17 *E. coli* isolates were genetically distinct. Furthermore, it is interesting to note that 16 of the 24 OXA-48-positive *E. coli* belonged to phylogenetic group D or B2, which mainly include virulent strains. The *E. cloacae* isolates were overall clonally diverse.

As suggested previously, the $bla_{OXA\text{-}48}$ gene was located on a 62 kb IncL/M plasmid in most of our isolates (n=99, 92.5%), indicating that current spread of OXA-48 beta-lactamase producers is mainly related to the diffusion of this plasmid. The dissemination of the $bla_{OXA\text{-}48}$ gene is also associated with the spread of different clones.

Interestingly, 20% of OXA-48-producing isolates collected in France were considered to be autochthonous, indicating that the *bla*$_{OXA-48}$ gene has already spread in the community in France. This latter result indicates ongoing diffusion of OXA-48-type genes in Europe.

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Silent hepatitis E virus infection in Dutch blood donors, 2011 to 2012

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In Europe, the dynamics of endemic hepatitis E virus (HEV) infection remain enigmatic. We studied the presence of silent HEV infection among Dutch blood donors. Using donations collected throughout the Netherlands in 2011 and 2012, 40,176 donations were tested for HEV RNA in 459 pools of 48 or 480 donations. Deconstruction of the reactive pools identified 13 viraemic donors. In addition, 5,239 donors were tested for presence of anti-HEV IgG and IgM and for HEV RNA when IgM-positive. Of the 5,239 donations, 1,401 (27%) tested repeat-positive for HEV IgG, of which 49 (3.5%) also tested positive for anti-HEV IgM. Four of the HEV IgM-positive donors tested positive for HEV RNA. HEV IgG seroprevalence ranged from 13% among donors younger than 30 years to 43% in donors older than 60 years. The finding of 17 HEV RNA-positive donations among 45,415 donations corresponds to one HEV-positive blood donation per day in the Netherlands. For 16 of the 17 HEV RNA-positive donors, genotyping succeeded, revealing HEV genotype 3, which is circulating among Dutch pigs. Apparently, silent HEV infection is common in the Netherlands, which possibly applies to larger parts of Europe.

Introduction

Hepatitis E virus (HEV) is a non-enveloped RNA virus, classified into four genotypes. HEV genotypes 1 and 2 have been found only in humans, whereas genotypes 3 and 4 have also been found in animals. The clinical features of hepatitis E are similar to those of viral hepatitis caused by other hepatotropic viruses. In Europe and North America, hepatitis E is known as an acute disease in travellers returning from tropical countries, but an endemic source of hepatitis E has always been suspected [1]. Recently it became clear that genotype 3 of hepatitis E virus (HEV gt-3) is widely spread among pigs in Europe, North America and Japan [2,3]. Surprisingly, infection of humans by HEV gt-3 seems to cause disease mainly in immunosuppressed persons, such as patients who have received organ transplants [4,5], who may develop chronic hepatitis E [6,7]. Blood donors can be silently infected with HEV, as indicated by plasma pools testing positive for HEV RNA [8,9] and by a high prevalence of antibodies to HEV among blood donors in the south west of France [10]. Cases of transfusion-transmitted hepatitis E have been reported [11-14]. The dynamics of HEV gt-3 infection and its implications for public health and the safety of blood are largely unknown. Which part of the population has signs of resolved or active infection? To estimate the infection pressure of HEV in the Netherlands, we determined the presence of HEV RNA and HEV antibodies in a large number of recent blood donations, collected throughout the country.

Methods

Collection and selection of samples

To estimate the presence of HEV in the donor population, 417 plasma pools of 48 blood donations each (representing 20,016 donations, collected from November 2011 through January 2012); and 42 plasma pools of 480 donations each (representing 20,160 donations, collected in April and May 2012) were tested for presence of HEV RNA. All Dutch blood collection centres participated, thus all regions of the Netherlands were represented. The switch to pools of 480 was made after performing PCR on the 417 pools of 48 donations, as it appeared that the level of viraemia in recent HEV infection was sufficient to be detected in pools of 480. Plasma pools testing positive for HEV RNA were deconstructed to identify and genotype individual HEV RNA-positive donations.

In addition, plasma samples from 5,239 consenting blood donors were collected on two days in March 2011 to determine HEV IgG seroprevalence, with subsequent testing for HEV IgM and HEV RNA in IgG-positive donations. All Dutch collection centres participated, thus all regions of the Netherlands were represented. Finally, for 391 donors testing positive for HEV IgG, archived samples of blood donations collected in 2009 were

Table 1

Characteristics of blood donors testing positive for hepatitis E virus RNA, the Netherlands, 2011–2012 (n=17)

Donor			Test results of index donation				Findings in serial donations		
			Anti-HEV					Interval in days between	
Donor	Sex and age (years)	Urbanisation	IgG	IgM	HEV-RNA (IU/mL)	HEV genotype	Anti-HEV seroconversion	first and last HEV RNA-positive donation	last and first HEV RNA-negative donation
1	M 36	3	+	–	$5.2x10^3$	3	Yes	NA	NA
2	M 26	5	++	++	$5.1x10^2$	3	Yes	58	NA
3	M 54	4	–	–	$4.7x10^5$	3	NA	NA	NA
4	F 25	1	–	–	$4.9x10^2$	3	Yes	NA	NA
5	M 63	4	–	–	$2.8x10^3$	3	Yes	NA	70
6	M 50	3	–	–	$9.8x10^2$	3	NA	NA	NA
7	M 58	2	+	+	$6.8x10^2$	3	Yes	56	84
8	F 51	3	–	–	$2.7x10^5$	3	NA	NA	NA
9	F 44	4	+	–	$1.8x10^2$	NA	NA	NA	NA
10	M 69	3	–	–	$3.5x10^4$	3	Yes	56	115
11	F 57	3	–	–	$3.0x10^4$	3	NA	NA	NA
12	M 65	5	–	–	$1.4x10^4$	3	Yes	27	91
13	M 41	3	–	–	$2.2x10^3$	3	Yes	NA	63
14	M 67	2	+++	+++	$3.7x10^4$	3	Yes	NA	201
15	M 64	2	+++	+	Pos<25	3	Yes	35	105
16	M 60	2	++	+	$4.5x10^2$	3	Yes	43	86
17	M 57	4	+++	+	Pos<25	3	Yes	42	83

F: female; HEV: hepatitis E virus; M: male; NA: no sample available; –: negative; + to +++: positive.

Donors 1–13: detected by PCR testing of 40,176 donations. Donors 14–17: detected by serological testing of 5,239 donations.

The level of urbanisation ranges from 1=highly urbanised (≥2500 addresses/km²) to 5=rural (<500 addresses/km²). IgG and IgM anti-HEV signals are categorised according to measured sample–to–cut-off (S/CO) ratios, as follows: S/CO<1.0= –; 1.0≤S/CO<5.0=+; 5.0≤S/CO<10.0=++; S/CO≥10.0=+++.

retrieved for retrospective testing, to estimate the incidence of HEV infection in previous years.

Detection and genotyping of hepatitis E virus RNA

Amplification of a 74 bp fragment of HEV ORF3 was adapted from Pas et al. [7]. RNA was extracted from 0.4 mL of plasma using the QIAamp MinElute Virus Spin Kit (QIAGEN) on a nucleic acid extractor (QIAcube, QIAGEN) and eluted in 50 µL according to the manufacturer's protocol. For repository samples, 60 µl of plasma was diluted into 400 µl before extraction. MS2 phage was added prior to extraction as an internal control. Amplification used 20 µL of eluate in a 50 µL volume containing 12.5 µL of TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems), 0.2 µM HEV probe (FAM-ATTCTCAGCCCTTCGC-MGB, Applied Biosystems), and 0.6 µM of HEV forward primer (CGGTGGTTTCTGGGGTGA, Invitrogen) and 0.9 µM HEV reverse primer (GCRAAGGGRTTGGTTGG, Invitrogen). PCR was performed using a real-time PCR system (LightCycler 480-II, Roche) and standard PCR conditions. Reactions were performed in duplicate, with and without MS2 detection using MS2-specific primers and a Hex/BHQ1 labelled Taqman probe [15]. The lower limit of detection (95% cut-off) of the assay is ca. 25 IU/mL HEV RNA. HEV viral loads were calculated retrospectively from the PCR Ct values, using a calibration curve based on the first World Health Organization (WHO) International Standard for HEV RNA [16], which later became available. HEV genotyping was performed by amplification and sequencing of a 326 bp fragment of the ORF2 region [17] using AMV RT and GoTaq DNA polymerase (Promega) according to the manufacturer's instructions. If the HEV-RNA load in the index donation was too low, genotyping was performed using earlier or later samples of the donor. Sequence analysis was performed using DNAstar and Geneious software, using HEV reference sequences as described by Baylis et al. [18] and additional HEV sequences from GenBank. Genetic distances were calculated using the Tamura-Nei model, the phylogenetic tree was constructed using the neighbour-joining method. The 16 blood donor HEV sequences obtained in this study are

available in GenBank via accession codes JX645320–JX645333, JX678984 and KC223601. For comparison, HEV sequences from seven Dutch endemic hepatitis E patients were included, diagnosed in 2011 or 2012 in our laboratory. No further patient information was available. The seven patient HEV sequences are available via GenBank accession codes JX645334–JX645340.

Serological testing

Samples were screened for IgG antibodies to HEV using an anti-HEV IgG enzyme immuno assay (EIA) (Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China) [5,10,19,20]. Positive samples were tested for presence of anti-HEV IgM antibodies by an anti-HEV IgM EIA (Wantai). Positive samples were considered anti-HEV positive if found positive upon repeated testing. The assays were performed following the manufacturer's instructions. IgM-positive samples were tested for presence of HEV RNA and, if PCR-positive, were subsequently genotyped. Archived serial samples from HEV RNA-positive donors were tested to confirm seroconversion and to determine the duration of viraemia.

Statistical analysis and geographical information

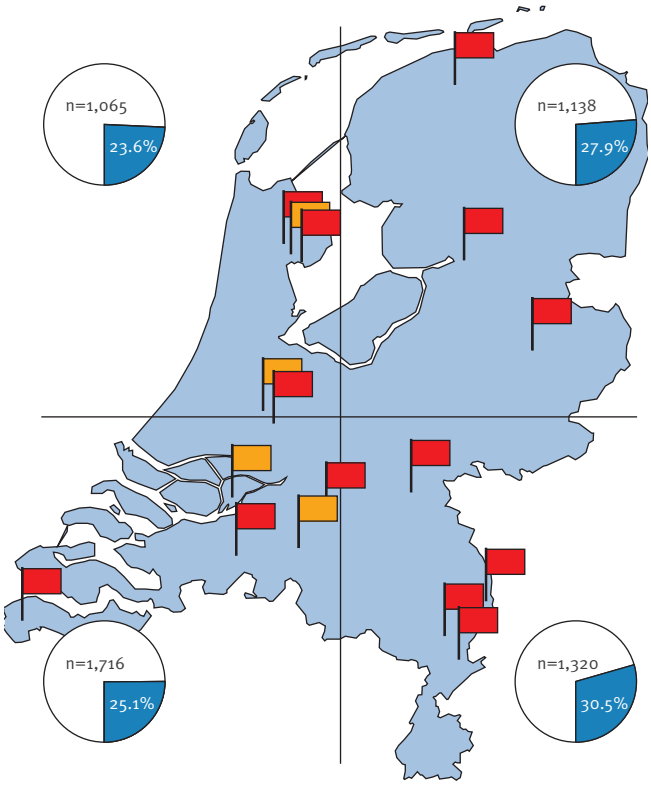
The chi-square test was used for statistical analysis. The Newcombe-Wilson method was applied to determine 95% confidence intervals (CIs). The age–seroprevalence curve was calculated using a second-degree polynomial fit. For incidence calculation, seroconversion was defined as conversion from seronegative to seropositive in subsequent donations, together with at least a threefold increase of the HEV IgG sample–to–cut-off EIA value. The degree of urbanisation for postal code areas was provided by the central bureau for statistics (CBS), using a five-point scale ranging from 1 (highly urbanised, ≥2,500 addresses/km²) to 5 (rural, <500 addresses/km²).

Results

The screening of 459 plasma pools, containing 40,176 blood donations, for presence of HEV RNA, resulted in the identification of 13 HEV viraemic donors (Donors 1 to 13 in Table 1, red flags in Figure 1). If possible, the presence of HEV infection was confirmed by detection of HEV RNA in the original plasma bag. Seven donors were detected among the 20,016 donations in pools of 48, six donors among the 20,160 donations in pools of 480. Nine of the 13 donors were in the early, seronegative phase of infection. For five of these seronegative donors a follow-up sample was available, and for three seropositive donors an earlier sample was available; all demonstrating seroconversion and confirming recent infection. An indication of the duration of viraemia was obtained as follows: In seven donors HEV was detectable in serial donations, 27 to 58 days apart (see 'first to last HEV RNA-positive donation' in Table 1). In nine donors, an HEV-negative donation was available before and after the viraemic donation, 83 to 201 days apart (see 'last to first HEV RNA-negative donation in Table 1).

Figure 1

Hepatitis E virus-infected blood donors in the Netherlands, 2011–2012 (n=17)



Flags indicate the residence of HEV RNA-positive donors (red: detected via PCR on plasma pools; orange: detected via serology). Pie diagrams show the IgG anti-HEV seroprevalence in the four quadrants of the country.

Screening of 5,239 donors for the presence of HEV antibodies revealed 1,401 donors who were repeat-reactive for anti-HEV IgG, resulting in a seroprevalence of 26.7% (95% CI: 25.6–28.0). Some regional variation was observed (Table 2 and Figure 1): the seroprevalence in the south-eastern part of the Netherlands (30.5%) was higher than in the rest of the country (p=0.0004), while the seroprevalence in the north-western part was lower (23.6%, p=0.009). Anti-HEV IgG seroprevalence strongly increased with age: after the age of 30 years, the seroprevalence increased linearly with 1.05% per year (Figure 2; R^2=0.98). The variation in seroprevalence in different parts of the Netherlands cannot be not explained by different age distributions of local donor populations. The overall seroprevalence in males was higher than in females (29.2 versus 23.1%), but this difference can be attributed to the higher average age of male donors (51.1 versus 45.5 years); no difference was observed when age-weighed seroprevalences were compared.

Table 2

Anti-hepatitis E virus IgG seroprevalence among blood donors, the Netherlands, 2011-2012 (n=5,239)

	Anti-HEV IgG status		Average age (years)	
	Positive/tested	Seroprevalence	IgG-positive	IgG-negative
Total	**1,401/5,239**	**26.7%**	**54.1**	**46.9**
Male	911/3,119	29.2%	55.6	49.3
Female	490/2120	23.1%	51.2	43.8
Region				
North-east	317/1,138	27.9%	53.3	47.9
South-east	402/1,320	30.5%	54.0	47.3
North-west	251/1,065	23.6%	54.7	45.8
South-west	431/1,716	25.1%	54.2	46.7
Level of urbanisation[a]				
1	196/827	23.7%	52.3	42.3
2	384/1,366	28.1%	54.8	47.0
3	263/995	26.4%	53.7	47.4
4	273/1,054	25.9%	54.6	47.7
5	263/916	28.7%	54.4	49.6

HEV: hepatitis E virus.

[a] The level of urbanisation ranges from 1=highly urbanised (≥2,500 addresses/km²) to 5=rural (<500 addresses/km²). For 81 donors, no information on the urbanisation level was available.

The anti-HEV IgG seroprevalence did not correlate with the level of urbanisation (Table 2). The lower seroprevalence in highly urban areas can be explained by the lower average age of urban donors. The HEV seroprevalence in the area with the highest density of pigs (surrounding the city of Eindhoven), was not different from the seroprevalence of the rest of the south-eastern part of the Netherlands (30.2% versus 30.6%).

The incidence of HEV infection in recent years was estimated by measuring anti-HEV IgG in earlier, archived samples collected in 2009 from the 391 donors who tested positive for anti-HEV IgG in 2011. The total time span, covered by the serial samples, was 571 years. Seventeen donors seroconverted during this period, indicating an incidence of 1.1% per person-year (95% CI: 0.65–1.7).

Forty-nine (3.5%) of the 1,401 anti-HEV IgG positive donors tested positive for IgM anti-HEV. Four of these IgM-positive donors tested positive for HEV RNA (Donors 14 to 17 in Table 1, and orange flags in Figure 1). Serial samples of all four donors showed IgM and IgG anti-HEV seroconversion, confirming recent infection.

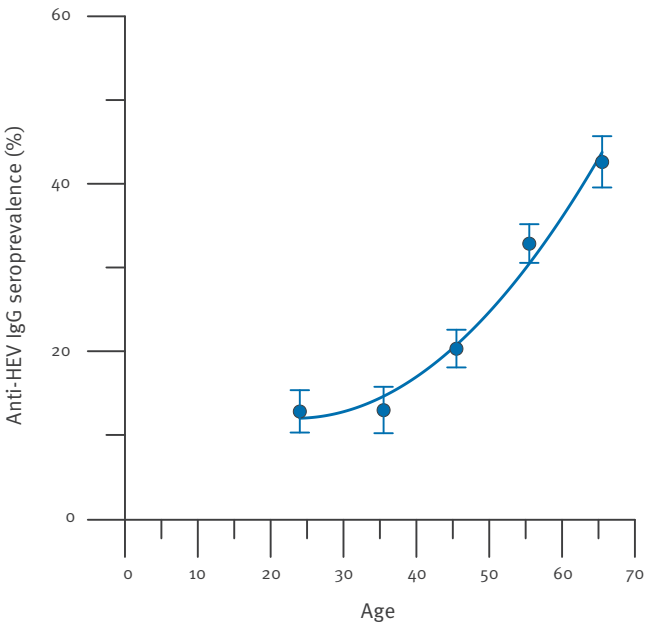
HEV viral loads in the 17 viraemic donors ranged from near the detection limit (<25 IU/mL HEV RNA) to more than 100,000 IU/mL HEV RNA. In seven donors HEV viraemia occurred in up to five serial donations, with a maximum recorded viraemic period of 58 days (Donor 2 in Table 1). In 16 of the 17 viraemic donors HEV genotyping succeeded, showing the presence of HEV genotype 3. Phylogenetic analysis of the HEV sequences suggested clustering with each other, with Dutch endemic hepatitis E patients and with HEV sequences obtained from Dutch pigs, see Figure 3.

Discussion

The detection of HEV viraemia in 17 of 45,415 recent Dutch blood donations demonstrates a high incidence of HEV infection in the Netherlands. Our serological screening suggested that roughly one quarter of the Dutch adult population experienced HEV infection. This proportion compares with the 16% of 500 donors in the south-west of Britain [19], and the 53% of 512 donors in the south-west of France [10], who recently tested positive for IgG anti-HEV, using the same antibody assay as employed in this study. Unfortunately, there is no gold standard for HEV antibody testing. A recent study reports an HEV IgG seroprevalence of only 2.6% in 7,072 Dutch samples, collected in 2006 and 2007, employing another brand of HEV antibody assay (MP/Genelabs) [21]. The Wantai assay used in the present study may suffer from a high level of non-specific reactivity. However, studies comparing the performance of the Wantai and the MP/Genelabs assay demonstrate a higher sensitivity and detection of more sera from PCR-proven cases by the Wantai assay [10,20]. Classical anti-HEV serology probably lacks sensitivity and seems unsuitable to confirm Wantai EIA test results. In addition, the frequent finding of HEV RNA-positive donors (Table 1), the striking age-related increase of HEV IgG seroprevalence (Figure 2), and the fact that already in 2005, HEV was found to circulate on 53 of 97 Dutch

Figure 2

Anti-hepatitis E virus IgG seroprevalence in 10-year age groups of blood donors, the Netherlands, 2011–2012 (n=5,239)



The first group represents donors between 18 and 29 years rather than a 10-year group. Error bars indicate the 95% confidence interval for each age group.

pig farms [2], suggest that the high seroprevalence as detected by the Wantai assay may be correct.

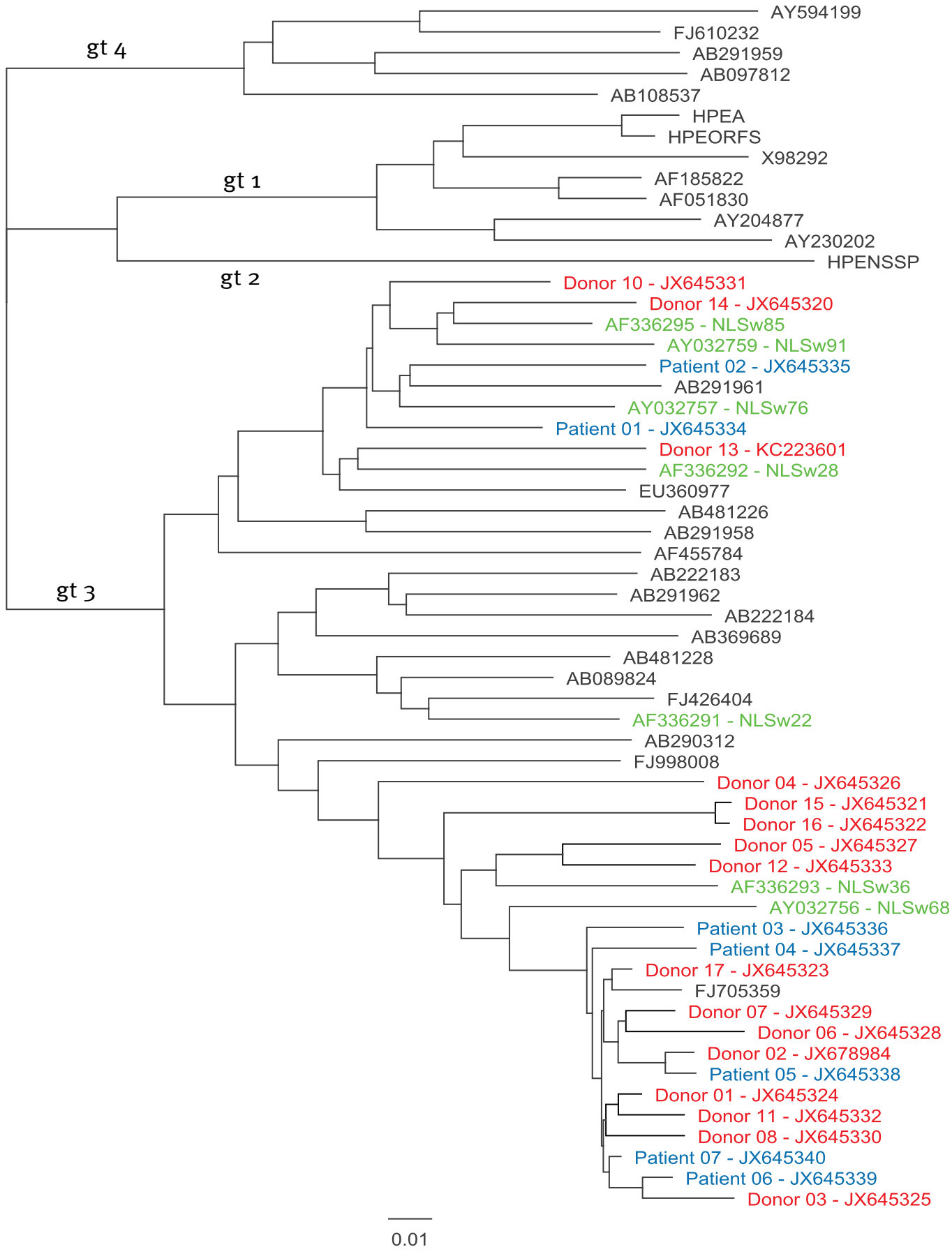
Unfortunately, seroprevalence data for persons under the age of 18 are not available. The striking age-related seroprevalence (Figure 2) is difficult to interpret. The age-dependent seroprevalence may simply reflect a long-standing, stable situation, in which people are evenly exposed to HEV in the course of their life. In that scenario, the current high number of viraemic and seroconverting donors may reflect a temporary elevation of HEV infection pressure. If endemic HEV infection were a recent phenomenon, the age-dependent seroprevalence could only be explained by age-dependent exposure or age-dependent susceptibility, which is difficult to imagine for a food-borne pathogen. Alternatively, the seroprevalence curve could reflect an age-cohort effect, caused by transient exposure of older generations in the past. Such a cohort effect has been described in England [22]. The HEV incidence found in this study of 1% per year, as well as the high number of HEV RNA-positive blood donors, seem to contradict transient exposure to HEV in the past; but endemic HEV may have returned after a long period of absence.

HEV transmission by blood transfusion has been reported in Saudi Arabia, Japan, France and the United Kingdom [11-14]. Our results suggest roughly one HEV viraemic donation per day in the Netherlands. This may be an underestimation, considering that the serological screening of 5,239 donors only detected antibody-positive donors in a later stage of infection (Donors 14 to 17 in Table 1), while the screening for HEV RNA of 40,176 pooled (diluted) donations detected donors in an early stage of infection, with high levels of HEV RNA and low or absent HEV antibodies (Donors 1 to 13). HEV RNA-positive blood may pose a threat to immunosuppressed blood recipients, such as recipients of organ transplants and patients with haematological malignancies; and possibly to pregnant women [23]. Because blood transfusion is only a minor source of HEV infection, the routine screening of blood donations for the presence of HEV does not yet seem warranted. Each immunosuppressed patient with unexplained elevated liver enzymes should be tested for the presence of HEV RNA, irrespective of exposure to blood products. Fortunately, it appears that chronic HEV infection in immunosuppressed patients can be cured by a temporary reduction of immunosuppression, or by antiviral treatment using ribavirin [5,24].

The source and transmission routes of HEV gt-3 infection have not yet been uncovered. In contrast to the Midi-Pyrénées region of France, where an association of HEV seropositivity with rural residence was found [10], the level of urbanisation and the vicinity of pig farms do not play a role in the Netherlands. Until recently, little was known of HEV transmission dynamics in European pig populations. Berto et al. describe the presence of HEV in 8 to 73% of stool samples collected from pig farms between 2007 and 2011 throughout Europe, and the presence of HEV in fattening pigs [25]. It is unclear whether pigs are the source of the current HEV infections. Intensive pig farming may have become the major amplifier of the virus, considering that millions of pigs are being reared in the Netherlands each year, of which each year again a large part probably acquires and sheds HEV. Subsequently, HEV may be spread via contaminated meat [26,27] or via faecally contaminated water used for irrigation [28]. Studies are needed to identify the transmission routes of HEV gt-3 to humans, so that appropriate measures can be taken. It seems likely that at this moment other Western countries also experience extensive, silent HEV infection.

Figure 3

Phylogenetic comparison of hepatitis E virus RNA sequences (a 326 bp fragment of ORF2), the Netherlands, 2011–2012 (n=30)



Red: Dutch blood donors; blue: Dutch hepatitis E patients; green: Dutch pigs. HEV reference strains from GenBank are shown in black. Genetic distances were calculated using the Tamura-Nei model, the phylogenetic tree was constructed using the neighbour-joining method.

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