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Detection of mcr-1 encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015

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The plasmid-mediated colistin resistance gene, *mcr-1*, was detected in an *Escherichia coli* isolate from a Danish patient with bloodstream infection and in five *E. coli* isolates from imported chicken meat. One isolate from chicken meat belonged to the epidemic spreading sequence type ST131. In addition to IncI2*, an incX4 replicon was found to be linked to *mcr-1*. This report follows a recent detection of *mcr-1* in E. coli from animals, food and humans in China.

Very recently, in November 2015, *Liu et al.* reported the finding of a transferable plasmid-mediated colistin resistance gene, *mcr-1*, detected in *Escherichia coli* isolates from animals, food and patients in China. Moreover, they found *mcr-1* in *Klebsiella pneumoniae* isolates from patients [1]. Horizontal gene transfer represents a paradigm shift in colistin resistance, which until then only was found to be mediated by chromosomal mutations and thus spread by vertical transmission.

National surveillance of antimicrobial resistance in food animals, food and humans in Denmark using whole genome sequencing

Since 2012, the national surveillance of antimicrobial resistance in food animals, food and humans in Denmark (www.DANMAP.org) has used whole-genome sequence (WGS) analysis for detection of resistance genes and multilocus sequence typing (MLST) using the open-access bioinformatic web-tools ResFinder and MLST, respectively from www.genomicepidemiology.org for characterisation of extended spectrum beta-lactamase (ESBL)- and AmpC-producing *E. coli* isolates [2-4]. The *mcr-1* sequence from China was added on 24 November 2015 to the ResFinder database as soon as it was available from The National Center for Biotechnology Information (NCBI).

Investigation of presence of *mcr-1* in *E. coli* isolates from food animals, food and human bloodstream infections

The updated version of ResFinder was used to analyse the WGS data from ESBL- and AmpC-producing *E. coli* isolates from food animals and food for the years 2012 to 2014, as well as ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections, and carbapenemase-producing organisms (CPOs) from humans, from January 2014 to beginning of November 2015 (Table 1), for the presence of *mcr-1*. Furthermore, fluoroquinolone resistance determinants were investigated by searching manually for mutations in the GyrA, ParC and ParE. [5].

The *mcr-1* gene was detected in one *E. coli* isolate from a human bloodstream infection from 2015 and in five *E. coli* isolates obtained from chicken meat of European origin imported to Denmark from 2012, 2013 and 2014 (Table 2). None of the CPOs were positive for *mcr-1* (Table 1).

The patient infected with the *mcr-1*-positive *E. coli* was an elderly man with prostate cancer and repeated urinary tract infections with ESBL-producing *E. coli* resulting in four positive urine samples over five month prior to the bloodstream infection, all resistant to third generation cephalosporins, gentamicin, sulfamethoxazole, trimethoprim and ciprofloxacin (these isolates were not

Table 1

Numbers of extended spectrum beta-lactamase and AmpC-producing *E. coli* isolates obtained and analysed by WGS from chicken meat, humans and carbapenemase-producing isolates from humans tested for *mcr-1* using ResFinder, Denmark, November 2015 (n=914)

Isolate origin	No. of isolates analysed by WGS	No. of sequences positive for *mcr-1*
ESBL- and AmpC-producing *E. coli* isolates from Danish chicken meat (2012–2014)	125	0
ESBL- and AmpC-producing *E. coli* isolates from imported chicken meat (2012–2014)	255	5
ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections (January 2014– beginning of November 2015)	417	1
Carbapenemase-producing isolates from humans (January 2014–beginning of November 2015)	117	0

ESBL: extended spectrum beta-lactamase; No: number; WGS: whole-genome sequence.

Table 2

Genotypic characterisation of *mcr-1*-positive *E. coli* isolates, Denmark, November 2015 (n=6)

Isolate name	Origin	Year of detection	MLST	Resistance genes detected by ResFinder besides *mcr-1*	Detection of chromosomal mutations encoding resistance to quinolones
0412016126	Chicken meat	2012	ST359	*aadA1*, *aadA5*, *aph(3')-Ic*, bla_{CMY-2}, bla_{TEM-1B}, *dfrA1*,*strA*, *strB*, *sul1*, *sul2*, *tet*(B)	GyrA (S83L, D87N) ParC (E62K)
0412044854	Chicken meat	2012	ST48	*aadA1*, bla_{CMY-2}, bla_{TEM-1B}, *dfrA1*, *mph(B)*, *strA*, *strB*, *sul1*, *sul2*, *tet*(A)	GyrA (S83L)
0412049521	Chicken meat	2012	ST131	*aadA1*, bla_{SHV-12}, bla_{CMY-2}, *strA*, *strB*, *sul1*, *dfrA1*, *tet*(A)	ND
0413040864	Chicken meat	2013	ST1112	*aadA1*, *aadA2*, bla_{SHV-12}, *cmlA1*, *sul3*, *tet*(A)	ND
14042624	Chicken meat	2014	ST2063	*aadA1*, *aadA2*, bla_{SHV-12}, *cmlA1*, *sul3*	ND
ESBL20150072	Human, bloodstream infection	2015	ST744	*aadA5*, bla_{CMY-2}, $bla_{CTX-M-55}$, bla_{TEM-1B}, *catA1*, *dfrA17*, *floR*, *fosA*, *mph*(A), *rmtB*, *strA*, *strB*, *sul1*, *sul2*, *tet*(A)	GyrA (S83L, D87N) ParC (E62K)

MLST: multilocus sequence typing; ND: not detected.

investigated further). He had been treated empirically with piperacillin/tazobactam and subsequently meropenem after susceptibility testing of the bloodstream isolate, but not with colistin according to the available patient data.

Besides *mcr-1*, the human isolate from the Danish patient contained 15 different resistance genes including $bla_{CTX-M-55}$ and bla_{CMY-2} conferring resistance to extended-spectrum beta-lactam antibiotics as well as two GyrA mutations (S83L, D87N) and a ParC mutation (E62K) leading to high-level fluoroquinolone resistance (Table 2). The human *mcr-1* positive *E. coli* isolate belonged to ST744, a rare sequence type in both humans and animals in Denmark. The patient had not been travelling abroad recently and the origin of the isolate is unknown.

The bla_{CMY-2} gene was detected in three of the five *mcr-1*-positive chicken meat isolates. In addition, three of the chicken meat *E. coli* isolates carried bla_{SHV-12} conferring resistance to extended-spectrum beta-lactam antibiotics excluding cephamycins.

One of the *mcr-1* positive *E. coli* isolates from chicken meat belonged to ST131. The other chicken meat isolates belonged to sequence types not frequently found in Denmark (Table 2). The human MCR-1-producing *E. coli* isolate was only susceptible to piperacillin/tazobactam, carbapenems and tigecycline according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [6], whereas the chicken meat isolates were less resistant (Table 3).

WGS analysis using the web-tool PlasmidFinder [9] identified an IncI2 replicon present in the human isolate as well as in three of the chicken meat isolates, but this replicon was not detected in the remaining two chicken meat isolates. De novo assembly using CLCbio Genomics Workbench (v8.5.1; Qiagen, Aarhus,

Table 3

Antimicrobial susceptibility profiles of the five MCR-1-producing *E. coli* isolates from chicken meat and the MCR-1-producing *E. coli* isolate from human bloodstream infection, Denmark November 2015

Origin	Human		Chicken meat									
Isolate name	ESBL20150072		14042624		0413040864		0412049521		0412016126		0412044854	
Antimicrobial agent	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R
Polymyxins												
Colistin	>4	R	>4	R	>4	R	>4	R	>4	R	>4	R
Polymyxin B[a]	4	R	>4	R	4	R	>4	R	>4	R	4	R
Beta-lactam/beta-lactam inhibitor combinations												
Ticarcillin/clavulanic acid	128/2	R	>128/2	R	≤16/2	S	64/2	R	64/2	R	≤16/2	S
Piperacillin/tazobactam	≤8/4	S	≤8/4	S	≤8/4	S	≤8/4	S	≤8/4	S	≤8/4	S
Cephalosporins												
Cefotaxime	>32	R	8	R	8	R	8	R	8	R	4	R
Ceftazidime	>16	R	>16	R	8	R	>16	R	16	R	8	R
Cefepime	>2	R	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Monobactams												
Aztreonam	>16	R	>16	R	>16	R	>16	R	8	R	8	R
Carbapenems												
Ertapenem	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Meropenem	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Imipenem	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Doripenem	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S
Aminoglycosides												
Gentamicin	>8	R	≤1	S	2	S	≤1	S	≤1	S	≤1	S
Tobramycin	>8	R	≤1	S	2	S	≤1	S	2	S	≤1	S
Amikacin	>4	R	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S
Fluoroquinolones												
Ciprofloxacin	>2	R	≤0.25	S	≤0.25	S	≤0.25	S	>2	R	≤0.25	S
Levofloxacin	8	R	≤1	S	≤1	S	≤1	S	>8	R	≤1	S
Tetracyclines												
Doxycycline[b]	8	I	≤2	S	16	R	8	I	16	R	8	I
Minocycline[b]	4	S	≤2	S	4	S	≤2	S	16	R	≤2	S
Glycylcyclines												
Tigecycline	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Folate pathway inhibitors												
Trimethoprim/ sulfamethoxazole	>4/76	R	≤0.5/9.5	S	≤0.5/9.5	S	>4/76	R	>4/76	R	>4/76	R

R/S according to the European Committee on Antimicrobial Susceptibility (EUCAST) clinical breakpoints [6].
I: intermediate; MIC: Minimal Inhibitory Concentration; R: resistant: S: sensitive.
[a] Breakpoint according to Société Francaise de Microbiologie [7]
[b] R/I/S according to The Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S25) [8].

Denmark) of the genomic data produced a direct link between the *mcr-1* gene and an IncX4 replicon in one of the two isolates not containing IncI2 replicons. An identical IncX4 replicon was detected in four of the chicken meat isolates including both isolates lacking an IncI2 replicon (but not in the human isolate).

Discussion and conclusion

Here we describe a MCR-1 producing *E. coli* isolate from a human infection coproducing both an ESBL (CTX-M-55) and an AmpC (CMY-2) cephalosporinase as well as five MCR-1 producing *E. coli* from chicken meat coproducing either and ESBL (SHV-12) or an AmpC (CMY-2) cephalosporinase, or both. Human and animal CTX-M-55-producing isolates are commonly reported from Asia [10,11], but are relatively rarely seen in Denmark. CTX-M-55-producing *E. coli* isolates were detected in only 3% of the *E. coli* from bloodstream infections in 2014 [4]. CMY-2-producing *E. coli* isolates have commonly been detected from chicken meat both in Denmark and other countries [2-4,12,13], but bla_{CMY-2} has been relatively rare in the Danish human bloodstream *E. coli* isolates. Similarly, only two of the 245 human bloodstream *E. coli* isolates from 2014 were

SHV-12-producing [4]. Based on antibiogram data it seems plausible that the bloodstream infection is related to the repeated urinary tract infections, but this will need to be confirmed by additional WGS analysis. At this point in time, the origin of the human isolate is unresolved.

MLST analysis did not show any close clonal relationship between any of the six isolates. However, one of the chicken meat isolates belonged to ST131. This sequence type is commonly associated with human *E. coli* urinary tract and blood infection isolates worldwide, but are rare in animal *E. coli* isolates [4,14,15]. The fact that a ST131 MCR-1-producing *E. coli* isolate was found is of special concern, since ST131 isolates have spread epidemically during the last decade [14,15] and the ability of *mcr-1* to be acquired by this sequence type has been demonstrated here.

The *mcr-1* gene was initially reported to be located on an IncI2 plasmid without other known resistance markers [1]. Here only four of the isolates were found to contain an IncI2 replicon, suggesting that the *mcr-1* gene was either located on the chromosome or on a plasmid type belonging to another group. In support of the latter is the fact that *de novo* assembly of the genomic data from one of the isolates produced a continuous DNA fragment containing both an IncX4 and the *mcr-1* gene, strongly suggesting that the *mcr-1* gene is not restricted to the IncI2 plasmid group, but additional studies are needed to clarify this further.

In conclusion, this study is to our knowledge, the first proof of colistin-resistant *mcr-1* positive *E. coli* outside China. The human isolate was only susceptible to very few antimicrobial classes such as carbapenems. Should an isolate like this acquire carbapenem resistance, it would leave very few, if any, suitable treatment options. Finally, our findings underline the importance of continuous microbiological surveillance programs and not the least the benefit of employing comprehensive WGS-based surveillance of antimicrobial resistance, as it allows for rapid re-analysis of large datasets *in silico* and thus make early detection and risk assessment possible when new resistance genes emerge.

*Authors' correction

Upon request of the authors, Christina Aaby Svendsen's name was corrected in the Acknowledgement section on 14 December 2015. In addition, the sentence "In addition to IncN2, an incX4 replicon was found to be linked to mcr-1." was corrected to read "In addition to IncI2, an incX4 replicon was found to be linked to mcr-1." on 16 December 2015 upon request of the authors.

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Conflict of interest

None declared.

Authors' contributions

HH and AMH collected the data and drafted the manuscript, HH, MS, PL, EZ and RK did the molecular analysis, FMA, FH, YA, RSH, LC, DSH, BO produced phenotypic data and participated in the coordination and concept of the manuscript, RLS coordinated and edited the manuscript.

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Colonisation with toxigenic *Corynebacterium diphtheriae* in a Scottish burns patient, June 2015

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On 12 June 2015, *Corynebacterium diphtheriae* was identified in a skin swab from a burns patient in Scotland. The isolate was confirmed to be genotypically and phenotypically toxigenic. Multilocus sequence typing of three patient isolates yielded sequence type ST 125. The patient was clinically well. We summarise findings of this case, and results of close contact identification and screening: 12 family and close contacts and 32 hospital staff have been found negative for *C. diphtheriae*.

Case report

On 9 June 2015, a 20 year old female patient had routine swabs taken from the discharge of recent wounds, following reconstructive surgery. The samples subsequently grew *Corynebacterium diphtheriae*.

The patient had contracted severe 30% burns to her face, neck, chest, arms, and thighs as a child. She was followed up regularly by the plastic surgery team for management of burn contractures and skin grafts, and had no other relevant past medical history. On examination her wounds were not erythematous or ulcerated, and appearances were consistent with satisfactory healing.

The patient's childhood vaccination history was fully up to date as per the UK (UK) childhood vaccination schedule recommended [1], and included vaccination for *C. diphtheriae* infection.

Further questioning revealed no history of foreign travel at all in preceding years, or of close contacts who had travelled abroad, and there was no known animal contact. The patient had no clinical features of cardiological, neurological or cutaneous manifestations of *C diphtheriae*. She reported a sore throat which was unremarkable on clinical examination with no membrane present. Following confirmation on 12 June of *C. diphtheriae* in swabs taken on 9 June, the patient was commenced on oral erythromycin 500mg, six hourly as per Public Health England (PHE) guidelines for the public health control and management of diphtheria. The treatment only started on 16 June, after some delays. The treatment continued for 14 days [2] and booster vaccinations were arranged. As the patient was very well, antitoxin was not administered.

Throat swabs and repeat skin swabs taken on 16 June, again grew toxigenic *C. diphtheriae*. Results were available on 19th June and were identified by using MALDI-TOF and Hoyle's Tellurite agar.

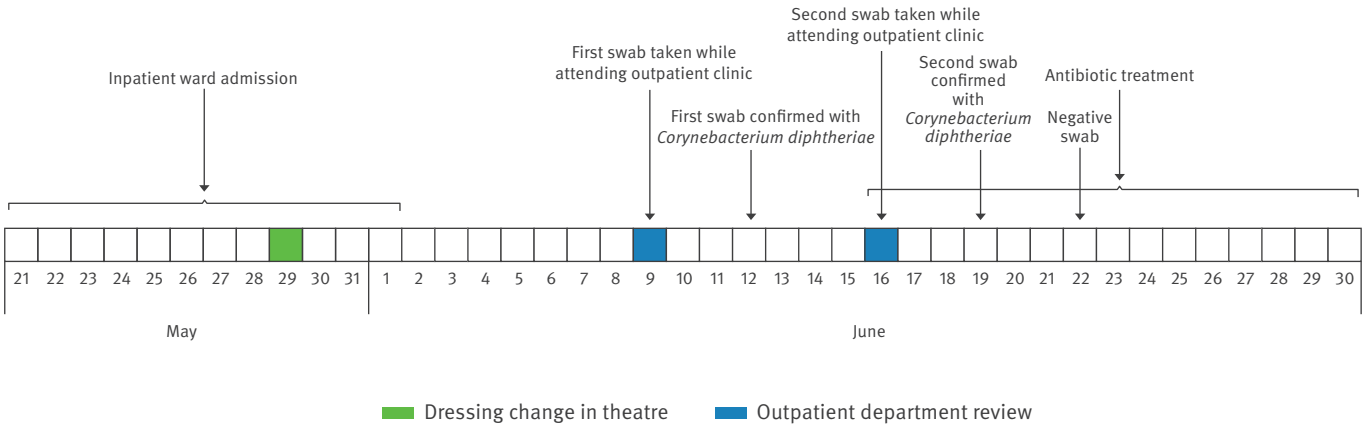
Nose, wound and throat swabs were taken on 22 and 23 June. The results of the swabs were available on 26 June and were all negative for *C. diphtheriae*.

Laboratory findings

The organism was initially identified on the 12 June, by matrix-associated laser desorption ionisation time-of-flight (MALDI-TOF) machine used for automated identification of microorganisms (Bruker); with a MALDI score of 99.9%. This test was repeated and a Gram-stain demonstrated Gram-positive bacilli. Hoyle's Tellurite agar and the API Coryne system (bioMérieux) were subsequently used to confirm the identity of the strain as *C. diphtheriae*. The isolate was then sent to the PHE diphtheria national reference laboratory in Colindale, where it was confirmed as PCR- positive for the *toxA* gene, and phenotypically positive for toxin production by the Elek immunodiffusion test. The two later isolates from the throat and wound swabs were also confirmed as *C. diphtheriae*, *tox-A* -positive and Elek-positive. The genotypic relationship of all three *C. diphtheriae* isolates was characterised using the multilocus sequence typing (MLST) scheme described by Bolt et al. [3] comprising the seven *C. diphtheriae* housekeeping genes *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, and *rpoB*. Allelic

Figure

Timeline for colonisation with toxigenic *Corynebacterium diphtheriae* in a Scottish burns patient, June 2015



profiles and sequence type (ST) designations for each strain were obtained via the PubMLST database curated by the Pasteur Institut, Paris, France (http://pubmlst.org/cdiphtheriae/). All three isolates had the alleleic profile 19,4,8,1,3,3,13 corresponding to ST 125.

Antibiotic susceptibility testing was undertaken using Clinical and Laboratory Standards Institute breakpoints [4] and the isolate tested sensitive to erythromycin, ciprofloxacin, meropenem, vancomycin, clindamycin, and clarithromycin. It was of intermediate sensitivity to penicillin. EUCAST breakpoints were not used due to lack of definitive breakpoints for *C. diphtheriae*.

Contact tracing

A timeline was prepared by our infection control team in conjunction with the clinical and public health teams, using the maximal incubation period of 10 days as a guide (Figure), with the caveat that the patient reported increased discharge from the wound before 31 May. The patient had spent most of this time as an outpatient at home, with the exception of being an inpatient for one day at the start of the lookback period, and presented to our clinic on 9 June 2015. Contact tracing was undertaken on all 12 family and other close contacts; this included friends, partner and relatives. Additionally, 32 hospital staff were identified as having had a relevant exposure during the patient's inpatient stay, such as being involved with changing wound dressings. Nose and throat swabs were taken from all of them, and Hoyle's Tellurite agar and MALDI-TOF techniques were used to screen specifically for the organism.

The vaccination histories of the patient's two year old child, family contacts as well as all hospital staff contacts, were checked and found to be complete as per UK recommendations. All identified family and close contacts and hospital staff were commenced on chemoprophylaxis with erythromycin at British National Formulary recommended doses, and excluded from work and encouraged to self-isolate until swab results were confirmed as negative. All contacts tested negative.

A memorandum was sent to all medical and nursing staff to enquire about recent foreign travel to exclude a hospital acquired infection.

The source of the organism remains unidentified.

Discussion

C. diphtheriae is an aerobic Gram-positive bacillus with a worldwide distribution. It causes diphtheria, a potentially life-threatening upper respiratory tract infection with a mortality rate of 5–10% in untreated cases [2,4,5]. As it is a vaccine preventable illness, it is now very rare in the European Union. Classic diphtheria is caused by bacteriophage-associated exotoxin-producing strains resulting in necrosis of infected cells and a classic fever, sore throat and tonsillar membrane. Non-toxigenic strains usually cause a milder illness or asymptomatic colonisation. Other manifestations of disease include cardiac syndromes (such as myocarditis), cutaneous lesions as well as neurological syndromes [5-7].

Humans are the main reservoir and transmit infection via respiratory droplets as well as direct contact. Respiratory and contact precautions are therefore required in an inpatient setting. The incubation period ranges from 1 to 10 days, and untreated individuals may be infectious for 2 to 4 weeks [2,5]. Treatment includes intravenous or oral macrolide therapy, intramuscular penicillin and antitoxin in severe illness.

Toxigenic *C. diphtheriae* is relatively rare in the UK and United States but prevalence is high in the Indian subcontinent and other parts of Asia, Eastern Europe, Africa, South and Central America. Numbers of toxigenic *C. diphtheriae* and *C. ulcerans* cases are one to six per year (2008–2014) in England [8]. Around 35 to 75 isolates of *C. diphtheriae* are submitted to the national reference laboratory per year (2010–2014)

and the majority of these are subsequently shown to be non-toxigenic [9,10].

Of 272 *C. diphtheriae* strains confirmed by the national reference laboratory from 2010 to 2014, 69% were *C. diphtheriae* biovar gravis, 26% biovar mitis, and 4% biovar belfanti [11]. The strains from this case were all biovar mitis. The main risk factor for infection with toxigenic *C. diphtheriae* infection in the UK is travel to an endemic area or contact with others returning from such an area. For toxigenic *C. ulcerans* infection, the main risk factor is contact with animals including companion animals [9,10]. Neither the case nor the contacts had travelled abroad or had any animal contact.

This case demonstrates the importance of team work, and required close coordination and communication between clinical, infection prevention and control, public health, community health and reference laboratory teams. The organism was first identified by the microbiology team in a clinical laboratory who then contacted the general practitioner, clinical, infection control and public health teams. The ensuing multidisciplinary investigation required parallel contact tracing within and outwith the hospital settings, clinical assessments of the case and contacts, infection control investigations within the hospital, as well as close liaison with GPs of the case and contacts to guide chemoprophylaxis regimes as well as exclusion from work. Lines of communication between different multidisciplinary team members included phonecalls, emails as well as a teleconference and this was crucial in ensuring a coordinated and thorough investigation. Nevertheless, the case raised some questions; for example, whether responsibility for liaison with the general practitioner as well as the patient directly lay with the microbiology team or local health protection unit. Additionally, would a Standard Operating Procedure or protocol be warranted to guide teams when such situations arise?

Given the relative rarity of toxigenic diphtheria, it may be best to handle each case on an individual basis. With the advent of new molecular or rapid diagnostic technologies, such as the MALDI-TOF, it is possible that we may detect a greater number of any pathogen (not restricted only to *C. diphtheriae*) which hitherto may have gone unidentified, and this may have both clinical and epidemiological implications. Indeed, if a Gram stain had been done in the first place (which would traditionally have been the case), it might have just been discarded as a diphtheroid contaminant.

Due to the highly contagious nature of *C. diphtheriae* and its relative rarity in Scotland, where only two cases were reported between 2000 and 2013 [12], this case is of particular interest, especially as the strain involved was a toxin producing strain. The ST of the three isolates from this patient (ST 125) was first designated for an isolate of *C. diphtheriae* from 2009 from France. As of 24 November 2015, no other isolates with ST 125 have been diagnosed and the two closest profiles have four of seven alleles in common; ST 232 (19,1,20,1,18,3,13) isolated in 2011 from Polynesia and ST 261 (13,4,8,44,3,23,13) isolated in 2009 from a patient in England [8], from a patient with a chronic leg ulcer from a prosthetic knee infection. Interestingly, this latter patient had themselves lived on a Polynesian island. The source of the organism for the case described here remains unclear, and little is known of the true epidemiology in Scotland.

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Conflict of interest

None declared.

Authors' contributions

Dr Ashutosh Deshpande: first main author, wrote first draft and co-ordinated subsequent redrafts;

Dr Teresa Inkster: second main author, wrote first draft, helped with all redrafts, provided infection prevention control information; Ms Kate Hamilton: prepared timeline figure, provided information regarding hospital contact tracing and other aspects of infection prevention and control;

Dr David Litt: supported redraft of article and performed MLST analysis; Dr Norman Fry: supported redraft of article and molecular testing of isolates; Dr Iain T R Kennedy: redrafted the article, provided epidemiological information including tracing of personal contacts and vaccination history, helped write discussion and management of case; Mrs Jacqueline Shookhye-Dickson: redrafted the article, provided epidemiological information including tracing of personal contacts and vaccination history, helped write discussion and management of case; Dr Robert L R Hill: helped to redraft the article and provide reference laboratory input.

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Middle East respiratory syndrome coronavirus (MERS-CoV) in dromedary camels in Nigeria, 2015

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Evidence of current and past Middle East respiratory syndrome coronavirus (MERS-CoV) infection in dromedary camels slaughtered at an abattoir in Kano, Nigeria in January 2015, was sought by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and serology. MERS-CoV RNA was detected in 14 (11%) of 132 nasal swabs and antibody in 126 (96%) of 131 serum samples. Phylogenetic analyses demonstrate that the viruses in Nigeria are genetically distinct from those reported in the Arabian peninsula.

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel lineage C betacoronavirus that can cause an acute viral respiratory disease in humans. As of 13 November 2015, there were 1,618 laboratory-confirmed cases of MERS reported to the World Health Organization (WHO), associated with 579 deaths [1,2]. Human disease is zoonotic in origin, although clusters of human-to-human transmission have been reported, especially within healthcare or family settings [1]. Dromedary camels in the Middle East have a high seroprevalence for MERS-CoV and MERS-CoV RNA has been consistently detected in these animals, especially in settings such as camel abattoirs, where camels from multiple origins are assembled. Camels are thus identified as a potential source of zoonotic MERS [3-6]. More than 60% of the global population of dromedary camels is distributed in African countries. Some of these countries are important camel exporters to the Arabian Peninsula [7]. MERS-CoV antibodies were also found with high prevalence in dromedary camels in these African countries with positive rates higher than 80% for the animals in Egypt, Ethiopia, Nigeria and Sudan, and of 30 to 54% in Tunisia [8]. However, zoonotic human disease has so far been reported only from countries in the Middle-East. The reason for the absence of zoonotic disease in Africa is unclear.

The genetic diversity of the virus in Africa is poorly defined. The only reports of genetic sequences from Africa have been those from viruses in Egypt and these were genetically distinct from those currently causing human disease in the Middle East [6]. However, phenotypic characterisation of these viruses revealed they have capacity to infect tissues of the human respiratory tract [9]. There is a need to better understand the ecology of MERS-CoV within Africa and the viral genetic diversity within this region. We report detection rates of MERS-CoV in dromedary camels in the abattoir in Kano, Nigeria, where around 55 camels are slaughtered per day, making it the largest camel abattoir in that country. Viral sequences derived from nasal swabs of these animals are also phylogenetically analysed.

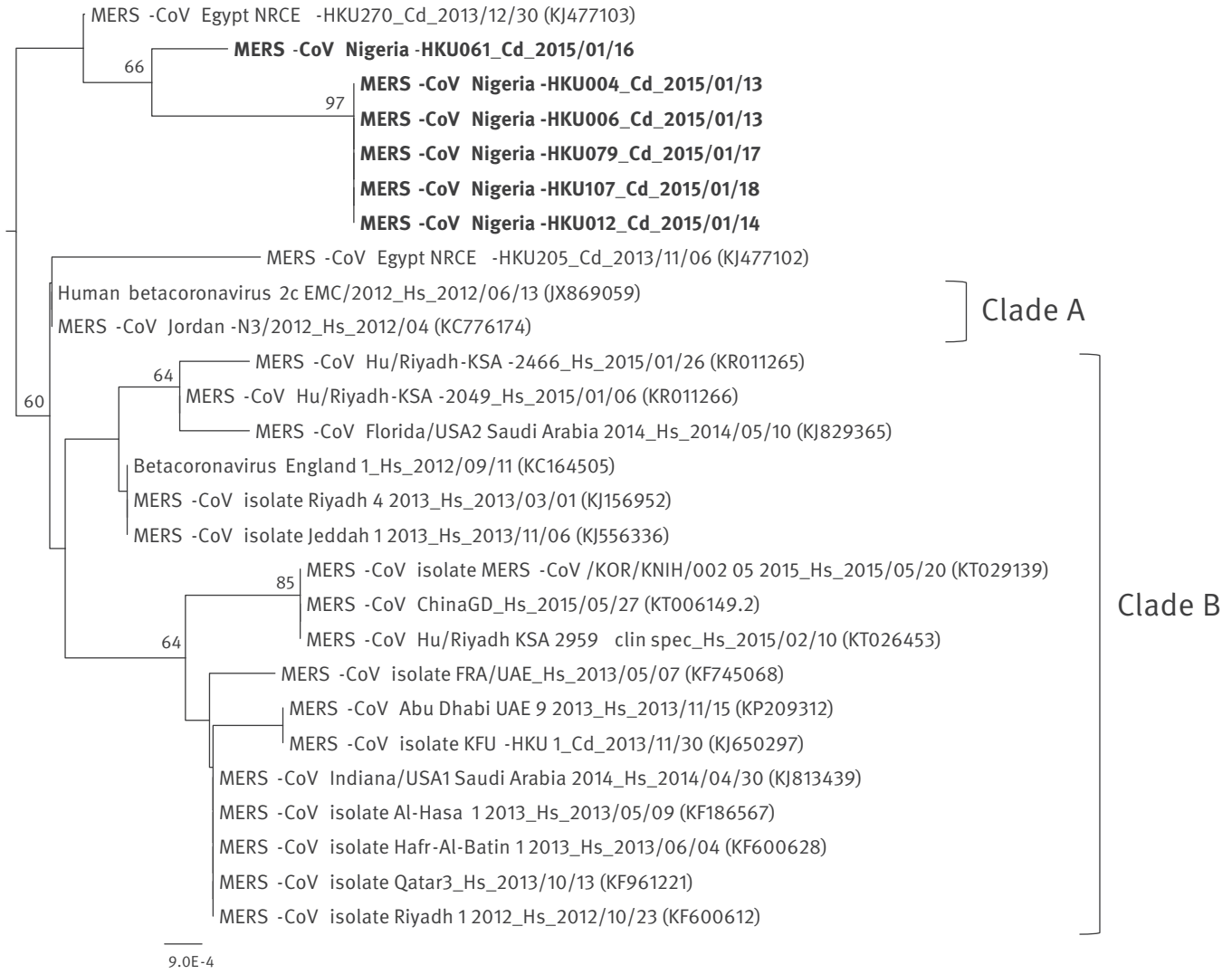
Methods

The study was carried out on seven consecutive days in January 2015. Nasal swabs and blood were collected from camels shortly after slaughter at the abattoir in Kano, Nigeria. Some of the animals slaughtered at this abattoir originate from northern Nigeria, as well as neighbouring African countries including Chad, Libya, Mali, Niger and Sudan. Nasal swabs were collected in RNALater (Ambion) or in virus transport medium and stored at -80°C. Blood samples were collected and serum separated. The age of the sampled camels was assessed by dental examination. Samples were shipped to the laboratory at The University of Hong Kong on dry ice for laboratory investigation.

Total nucleic acid was extracted from swab samples using EasyMag (Biomerieux), and tested for the presence of MERS-CoV RNA by upstream of the Envelope gene (UpE) reverse transcription-quantitative polymerase chain reaction (RT-qPCR) hydrolysis probe assay. All positive specimens were confirmed by a second RT-qPCR assay targeting the open reading frame (ORF)1a region of the genome [6]. The S2 region of

Figure 1

Phylogenetic analysis of the Middle East respiratory syndrome coronaviruses (MERS-CoVs) recovered from Nigeria



Phylogenetic tree of S2 (600 bp) gene sequences obtained by neighbour-joining method with bootstrap values >60 indicated. The tree is mid-point rooted. Sequences generated from this study are in bold with GenBank accession numbers: KU201953–58. Accession numbers of other sequences retrieved from GenBank are shown in brackets.

the positive samples were amplified and sequenced for phylogenetic characterisation [10]. The full spike gene and 5kb of the ORF1b region of one virus were sequenced for more detailed analysis.

Sera were screened at a dilution of 1:20 for the detection of MERS-CoV antibody using a validated MERS-spike pseudoparticle neutralisation test (ppNT) as previously described [5].

Results

Overall, 132 of the 385 animals slaughtered during this period (34%; daily range: 24%–43%) were sampled shortly after slaughter. Nasal swabs were collected from all 132 camels, while serum samples were obtained from 131.

MERS-CoV RNA was detected by both UpE and ORF-1a RT-qPCR assays in 14 (11%), nasal swabs with positive samples being found in five of the seven days sampled (Table). Threshold cycles of the positives by UpE assay ranged from 23.8 to 37.4, which represented $3.54x10^2$ to $2.60x10^6$ copies of virus RNA per 1 mL of original swab sample. Ages of the MERS-CoV positive animals ranged from three to 12 years-old.

On a daily basis, the seroprevalence ranged from 82% to 100%, giving an overall seroprevalence of 95% (Table). All 14 animals positive by RT-qPCR were also positive for MERS-CoV antibody by ppNT. The highest MERS-CoV RNA positive rate by RT-qPCR was detected on the days with lowest seroprevalence.

We sequenced a 600 bp region in S2 gene of six selected RT-qPCR positive samples, representing at

Figure 2

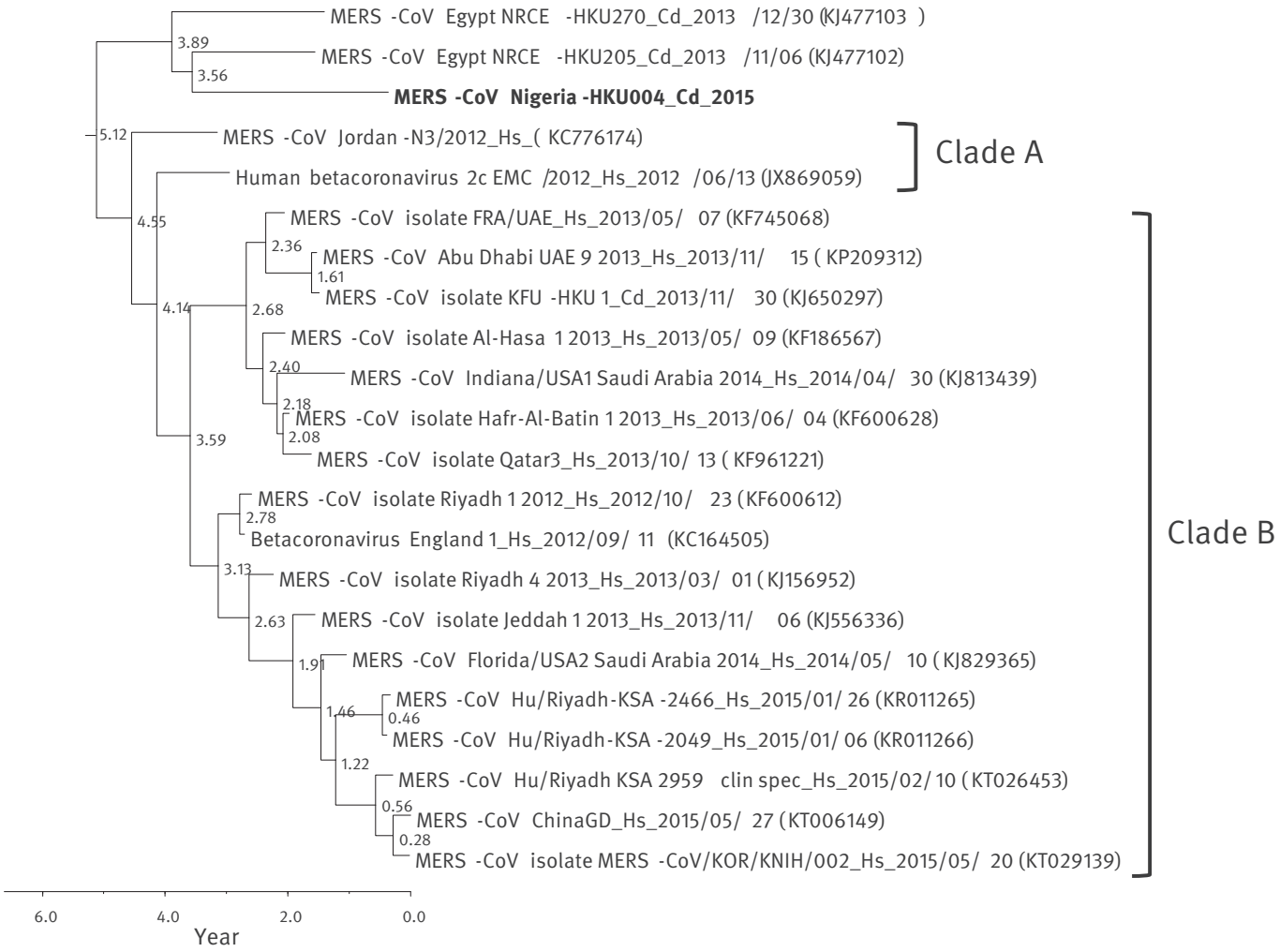
Pairwise similarities between nucleotide sequences of spike S2 region of Middle East respiratory syndrome coronaviruses, including several recovered from Nigeria and other representative viruses

	Virus clade	Nigeria-HKU004	Nigeria-HKU006	Nigeria-HKU012	Nigeria-HKU061	Nigeria-HKU079	Nigeria-HKU107	NRCE-HKU205	NRCE-HKU270	HCoV_2c_EMC	Jordan-N3/2012	Abu_Dhabi_UAE_9	Florida/USA2	England_1	KFU-HKU_1	Hafr-Al-Batin_1	Riyadh_4	FRA/UAE	KOR/KNIH/002	Hu/Riyadh-2049	Hu/Riyadh-2466	ChinaGD
Nigeria-HKU004	Undefined	ID																				
Nigeria-HKU006	Undefined	1	ID																			
Nigeria-HKU012	Undefined	1	1	ID																		
Nigeria-HKU061	Undefined	0.993	0.993	0.993	ID																	
Nigeria-HKU079	Undefined	1	1	1	0.993	ID																
Nigeria-HKU107	Undefined	1	1	1	0.993	1	ID															
Egypt NRCE-HKU205	Undefined	0.986	0.986	0.986	0.989	0.986	0.986	ID														
Egypt NRCE-HKU270	Undefined	0.993	0.993	0.993	0.996	0.993	0.993	0.993	ID													
Human_CoV_2c_EMC	A	0.991	0.991	0.991	0.994	0.991	0.991	0.994	0.998	ID												
Jordan-N3/2012	A	0.991	0.991	0.991	0.994	0.991	0.991	0.994	0.998	1	ID											
Abu_Dhabi_UAE_9	B	0.986	0.986	0.986	0.989	0.986	0.986	0.989	0.993	0.994	0.994	ID										
Florida/USA2_Saudi_Arabia	B	0.986	0.986	0.986	0.989	0.986	0.986	0.989	0.993	0.994	0.994	0.989	ID									
England_1	B	0.989	0.989	0.989	0.993	0.989	0.989	0.993	0.996	0.998	0.998	0.993	0.996	ID								
KFU-HKU_1	B	0.986	0.986	0.986	0.989	0.986	0.986	0.989	0.993	0.994	0.994	1	0.989	0.993	ID							
Hafr-Al-Batin_1	B	0.988	0.988	0.988	0.991	0.988	0.988	0.991	0.994	0.996	0.996	0.998	0.991	0.994	0.998	ID						
Riyadh_4	B	0.989	0.989	0.989	0.993	0.989	0.989	0.993	0.996	0.998	0.998	0.993	0.996	1	0.993	0.994	ID					
FRA/UAE	B	0.989	0.989	0.989	0.989	0.989	0.989	0.989	0.993	0.994	0.994	0.996	0.989	0.993	0.996	0.998	0.993	ID				
KOR/KNIH/002	B	0.984	0.984	0.984	0.988	0.984	0.984	0.988	0.991	0.993	0.993	0.994	0.991	0.991	0.994	0.996	0.991	0.994	ID			
Hu/Riyadh-KSA-2049	B	0.988	0.988	0.988	0.991	0.988	0.988	0.991	0.994	0.996	0.996	0.991	0.998	0.998	0.991	0.993	0.998	0.991	0.993	ID		
Hu/Riyadh-KSA-2466	B	0.986	0.986	0.986	0.989	0.986	0.986	0.989	0.993	0.994	0.994	0.989	0.996	0.996	0.989	0.991	0.996	0.989	0.991	0.998	ID	
ChinaGD	B	0.984	0.984	0.984	0.988	0.984	0.984	0.988	0.991	0.993	0.993	0.994	0.991	0.991	0.994	0.996	0.991	0.994	1	0.993	0.991	ID

ID: identical virus.

Figure 3

Time resolved phylogenetic tree of open reading frame (ORF)1b of Middle East respiratory syndrome coronaviruses (MERS-CoVs) recovered in Africa and the Middle East, 2012–2015



The time resolved phylogenetic tree was constructed using Bayesian evolutionary analysis by sampling trees (BEAST) with an uncorrelated lognormal relaxed clock based on open reading frame (ORF)1b (5,347 bp) gene sequences of representative MERS-CoVs. Median ages of the nodes with respective to MERS-CoV ChinaGD (the latest virus included) are shown. The tree is rooted to bat coronavirus Neoromicia. The sequence generated from this study (Nigeria-HKU004) is in bold and has GenBank accession number KU201959. GenBank accession numbers of other sequences retrieved for the phylogenetic analysis are shown in brackets.

least one positive sample from each day with PCR-positive samples. Phylogenetic analysis showed that these six viruses formed two distinct lineages, the virus from a camel sampled on 16 January being distinct from the others (Figure 1).

Both of the Nigerian virus groups were distinct from previously known virus lineages. The Nigerian camel viruses were most closely related to virus NRCE-HKU270 previously found in Egypt with nt sequence similarity of 99.3 to 99.6%. MERS-CoV in Nigeria were genetically distinct from viruses found in camels in the Middle East and viruses detected in humans in more recent years (nt sequence similarity ranging from 98.4 to 99.4%) (Figure 2).

The time-resolved phylogeny of the ORF1b region of one virus confirms these overall findings (Figure 3).

We derived the nt sequence of the full spike gene of one virus Nigeria HKU004 and the deduced amino acid sequence is aligned and compared to those of other human and animal MERS-CoV in Figure 4. This reveals that the receptor binding domain, which is crucial for virus host range and tropism, is fully conserved. There are only two amino acid residues not previously reported in other MERS-CoV spike proteins; these being S 656 T and L 1200 F.

Discussion

We report high MERS-CoV seroprevalence in camels gathered in an abattoir in Nigeria with relatively high detection rates of virus in nasal swabs. Phylogenetic analysis revealed that these viruses are genetically distinct from viruses reported from camels or humans in the Arabia peninsula and cluster with, but are clearly distinct from those reported from Egypt. However, it is

Figure 4

Alignment of the full spike protein amino acid sequence of a Middle East respiratory syndrome coronavirus (MERS-CoV) found in Nigeria with those of other MERS-CoVs

Viurus clade	Virus sequences compared	Spike region and spike protein amino acid position																																			
		S1	S1	S1	S1	S1	S1	S1	S1	COR	COR	COR	COR	COR	RBD	RBD	RBD	S1	S1	S1	S1	S1	S1	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
		23	26	94	125	137	158	194	301	411	431	434	457	460	506	509	530	656	666	677	683	696	744	756	773	810	833	856	886	888	914	918	1,020	1,158	1193	1,200	1,208	1,314	1,333
A	MERS-CoV ChinaGD	P	V	G	N	S	D	H	R	L	A	A	S	S	L	D	V	S	E	V	S	M	T	E	L	V	Q	S	A	S	Q	A	R	A	A	L	Q	V	C
A	MERS-CoV isolate MERS-CoV/KOR/KNIH/002	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV Hu/Riyadh KSA 2959 clin spec	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV Abu Dhabi UAE 9 2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	I	.	.	.	.	.	.	.	.	S	.	.	.	.
A	MERS-CoV Florida/USA2 Saudi Arabia 2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV Indiana/USA1 Saudi Arabia 2014	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV isolate Al-Hasa 1 2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.
A	Human Betacoronavirus England 1	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.
A	MERS-CoV isolate KFU-HKU 1	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	F	I	.	.	.	.	.	.	.	.	S	.	.	.	.
A	MERS-CoV isolate Hafr-Al-Batin 1 2013	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV isolate Qatar3	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV isolate Riyadh 1 2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MERS-CoV isolate Riyadh 4 2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.
A	MERS-CoV isolate FRA/UAE	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	S	.	.	.	.
A	MERS-CoV isolate Jeddah 1 2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.
A	MERS-CoV Hu/Riyadh-KSA-2049	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
A	MERS-CoV Hu/Riyadh-KSA-2466	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B	Human betacoronavirus 2c EMC/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.
B	MERS-CoV Jordan-N3/2012	.	.	V	.	.	.	Y	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	S	.	.	.	.	.
UD	MERS-CoV Egypt NRCE-HKU205	L	A	.	.	.	.	Y	.	.	.	S	.	.	.	.	.	.	K	.	.	T	.	Q	.	.	.	.	S	I	.	S	Q	S	.	.	.	.	R
UD	MERS-CoV Egypt NRCE-HKU270	.	A	.	.	.	Y	Y	.	.	T	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	Y	.	.	.	.	Q	S	.	.	.	.	.
UD	MERS-CoV Nigeria HKU004	.	A	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	Y	.	.	H	.	Q	L	.	F	.	.	.

GenBank accession number for MERS-CoV Nigeria HKU004 was KU201953.
RBD: receptor binding domain; UD: undefined.

Table

Swabs and sera collected from dromedary camels and results of reverse transcription-quantitative polymerase chain reaction and serology for Middle East respiratory syndrome coronavirus, Nigeria, January 2015

Sampling date	Number of camels slaughtered	Swabs sampled	Swabs positive n (%)	Sera sampled	Sera positive n (%)
13 Jan 2015	45	11	4 (36)	11	9 (82)
14 Jan 2015	54	17	6 (35)	17	16 (94)
15 Jan 2015	50	18	0 (0)	18	18 (100)
16 Jan 2015	48	15	1 (7)	15	15 (100)
17 Jan 2015	70	30	2 (7)	30	28 (93)
18 Jan 2015	55	19	1 (5)	19	19 (100)
19 Jan 2015	63	22	0 (0)	21	20 (95)
Total	**385**	**132**	**14 (11)**	**131**	**125 (95)**

important to note that these Nigerian viruses remain genetically closely related to other MERS-CoV with an overall nt similarity of›0.98 for the S2 gene. Thus these are almost certainly pertaining to one viral species, and very likely (though not directly studied in the present work) also form one serotype.

Although the animals in this study were sampled in Kano, Nigeria, some animals originated from neighbouring African countries. The camels are kept within the abattoir for a number of days before slaughter allowing opportunity for virus cross-infection and amplification. Thus the viruses detected in this study may reflect viruses circulating in this wider region.

A limitation of this study is the short period within which the sampling was carried out. Thus it is unclear whether the high rate of virus detection reflects an all year-round pattern or if there are seasonal differences in virus activity. It has been suspected that virus activity in camels may increase during the calving season. In Nigeria, as in the Arabian peninsula, camel calving peaks during the January to March period. Thus it is possible that the rate of virus detection observed in this study reflects a seasonal peak. Indeed this was the rationale for targeting sampling within this time frame. Even if the observations of this study reflect a seasonal peak, the detection of MERS-CoV in camels slaughtered on five of seven days sampled, suggests intense exposure of humans to virus infected camels, whether seasonally or year round, and thus potentially a significant zoonotic threat.

It is not known if the MERS-CoV in Nigeria has similar zoonotic potential to viruses currently circulating in the Arabian peninsula. Other explanations for the lack of zoonotic MERS in Africa include differences in patterns of exposure to infected animals or animal products or differences in human susceptibility to MERS-CoV. Alternatively, MERS may indeed be occurring but unrecognised in Africa due to lack of awareness or diagnostic testing.

A second limitation of this study is that there were no virus isolates available for phenotypic characterisation and full genome sequence data were not available for genetic comparison across the whole genome with viruses known to infect humans. Comparison of the sequence of the spike gene with other MERS-CoV suggests that the virus isolated in Nigeria appears to have competence to bind the dipeptidyl peptidase (DPP)-4 receptor. Viruses isolated from Egypt, though genetically distinct, had comparable tropism and replication competence in ex vivo cultures of the human bronchus and lung to those viruses isolated from humans in Saudi Arabia [9]. Thus zoonotic potential of these viruses cannot be excluded. Further studies to determine the genetic diversity and biological characterisation of MERS-CoV across Africa are urgently needed. Studies looking into seroprevalence of humans exposed to settings associated with such high levels of exposure to MERS-CoV such as camel abattoirs across Africa are also important to assess the extent of zoonotic spillover, if any.

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Conflict of interest

None declared.

Authors' contributions

JOO and MP designed the study, JOO and SAK carried out the field work, DKWC, RAPMP, SMSC and LLMP supervised and carried out the laboratory analysis, DKWC and MP drafted the manuscript. All authors critically reviewed the manuscript.

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Research article

Tuberculosis treatment outcome in the European Union and European Economic Area: an analysis of surveillance data from 2002–2011

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Monitoring the treatment outcome (TO) of tuberculosis (TB) is essential to evaluate the effectiveness of the intervention and to identify potential barriers for TB control. The global target is to reach a treatment success rate (TSR) of at least 85%. We aimed to assess the TB TO in the European Union and European Economic Area (EU/EEA) between 2002 and 2011, and to identify factors associated with unsuccessful treatment. Only 18 countries reported information on TO for the whole observation period accounting for 250,854 new culture-confirmed pulmonary TB cases. The 85% target of TSR was not reached in any year between 2002 and 2011 and was on average 78%. The TSR for multidrug-resistant (MDR)-TB cases at 24-month follow-up was 49%. In the multivariable regression model, unsuccessful treatment was significantly associated with increasing age (odds ratio (OR) = 1.02 per a one-year increase, 95% confidence interval (CI): 1.02–1.02), MDR-TB (OR = 8.7, 95% CI: 5.09–14.97), male sex (OR = 1.40, 95% CI: 1.28–1.52), and foreign origin (OR = 1.32, 95% CI: 1.03–1.70). The data highlight that special efforts are required for patients with MDR-TB and the elderly aged ≥65 years, who have particularly low TSR. To allow for valid monitoring at EU level all countries should aim to report TO for all TB cases.

Introduction

In 1991, the 44th World Health Assembly set targets to detect at least 70% of new tuberculosis (TB) cases and to cure at least 85% of those detected [1]. The Stop TB Partnership developed the *Global Plan to Stop TB 2006–2015* to achieve these targets set for 2015 within the context of the Millennium Development Goals [2]. Monitoring the outcome of TB treatment is essential to evaluate the effectiveness of the intervention and to identify the potential barriers for TB control.

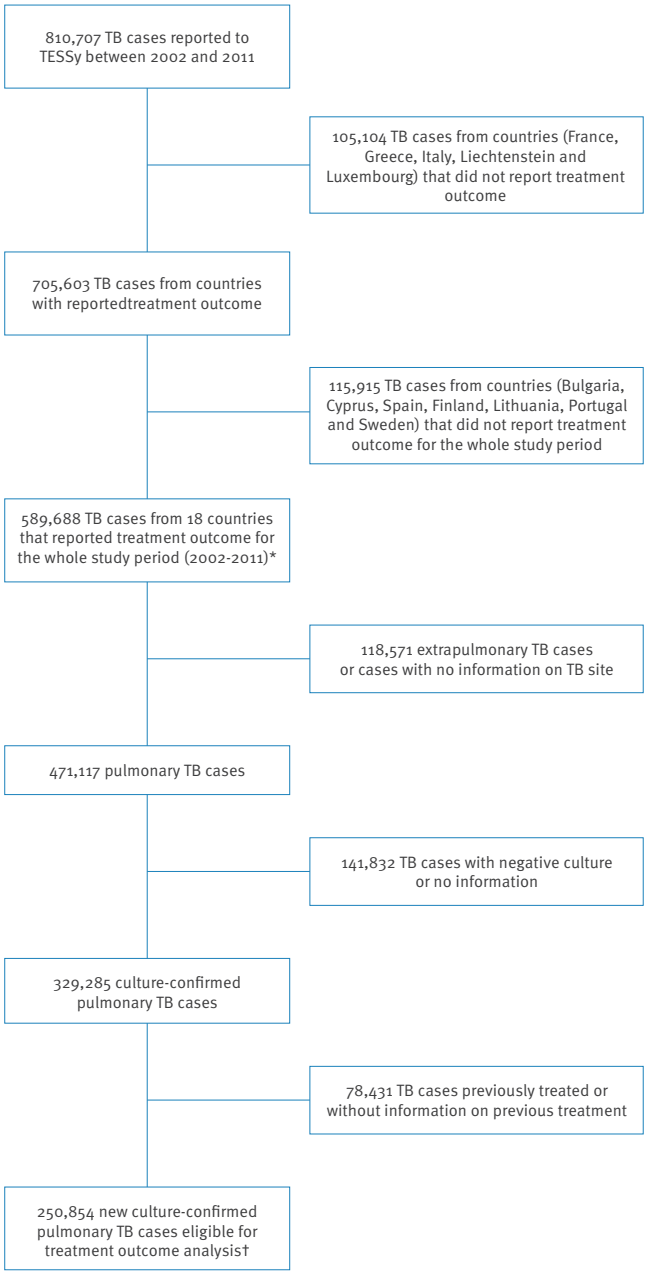
In Europe, a Working Group of the World Health Organization (WHO) and the International Union against Tuberculosis and Lung Disease (IUATLD) published recommendations for uniform reporting by TB surveillance and cohort analysis of treatment outcome (TO) across Europe [3,4]. A minimal set of six exclusive categories of TO was recommended as standard: cured, completed, failed, died, interrupted (defaulted) and transferred out. Furthermore, analysis of TO should be separate for new and retreatment cases [4]. In 2008, the European Centre for Disease Prevention and Control (ECDC) published the *Framework Action Plan to fight Tuberculosis in the European Union* following the WHO/IUATLD recommendations; including a core indicator of 85% treatment success rate for new pulmonary culture-confirmed TB cases and 70% for new pulmonary culture-confirmed multidrug-resistant (MDR) TB cases [5].

In 2013, in the European Union and European Economic Area (EU/EEA), the TB notification rate was 12.7 per 100,000 population [6]. Notification rates were heterogeneous: five countries had incidence rates ≥ 20 and 24 countries had incidence rates < 20 cases per 100,000 population in 2013) [6]. In the majority of countries the trend in case notification rate showed a sustained decline during the period 2009-2013. In 2013, the overall treatment success rate was 73.5%.

In this study, we aimed to assess the TB TO in the EU/EEA and to identify factors associated with unsuccessful treatment applying the WHO/IUATLD recommendations for the EU/EEA for cohort analysis over a 10-year observation period.

Figure 1

Algorithm for inclusion of cases in the analysis on treatment outcome of new culture-confirmed pulmonary tuberculosis, EU/EEA, 2002–2011 (n=589,688) Algorithm for inclusion of cases in the analysis on treatment outcome of new culture-confirmed pulmonary tuberculosis, EU/EEA, 2002–2011 (n=589,688)



EEA: European Economic Area; EU: European Union

a Including Austria, Belgium, Czech Republic, Denmark, Estonia, Germany, Hungary, Ireland, Iceland, Latvia, Malta, the Netherlands, Norway, Poland, Romania, Slovenia, Slovakia and the United Kingdom.

b Denmark did not report information on previous treatment in 2005. These cases were coded as missing in the cohort (n=428).

Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

Methods

Data source

Since January 2008, all EU countries (27; since February 2015 28 countries, after Croatia has joined) and the EEA countries Iceland and Norway (Liechtenstein has not reported data since 2007) report their available data on TB to The European Surveillance System (TESSy) hosted by ECDC [6]. For years between 1996 and 2007, data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe. In this paper, we analysed data of TB TO during 2002-2011 and extracted from TESSy on 2 October 2013.

Since the reporting year 2002, case-based TO data have been provided by EU/EEA countries for cases notified one year before the year of TO reporting. Since 2005, TO data of MDR-TB cases have been reported for cases notified two years earlier [6]. Thus, for TO analysis of MDR-TB cases data reported between 2005 and 2010 were used in this study.

Definitions

In line with the WHO/IUATLD recommendation for EU/EEA countries [3,4] the cohort eligible for the TO analysis included new culture-confirmed pulmonary TB cases. Cases are observed until the first outcome up to a maximum of 12 months after the start of treatment and MDR-TB cases for 24 months. TO was categorised based on the WHO/IUATLD recommendations [4] with two additional categories 'Still on treatment' and 'Unknown' (Table 1) [6,7].

A new TB case was defined as a case that never previously received drug treatment for active TB, or received anti-TB drugs for less than one month. Five countries (Belgium, Denmark, Ireland, Norway and the United Kingdom (UK)) did not report information about previous treatment and for those previous diagnosis was used as a proxy for defining new and previously treated cases.

The term native case refers to cases born in or, if this information was unavailable, having citizenship in the reporting country. Foreign origin refers to cases born in (or citizens of) a country different from the reporting country.

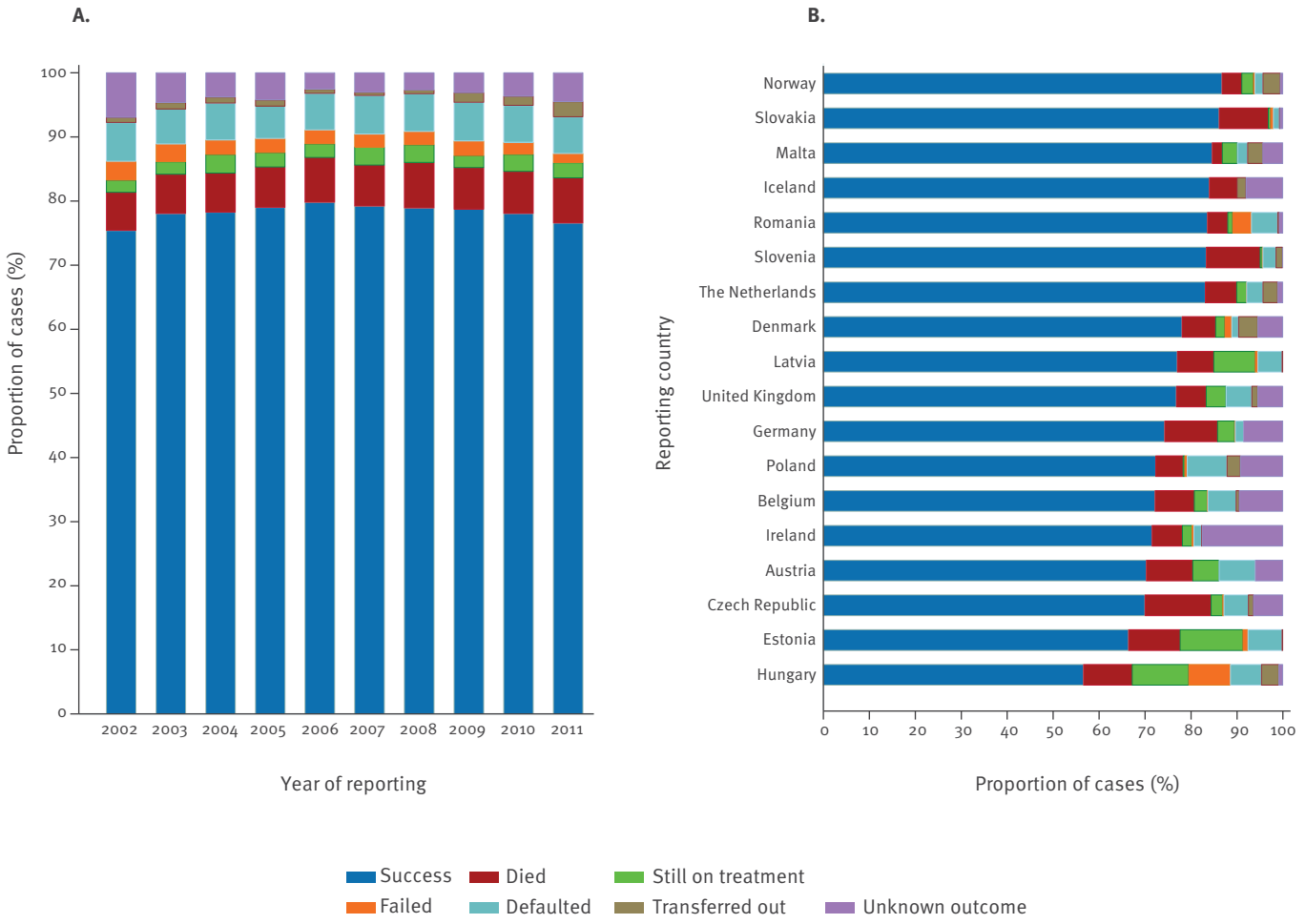
MDR-TB was defined as TB with resistance to at least rifampicin and isoniazid. A low TB incidence country was defined as country with TB incidence rate <20 cases per 100,000 population and a high TB incidence country with TB incidence rate ≥ 20 cases per 100,000 population.

Statistical analysis

Data were described by totals and percentages. The chi-squared test was used to assess differences in categorical variables. Only cases from countries

Figure 2

Treatment outcome of new culture-confirmed pulmonary tuberculosis cases in the EU/EEA by A. Reporting years B. Reporting countries, 2002–2011 (n=250,854)



Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

that reported on TO for the whole study period were included in TO calculation and the statistical model.

Trend analysis of treatment success rate was conducted using the nonparametric test for trend across ordered groups i.e. the reporting years. A logistic regression model was used to examine the association between the TO, successful vs unsuccessful, and potential predictor variables age, sex, geographical origin, MDR-TB and reporting year. In the regression model, TB TO was categorised as treatment success vs unsuccessful treatment. Independent variables were systematically investigated in the univariate analysis and in the multivariable model for adjusting to possible confounders. To correct for the clustering within countries, we specified that the standard errors allow for intragroup correlation, assuming that the observations are independent across groups (clusters) but not necessarily within groups. Based on the regression coefficient of the model, odds ratios (OR) with their 95% confidence interval (CI) were also calculated to assess the strength of association.

All tests were two-sided and considered significant if $p<0.05$. All analyses were performed using STATA (version12, StataCorp, LP, TX, US) software.

Ethical statement

This study was based on national surveillance data submitted to ECDC. Therefore, written informed consent from the patients was not required due to the anonymous nature of the data.

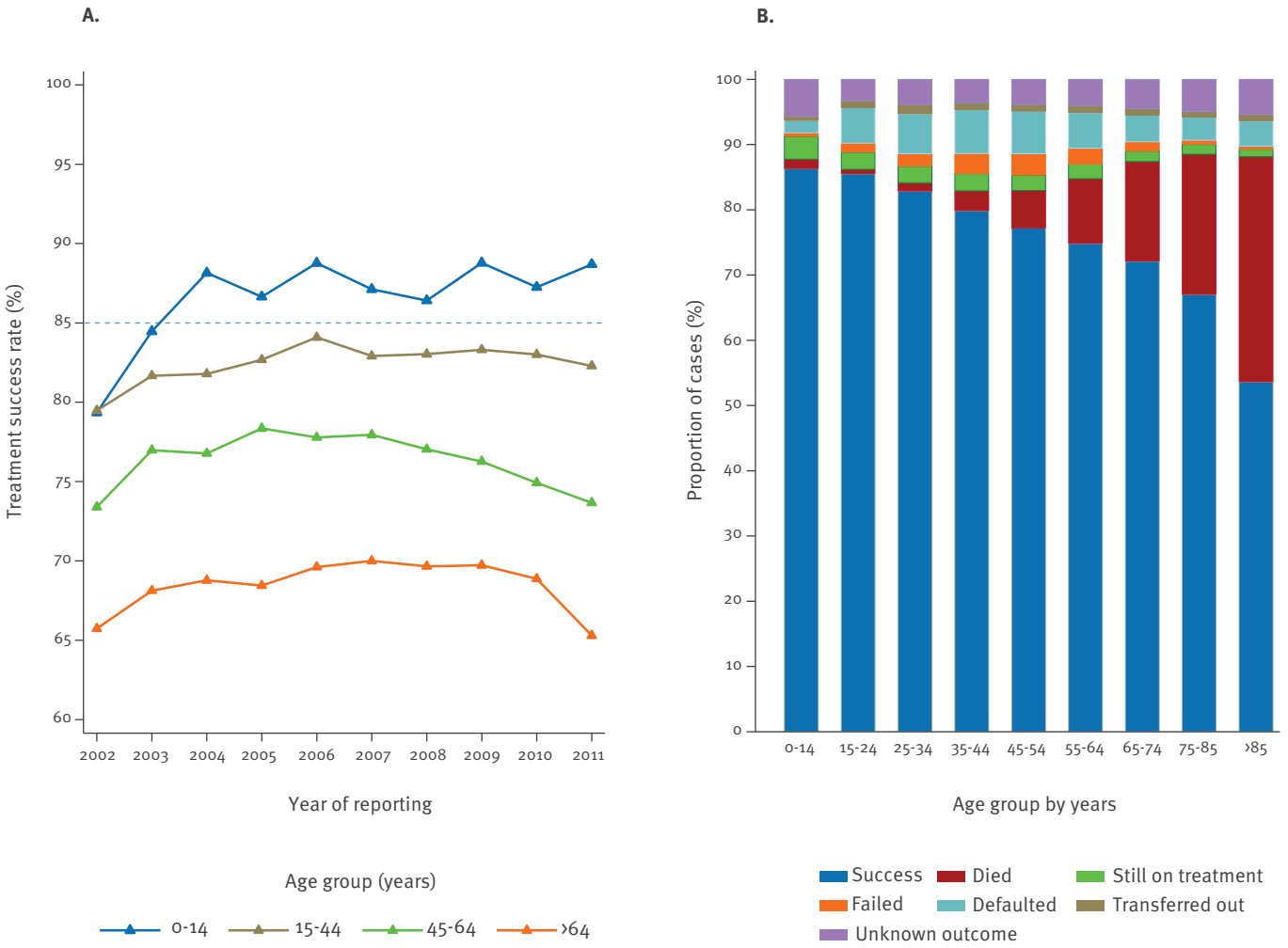
Results

Treatment outcome reporting

Between 2002 and 2011, a total of 810,707 TB cases were notified. Of these, 83.3% (n=675,627) had information on TO. The number of countries, that reported information on TO, increased from 21 countries in 2002 to 25 countries in 2011. Five countries (France, Greece,

Figure 3

Treatment success rate of new culture-confirmed pulmonary tuberculosis in the EU/EEA. 3A. Age group 3B. Treatment outcome by reporting year, 2002–2011 (n=250,810)



EU: European Union; EEA: European Economic Area

Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

Italy, Liechtenstein and Luxembourg) did not report on TO in any year between 2002 and 2011. Eighteen countries (Austria, Belgium, Czech Republic, Denmark, Estonia, Germany, Hungary, Ireland, Iceland, Latvia, Malta, the Netherlands, Norway, Poland, Romania, Slovenia, Slovakia and the UK) provided information on TO for the whole 10-year study period (2002–2011), corresponding to 589,688 TB cases (72.7% of all reported cases). Of these 589,688 TB cases, 42.5% (n=250,854) were new culture-confirmed pulmonary TB cases eligible for our analysis (Figure 1).

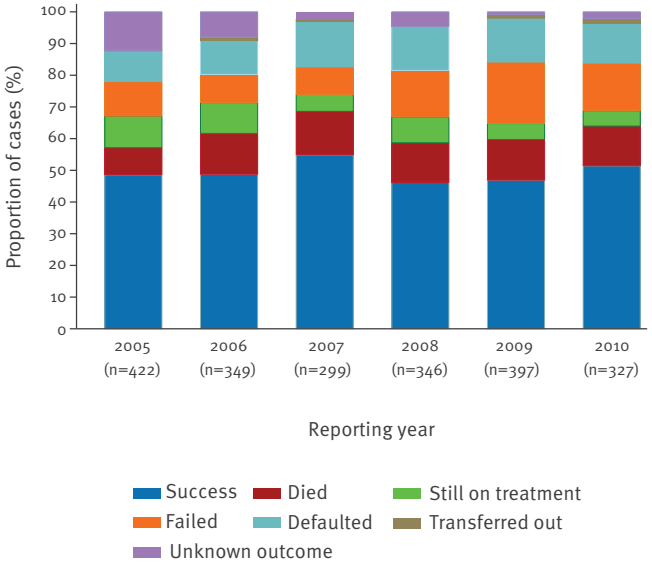
Significant differences were observed in the demographic characteristics between included and excluded cases. Compared with the excluded cases, the included new culture-confirmed pulmonary TB cases were more likely to be men (67.2% vs 63.7%; p<0.001), of native origin (84.4% vs 78.7%; p<0.001) and non-MDR-TB (98.6% vs 97.9%, p<0.001) respectively. Also, the treatment success rate was higher in included cases compared with excluded ones (78.2% vs 71.1%; p<0.001).

Cohort characteristics

The majority of cohort cases were reported by Romania, Poland, Germany and the UK (45.1%, 17.8%, 9.7% and 9.5% respectively). The median age of cases was 45 years (interquartile range (IQR) 31–57). Of cohort cases 67.2% were male and 14.9% were of foreign origin. MDR-TB was reported in 1.4% (n=3,597) of the cohort cases, mainly from Romania (n=1,219), Latvia (n=802) and Estonia (n=460). Information of drug susceptibility

Figure 4

Treatment outcome at 24-month follow-up for new culture-confirmed pulmonary MDR-TB cases in the EU/EEA, 2005–2010 (n=2,140)



EU: European Union; EEA: European Economic Area; MDR: multidrug-resistant; TB: tuberculosis.

Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

testing results for isoniazid and rifampicin was missing in almost half of the cases (48.4%) (Table 2).

Twelve-month treatment outcome

The overall proportion of cases with reported treatment success was 78.2% (country range 56.6% in Hungary to 86.8% in Norway). Treatment success rate was higher among female cases compared with male cases (81.6% vs 76.5% respectively; p<0.01) overall and in each respective reporting year, age group and geographical origin (data not shown). About 6.5% of TB cases died (country range 2.2% in Malta to 14.4% in Czech Republic). On average, 2.4% of cases were still on treatment at the end of the 12-month follow-up period (country range 0% in Iceland to 13.7% in Estonia); 32.7% of these cases were MDR-TB. The proportion of cases with unsatisfactory outcome 12 months after start of treatment was 8.9% (country range 2.0% in Iceland and Germany to 19.6% in Hungary). Of them 2.2% were reported as treatment failure, 5.7% as defaulted and 1.0% as transferred out. Cases with unknown TO accounted for 4.0% (country range 0% in Latvia and Estonia to 17.6% in Ireland) (Figure 2).

The treatment success rate decreased with increasing age (85.3% treatment success in cases aged<15 years vs 68.4% in cases aged>64 years; p<0.001) (Figure 3a,b). The death rate rose substantially with increasing age of cases (1.8% death rate in cases aged<15 years vs 19.4% in cases aged>64 years; p<0.001) (Figure 3b).

A higher proportion of cases with unknown outcome was noticed among cases aged<15 years compared with other age groups.

The treatment success rate was slightly higher among native cases than among cases of foreign origin (78.7% vs 77.3% respectively; p=0.05). In native cases, a higher proportion of deaths (6.9% in native cases vs 4.3% in cases of foreign origin; p<0.001) and treatment failure (2.7% in native cases vs 0.2% in cases of foreign origin; p<0.001) was observed. Also the proportion of cases with advanced age was higher in native cases than cases of foreign origin (cases aged>64 years accounted for 17.2% in native cases vs 10.8% in cases of foreign origin; p<0.001). The proportion of cases with unknown outcome was markedly higher in cases of foreign origin than in native cases (3.0 vs 6.5% respectively; p<0.001).

Adjusting for age and country of origin revealed no significant differences in treatment success rate between countries with low or high incidence (OR 0.7; p=0.1). Noticeably, the proportion of elderly aged >64 years was higher in low incidence countries compared with high incidence countries (cases aged>64 years accounted for 20.9% vs 14.6% respectively; p<0.001). Similarly, cases of foreign origin were more presented in low incidence countries (44.7% vs 0.8% respectively; p<0.001).

Twenty-four-month treatment outcome of multidrug-resistant tuberculosis

Between 2005 and 2010, 2,140 MDR-TB cases were reported from the 18 EU/EEA countries included in the analysed cohort. The overall treatment success rate for MDR-TB cases at 24-month follow-up was 49.2% and did not reach the 70% target of treatment success rate for MDR-TB cases in any year of the observation period. About 12.0% of MDR-TB cases died and 7.3% were still on treatment at the end of 24-month follow-up. The proportion of cases with unsatisfactory outcome was 26.3% (including 13.1% failed, 12.4% defaulted and 0.8% transferred out). About 5.2% of the MDR-TB cases were reported with unknown TO (Figure 4).

Trend analysis

The 85% target of overall treatment success rate was not reached in any year between 2002 and 2011. Analysis of trends shows that the treatment success rate increased from 75.4% in 2002 to 79.8% in 2006 (p=0.04), then subsequently decreased to 76.6% in 2011 (p=0.07). No change in the cohort profile regarding proportions of age groups, sex and MDR-TB was observed over time. The proportion of cases of foreign origin increased from (N=3672; 14.5%) in 2006 to (N=4081; 18.5%) in 2011 (p<0.001). Some countries (Belgium, Germany, Hungary, Romania and the UK) showed a continuous increase in the treatment success rate throughout the observation period, while for other countries (Austria, Denmark, Estonia, Netherlands and Poland) a decline was observed (data not shown).

Table 1

Summary of tuberculosis treatment outcome categories according to the WHO/IUATLD recommendations and the ECDC surveillance report 2015

Categories	Definitions
Cured	Treatment completion and culture-negative samples taken at the end of treatment and on at least one previous occasion.
Completed	Treatment completed, but case does not meet the criteria to be classified as cured or treatment failure.
Failed	Culture or sputum smear remaining positive or becoming positive again five months or later into the course of treatment.
Died	Death before cured or treatment completion, irrespective of cause.
Defaulted	Treatment interrupted for two months or more, not resulting from a decision of the care provider.
Transferred out	Patient referred to another clinical unit for treatment and information on outcome not available.
Still on treatment	Patient still on treatment at 12 months and at 24 months for MDR-TB cases without any other outcome during treatment.
Unknown outcome	Information on outcome not available, for cases not known to have been transferred.
Groups created for the purpose of this study	
Unsatisfactory outcome	The percentage of cases that interrupted treatment, were transferred or failed treatment out of all notified.
Successful outcome/treatment success	The percentage of cases that were cured or completed treatment out of all notified.
Unsuccessful treatment	The percentage of cases that were not cured or completed treatment out of all notified (i.e. unsatisfactory outcome, death, still on treatment and unknown outcome).

ECDC: European Centre for Disease Prevention and Control; IUATLD: International Union against Tuberculosis and Lung Disease; WHO: World Health Organization.

Factors associated with unsuccessful treatment

In the multivariable logistic regression model, unsuccessful treatment was significantly associated with increasing age of the case (adjusted OR (aOR) = 1.02 per a one-year increase, 95%CI: 1.02–1.02), male sex (aOR = 1.40, 95%CI: 1.28–1.52) and foreign origin (aOR = 1.32, 95%CI: 1.03–1.70). The strongest association with unsuccessful treatment was observed with MDR-TB (aOR = 8.7, 95%CI: 5.09–14.97). No association was found between unsuccessful treatment and reporting period (aOR = 0.96, 95%CI: 0.71–1.30) (Table 3). In a separate analysis using a multivariable multinomial model, we did not find any difference in factors associated with death and those associated with the other unsuccessful treatment outcomes i.e. unsatisfactory outcome, still on treatment and unknown outcome (data not shown).

Discussion

This study investigated TB TO and factors associated with unsuccessful treatment in the EU/EEA over 10 years following the WHO/IUATLD recommendations for cohort analysis. It shows that the overall treatment success rate of new culture-confirmed pulmonary TB cases in the EU/EEA was 78.2% and failed to reach the 85% target in any year between 2002 and 2011. The study also indicates that main factors associated with unsuccessful treatment were increasing age, male sex, foreign origin and MDR-TB.

Still some countries do not provide information on TO to TESSy and for those who reported on TO, the proportion of cases with unknown outcome remained high at 4.0% overall. It is true that an unknown TO does not necessarily represent a negative one, yet from a programmatic perspective, lack of knowledge about TO deprives the programme from important information to guide TB control. Moreover, no significant improvement in treatment success rate was observed over the 10-year study period. These findings demonstrate a programmatic weakness within TB control in the EU/EEA and highlight the urgent need for strengthening the monitoring and evaluation process at country level [8].

Another analysis of data in TESSy reported by 22 EU/EEA countries in 2007, showed a treatment success rate of 79.5% for new culture-confirmed pulmonary cases [8]. A meta-analysis of published reports of TB TO in Europe found that 74.4% of outcomes were successful [9]. Another study conducted in 18 EU/EEA countries in 2005, showed a mean success rate of only 69% [10]. These results need to be compared with caution due to the different definition of cohort used and the different study periods [11]. Higher treatment success rates were reported in low incidence countries in North America, with 89% in the United States (US) in 2013, and with 86% in 2012 in Canada [12,13].

In the multivariable regression model, male sex was independently associated with unsuccessful treatment. The lower treatment success rate in men compared

Table 2 A*

Characteristics of new pulmonary culture-positive tuberculosis cases in the EU/EEA[a], 2002–2011 (n=250,854)

Demographic and clinical features of TB cases	Number of cases (n = 250,854 cases)	Percent
Sex		
Female	82,336	32.8
Male	168,442	67.2
Unknown	76	0.03
Age group		
<15	3,477	1.4
15–44	125,800	50.2
45–64	79,815	31.8
>64	41,718	16.6
Unknown	44	0.02
Geographical origin		
Native cases	202,129	80.6
Cases of foreign origin	37,471	14.9
Unknown	11,254	4.5
HIV status		
Positive	623	0.3
Negative	14,889	5.9
Unknown	235,342	93.8
Multidrug-resistance		
No	125,880	50.2
Yes	3,597	1.4
Unknown	121,377	48.4
EU/EEA countries		
Countries with low TB incidence rate[b]	82,711	33.0
Countries with high TB incidence rate[c]	168,143	67.0
Countries reporting treatment outcome for the whole study period		
Austria	4,030	1.6
Belgium	4,922	2.0
Czech Republic	4,911	2.0
Denmark	1,928	0.8
Estonia	2,771	1.1
Germany	24,195	9.7
Hungary	6,347	2.5
Ireland	1,704	0.7
Iceland	50	0.02
Latvia	7,727	3.1
Malta	91	0.1
The Netherlands	5,205	2.1

EU: European Union; EEA: European Economic Area; TB: tuberculosis; UK: United Kingdom.

[a] Including 18 countries that reported treatment outcome for the whole study period: Austria, Belgium, Czech Republic, Denmark, Estonia, Germany, Hungary, Ireland, Iceland, Latvia, Malta, the Netherlands, Norway, Poland, Romania, Slovenia, Slovakia and the United Kingdom.

[b] Countries with low TB incidence rate (<20,000/100,000 population) included Austria, Belgium, Czech Republic, Denmark, Germany, Hungary, Ireland, Iceland, Malta, the Netherlands, Norway, Slovenia, Slovakia and UK.

[c] Countries with high TB incidence rate (≥20,000/100,000 population) included Estonia, Latvia, Poland and Romania.

Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

Table 2 B*

Characteristics of new pulmonary culture-positive tuberculosis cases in the EU/EEA[a], 2002–2011 (n=250,854)

Demographic and clinical features of TB cases	Number of cases (n = 250,854 cases)	Percent
Countries reporting treatment outcome for the whole study period		
Norway	1,216	0.5
Poland	44,577	17.8
Romania	113,068	45.1
Slovenia	1,679	0.7
Slovakia	2.553	1.0
UK	23,880	9.5
Treatment outcome		
Success (cured or treatment completed)	196,105	78.2
Died	16,222	6.5
Still on treatment (12 months follow-up)	5,914	2.4
Failed	5,583	2.2
Defaulted	14,392	5.7
Transferred out	2,653	1.0
Unknown outcome	9,985	4.0

EU: European Union; EEA: European Economic Area; TB: tuberculosis; UK: United Kingdom.

[a] Including 18 countries that reported treatment outcome for the whole study period: Austria, Belgium, Czech Republic, Denmark, Estonia, Germany, Hungary, Ireland, Iceland, Latvia, Malta, the Netherlands, Norway, Poland, Romania, Slovenia, Slovakia and the United Kingdom.

[b] Countries with low TB incidence rate (<20,000/100,000 population) included Austria, Belgium, Czech Republic, Denmark, Germany, Hungary, Ireland, Iceland, Malta, the Netherlands, Norway, Slovenia, Slovakia and UK.

[c] Countries with high TB incidence rate (≥20,000/100,000 population) included Estonia, Latvia, Poland and Romania.

Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

with women was mostly attributed to a higher proportion of died, failed and defaulted in men compared with women, which persisted when stratifying by reporting year, country of origin and age group (with exception among cases aged <15 years). Another study done in the EU reported also that the success rate was higher in women and this was due to a greater occurrence of defaulted and treatment failure in men [10]. A study from South Africa found that men were less likely to adhere to their treatment than women and male sex was associated with the risk of treatment interruption [14]. The poorer TO in men can be attributed to some behavioural components such as alcohol and drug abuse, which are still predominant among men. Studies done in Paris [15] and Hamburg [16] indicated that unsuccessful TB treatment was associated with alcohol and injecting drug use. A Spanish study showed that injecting drug use was associated with treatment default; while alcoholism was associated with death during TB treatment [17]. According to the European

Table 3

Factors associated with treatment outcome among new culture-confirmed pulmonary tuberculosis cases, 2002–2011 (n=250,854)

Predicting factors	Treatment success (totals)		Univariable analysis[a]		Multivariable analysis[a,b]	
	Yes	No	OR (95% CI)	p value	AOR (95% CI)	p value
Increase in age per year (continues variable)	196,895	53,959	1.02 (1.01–1.02)	<0.001	1.02 (1.02–1.02)	<0.001
Sex						
Female	67,512	14,824	1	<0.001	1	<0.001
Male	129,323	39,119	1.38 (1.27–1.49)		1.40 (1.28–1.52)	
Country of origin						
Native cases	159,617	42,512	1	0.72	1	0.03
Cases of foreign origin	29,151	8,320	1.07 (0.74–1.56)		1.32 (1.03–1.70)	
Multidrug-resistance						
No	99,666	26,214	1	<0.001	1	<0.001
Yes	1,252	2,345	7.12 (4.07–12.44)		8.7 (5.09–14.97)	
Unknown	95,977	25,400	1.00 (0.77–1.32)	0.96	1.08 (0.86–1.36)	0.51
Reporting year						
2002 to 2006	105,566	29,445	1	0.73	1	0.81
2007 to 2011	91,329	24,514	0.96 (0.77–1.19)		0.96 (0.71–1.30)	

EU: European Union; EEA: European Economic Area; MDR: multidrug-resistant; TB: tuberculosis.

[a]Univariable and multivariable analysis were performed using logistic regression models specifying that standard errors allow for intra-country correlation (controlling for clustering within countries).

[b]Tuberculosis cases with available information for all predicting factors were included in the multivariable analysis (n = 239,485/250,854).

The logistic regression models were based on 12-month outcomes for non-MDR TB cases and on 24-month outcomes for MDR-TB cases.

Source: The European Surveillance System (TESSy). TESSy was operational since 2008 and for data from 2002-2007 data were transferred into TESSy from the historical database of the former EuroTB project for the TB surveillance activities in Europe.

Monitoring Centre for Drugs and Drug Addiction, drug use and alcohol consumption are considerably more common among men than women in EU countries [18]. Biological factors may also contribute to the different TB TO between males and females. Animal models showed that male mice developed more severe TB disease, while females were more resistant and exhibit more robust immune responses to infection [19].

In our study, the proportion of cases with unsatisfactory outcomes was 8.9%; thus below the maximum proportion of 10% in the WHO/IUATLD recommendations. This threshold aims to serve National TB Programmes as an orientation to direct their efforts in improving TO [4]. Three countries: Hungary, Poland and Romania, exceeded the 10% proportion of unsatisfactory outcomes. The proportion of deaths (any cause) was on average 6.5%. A high proportion of deaths among pulmonary TB cases (10%) was also reported by Canada in 2012 [13].

An inverse relationship between treatment success rate and increasing age was observed in this study. This can be partially explained by the increasing death rate with advancing age; as a result of demographic changes with an ageing population. However, studies from high- and low-incidence countries reported that older age also increases the risk of unsuccessful TB treatment [20-22]. The elderly are at increased risk for missed diagnosis [23] due to the fact that a diagnosis of TB among the elderly is often difficult due to atypical, non-specific clinical manifestation and may be confused with other concomitant age-related diseases [24,25]. This can lead to a delayed diagnosis and more advanced disease at presentation which in turn leads to increased mortality among the elderly [24,25]. A study in Norway demonstrated that deaths occurred mainly because TB diagnosis was established too late and half of the cases were only detected at autopsy [26]. Also a study from the US found that advanced age was strongly associated with unrecognised pulmonary TB leading to premature death [27]. Therefore, a high index of suspicion for TB in the elderly is undoubtedly justifiable [23]. Most recently, the *WHO Framework towards TB elimination in low-incidence countries* highlighted the particular challenges in treating active TB in the elderly and the importance of early diagnoses among them [28]. Furthermore, screening for active TB among the elderly has been suggested by WHO depending on the local TB epidemiology and risk-benefit assessments [28]. At present in Japan, early case detection and treatment are considered as the most effective TB control measures among the elderly in order to protect them from TB death [29].

In our analysis, MDR-TB was the strongest risk factor for unsuccessful treatment and treatment success rate of MDR-TB cases at 24-month follow-up was 49.2%, far

below the 70% target, a sign of the seriousness of the MDR-TB epidemic in the EU/EEA. It is well documented that treatment failure and mortality are higher among MDR-TB cases than among susceptible cases [30,31].

Although we found only a slight difference in treatment success rate in native and foreign cases, being of foreign origin was a significant risk factor for unsuccessful TO in our analysis. Markedly, a high proportion of cases of foreign origin had unknown outcome. It is unclear to which extent this might be due to migration for medical reasons. Overall, this result suggests that programmatic issues may play a role including access to healthcare in the context of mobility e.g. patients returning to their country of origin before the treatment is completed, as was observed in a study from London [32], and challenges in cross-border collaboration. Thus, the 61st World Health Assembly in 2008 called on countries to address migrant health issues in a more integrated, harmonised approach [33]. Foreign origin may be a proxy for other unmeasured indicators related to migration. In the EU, migrants have been reported to be at risk of not receiving the same level of healthcare in the preventive, diagnostic and treatment services as the native communities [34]. This might be due to a combination of factors including legal and working status, social exclusion, substandard economic condition and barriers in accessing healthcare services [34].

Limitations

This study has a number of limitations. A considerable proportion of reported cases was without information on TO and was therefore excluded from the analysis. Following the WHO/IUATLD recommendation for TO monitoring in the EU/EEA, only new culture-confirmed pulmonary TB cases were eligible for the analysis cohort, accounting for 42% of reported cases. Furthermore, a higher treatment success rate was found among included cases compared to excluded ones. Our results thus reflect the situation of the defined cohort and might overestimate the overall treatment success rate in the EU/EEA. A possible selection bias related to reporting countries can be noticed; some countries were overrepresented in our cohort e.g. Romania accounted for 45% of the cases included. Information on drug susceptibility testing results was missing for almost half of cases, some of whom may have been MDR-TB. Due to the unavailability of cause of death information in the database, we could not distinguish between cases who died from TB or due to other causes during TB treatment. This would be of particular importance for analysing TO among the elderly. With the data currently reported to TESSy, it is not possible to investigate some factors known to be associated with TB TO such as comorbidity (like HIV infection), drug or alcohol abuse, homelessness and other socioeconomic factors. These underlying factors are important for mapping risk groups in order to improve TB prevention and access to TB services, since TB is concentrated in certain at-risk groups in most low-incidence countries [28]. Poor implementation of TB programme guidelines in some EU/EEA countries might partially account for suboptimal TO, especially among MDR-TB cases. However, the degree of implementation of guidelines within countries and the appropriateness of the guidelines are beyond the scope of this paper.

Conclusion

In conclusion, shortcomings in TO reporting remain a challenge in monitoring TB control in the EU/EEA. The treatment success rate was 78%, below the global target of 85% for the decade after 2002. Special efforts are required for patients with MDR-TB, who have particularly low treatment success rates. For elderly patients, a high index of clinical suspicion for TB is required to ensure early diagnosis and treatment of TB [24], and improve TO among them. Finally, to allow for valid monitoring at EU/EEA level, all countries should aim to report TO for all TB cases. Collecting additional variables on social determinants, alcohol and drug abuse, and co-morbidities could increase our understanding of the factors related to TB TO and support the design of appropriate interventions.

*Erratum

Table 2 had to be replaced due to technical issues on 11 December 2015.

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Conflict of interest

The authors declare that they have no competing interests.

Authors' contributions

Concept and design (BK, WH, BH, MvdW), statistical analysis (BK), interpretation of the data (BK, VH), drafting the manuscript (BK) and critical revision of the manuscript for important intellectual content (BK, WH, BH, MvdW, VH, LF). All authors read and approve the final manuscript.

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Research article

Meningococcal vaccine antigen diversity in global databases

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The lack of an anti-capsular vaccine against serogroup B meningococcal disease has necessitated the exploration of alternative vaccine candidates, mostly proteins exhibiting varying degrees of antigenic variation. Analysis of variants of antigen-encoding genes is facilitated by publicly accessible online sequence repositories, such as the *Neisseria* PubMLST database and the associated Meningitis Research Foundation Meningococcus Genome Library (MRF-MGL). We investigated six proposed meningococcal vaccine formulations by deducing the prevalence of their components in the isolates represented in these repositories. Despite high diversity, a limited number of antigenic variants of each of the vaccine antigens were prevalent, with strong associations of particular variant combinations with given serogroups and genotypes. In the MRF-MGL and globally, the highest levels of identical sequences were observed with multicomponent/multivariant vaccines. Our analyses further demonstrated that certain combinations of antigen variants were prevalent over periods of decades in widely differing locations, indicating that vaccine formulations containing a judicious choice of antigen variants have potential for long-term protection across geographic regions. The data further indicated that formulations with multiple variants would be especially relevant at times of low disease incidence, as relative diversity was higher. Continued surveillance is required to monitor the changing prevalence of these vaccine antigens.

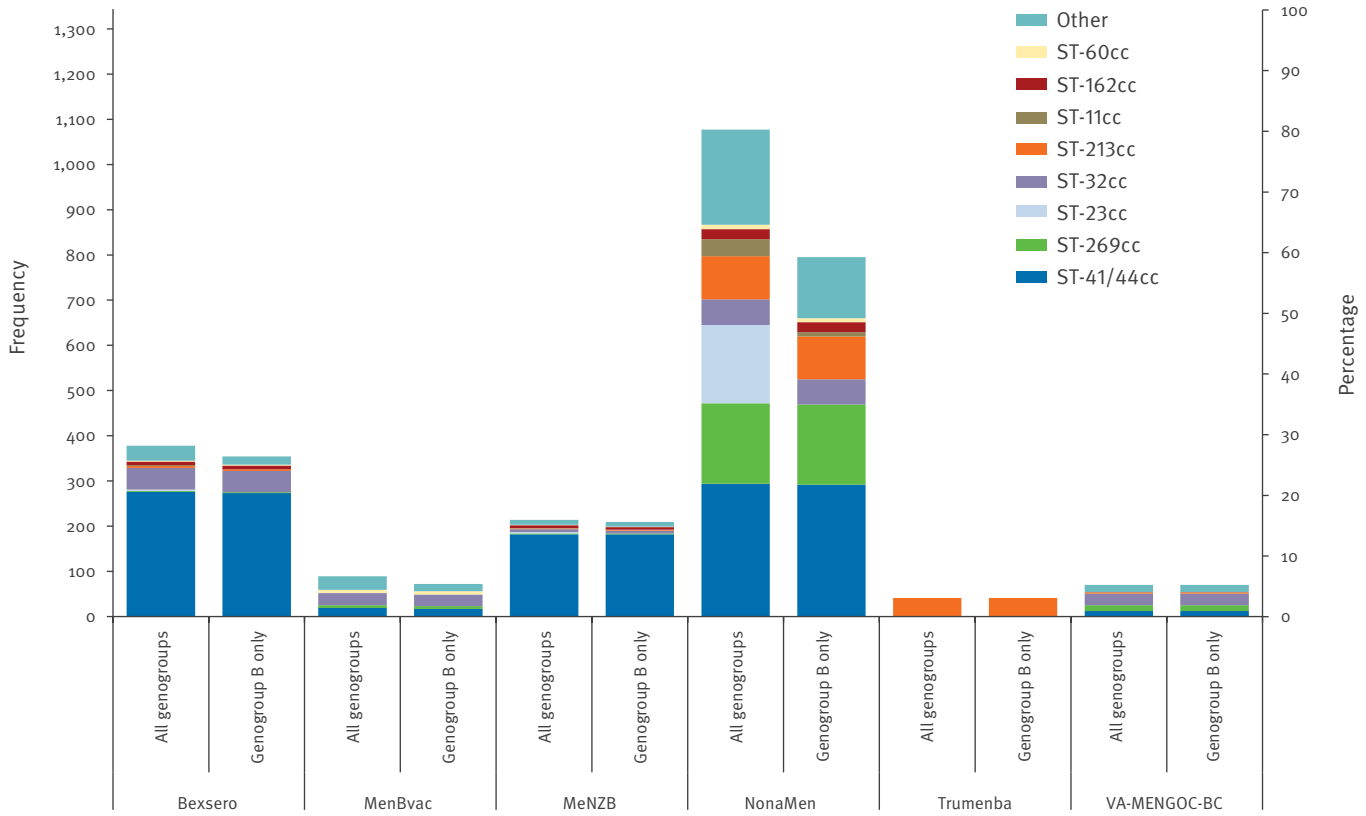
Introduction

Neisseria meningitidis, the meningococcus, a Gram-negative diplococcus, is a globally important causative agent of meningitis and septicaemia (severe sepsis), accounting for a significant amount of morbidity and mortality worldwide. However, it is frequently carried harmlessly in the human nasopharynx and can be considered part of the normal human commensal microbiota. Currently, no comprehensive vaccine exists against meningococcal disease due in large part to the structural similarity of serogroup B polysaccharide to polysaccharides associated with the human neural cell adhesion molecule (NCAM). This is thought to account for the poor human immune response against group B polysaccharide and also raises safety concerns [1]. Many subcapsular vaccine antigen candidates, especially proteins, have therefore been investigated, with the intention of producing serogroup B substitute formulations. Several such antigens have been incorporated into vaccine formulations that are in various stages of development.

First developed in the 1980s, outer membrane vesicle (OMV) vaccines were created to counter higher levels of disease incidence caused by particular serogroup B meningococci. These OMV vaccines contained the respective epidemic antigen variants of the outer membrane protein PorA of these meningococci as major immunogens and were successfully deployed in Norway (MenBvac), Cuba (VA-MENGOC-BC), and New Zealand (MeNZB) [2-4]. In the last decade however, many high-income regions such as Europe and North America have experienced a period of relatively low incidence of serogroup B meningococcal disease [5,6]. In such periods, when disease incidence is lower, but caused by more diverse meningococci, vaccines should ideally contain several components in order to attain the widest possible strain coverage. An example is the proposed NonaMen (RIVM, the Netherlands) vaccine formulation comprising nine PorA variants corresponding to the most prevalent disease-associated strains [7]. Alternatively, Bexsero, developed by Novartis, is a supplemented OMV vaccine which contains four components: PorA P1.7–2, 4, fHbp subvariant 1.1, NHBA variant 2, and NadA-3.8 subvariant [8]. This was licenced in Europe in 2013 and in the United States (US) in 2015 and has been included in the infant immunisation schedule in the United Kingdom (UK) since September 2015. The rLP2086 vaccine, Trumenba developed by Pfizer, which was licenced by the Food and Drug Administration in the US in 2014, is a bivalent recombinant vaccine based on two fHbp antigens from

Figure 1

Distribution of clonal complexes in meningococcal disease isolates in the MRF-MGL collection with exact peptide matches to at least one antigen of various vaccines, England and Wales, 2010/11–2012/13 (n = 1,344)



MRF-MGL: Meningitis Research Foundation Meningococcus Genome Library.

Invasive meningococcal disease isolates from England and Wales from epidemiological years 2010/11 to 2012/13 inclusive of all genogroups or genogroup B only. 'Other' includes uncommon ccs and unassigned STs. Secondary y-axis indicates percentage of total number of isolates.

subfamily A and B (subvariants 3.45 and 1.55 respectively) [9].

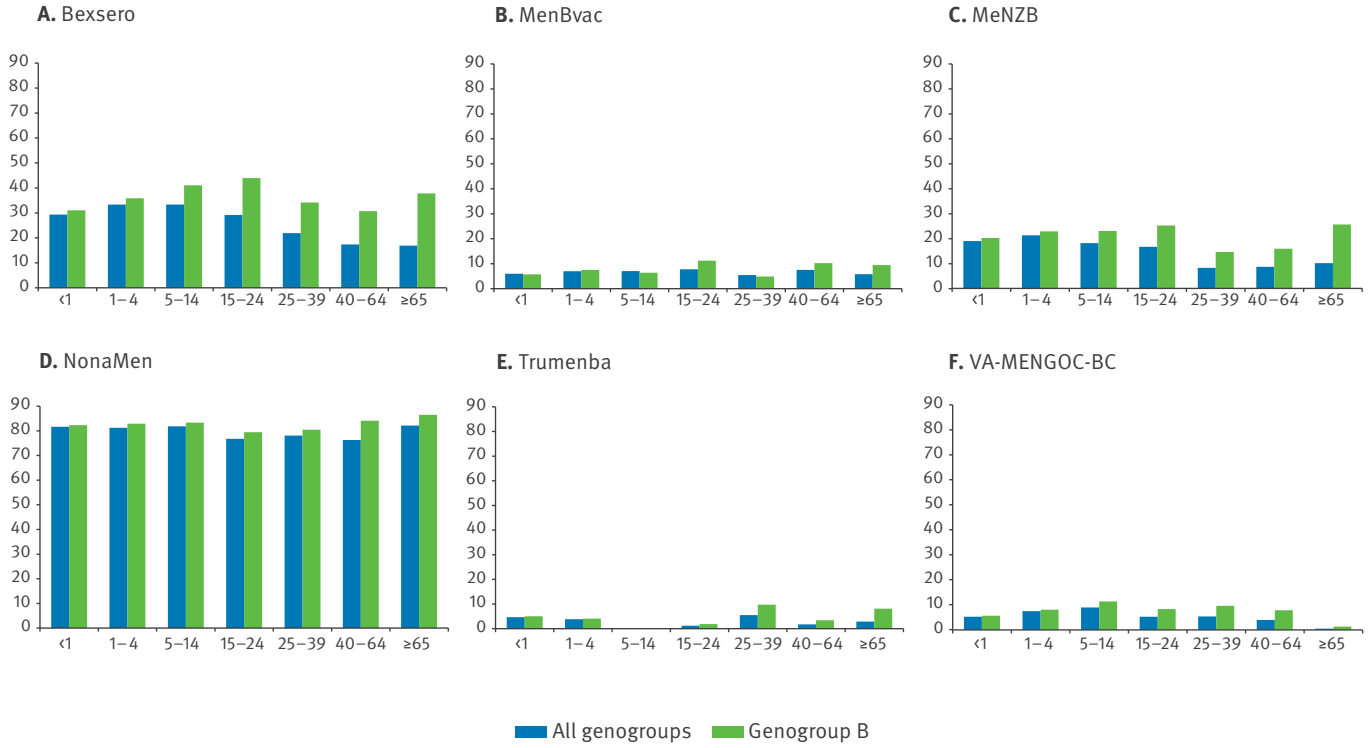
Over the past two decades, sequence-based molecular typing has become an intrinsic part of meningococcal disease surveillance and standardised typing methods and schemes have allowed for more comparability across reference and research laboratories in different countries [10-12]. For example, the European surveillance system (TESSy) of the European Centre for Disease Control (ECDC) (http://www.ecdc.europa.eu/en/activities/surveillance/Tessy) and the European Meningococcal Epidemiology in Real Time (EMERT) database (http://emgm.eu/emert/) include two typing antigens which are also vaccine candidates, PorA and FetA. Following the advent of whole genome sequencing (WGS) and its rapidly reducing costs, comprehensive investigations of the likely and actual impact of available or potential interventions may be made more easily. Publicly accessible online resources such as the *Neisseria* PubMLST database (http://pubmlst.org/neisseria/) and the Meningitis Research Foundation Meningococcus Genome Library (MRF-MGL) (http://www.meningitis.org/research/genome), which contain molecular typing information from single genes up to many hundreds, and for many thousands of isolates, allow fine-scaled analyses, including investigation of the distribution of vaccine components. In this study, the PubMLST and MRF-MGL databases were used in an investigation of the distribution of vaccine components in Bexsero, Trumenba, NonaMen, MenBvac, MeNZB and VA-MENGOC-BC.

Methods

This study made use of the public *Neisseria* PubMLST database http://pubmlst.org/neisseria/ and the MRF-MGL http://www.meningitis.org/research/genome which is hosted within it. The databases were accessed in August 2014. The MRF-MGL contained 1,344 *N. meningitidis* isolates which were all from England and Wales, covering all culture-confirmed cases of invasive meningococcal disease (IMD) from the epidemiological years 2010/11 to 2012/13. From the PubMLST database, we included 1,717 *N. meningitidis* isolates within the database which had assembled sequence data of at least 0.5 Mbp. This is the minimum amount of assembled sequence data that allows as complete an analysis of vaccine antigen distribution as possible. This

Figure 2

Percentage distribution of age groups in meningococcal disease isolates in the MRF-MGL collection with exact peptide matches to at least one antigen of various vaccines, England and Wales, 2010/11–2012/13 (n = 1,344)



MRF-MGL: Meningitis Research Foundation Meningococcus Genome Library.

Invasive meningococcal disease isolates from England and Wales from epidemiological years 2010/11 to 2012/13 inclusive of all genogroups and genogroup B only.

multinational collection contained data on disease and carriage isolates from the years 1937 to 2014, from 53 countries, six continents and excluded MRF-MGL isolates (Table 1).

The PubMLST database is a publicly-accessible repository of isolate and typing information for several species including *N. meningitidis*. It contains a large set of global records of *Neisseria* genus isolates (33,019 in March 2014) spanning 100 years. It is a complete catalogue of known genotypic and phenotypic variation, date and location of isolation, and permits an estimation of the minimum lifespan of particular genotypes and deduced antigenic types. While the collection is not an epidemiologically coherent sample set as a whole, it does include several such datasets (including the MRF-MGL), and permits several types of investigation into the evolution and population biology of *N. meningitidis*.

The presence of components of each of the fHbp-containing vaccines, were analysed in the collections [8,9]. Before the development of a unified nomenclature scheme in which each unique allele is assigned a unique numerical identifier [13], separate schemes were developed which divided fHbp into either two subfamilies (subfamily A and B) or three variant families (variant families 1, 2 and 3) according to nomenclature system [14,15]. These schemes can be cross-referenced online (http://pubmlst.org/neisseria/fHbp/). Briefly, subfamily B is equivalent to variant family 1 and subfamily A incorporates both variant families 2 and 3. Peptides are then numbered with the variant family/subfamily name e.g. fHbp 1.1 is variant family 1 peptide 1 [13]. This is the fHbp nomenclature used throughout this paper. The recombinant vaccine Trumenba contains two fHbp antigen subvariants: peptide 45 (Pfizer nomenclature A05, subfamily A/variant family 3) and peptide 55 (Pfizer nomenclature B01, subfamily B/variant family 1). As well as Bexsero and Trumenba, other vaccine formulations analysed were: NonaMen, which contains nine PorA antigens variants (P1.7,16; P1.5–1,2–2; P1.19,15–1; P1.5–2,10; P1.12–1,13; P1.7–2,4, P1.22,14; P1.7–1,1; P1.18–1,3), MenBvac, (P1.7,16), MeNZB (P1.7–2,4) and VA-MENGOC-BC (P1.19,15) [4,7,16,17]. Analysis of the distribution of vaccine components among clonal complexes and patient age groups in the MRF-MGL was based on an exact match of deduced peptide sequences ('sequence match') to at least one component of each vaccine formulation investigated. The analysis was carried out on isolates of all genogroups (organisms with a *cps* region, encoding a capsule) and genogroup B isolates only (those containing a *cps* region encoding the group B polysaccharide capsule). For the purposes of

this analysis is was assumed that such meningococci either had the capacity to express a capsule or had a very closely related ancestor which could [18]. It should be noted that OMV vaccines include other potentially immunogenic proteins not assessed here, although WGS allows for such analyses.

Simpson's index of diversity (*D*) was used to determine the diversity of each Bexsero vaccine antigen by age group in the MRF-MGL. The value of the index ranged from 0 to 1, with values nearer to 1 indicating greater diversity. Calculation of the index was performed as described previously [19]. The 95% confidence intervals (CIs) for the index were calculated as described previously [20].

Results

Current vaccine antigens in the MRF-MGL collection: invasive meningococcal disease isolates from England and Wales, 2010/11 to 2012/13

Within the MRF-MGL collection, NadA was significantly the least diverse peptide antigen examined ($D = 0.39$; 95% CI: 0.36–0.42) compared with: PorA variable region 1 (VR1) ($D = 0.87$; 95% CI: 0.86–0.88), PorA VR2 ($D = 0.92$; 95% CI: 0.92–0.93), fHbp ($D = 0.91$; 95% CI: 0.91–0.92) and NHBA ($D = 0.91$; 95% CI: 0.91–0.92). However, a total of 1,025 (76.3%) isolates were inferred to be missing the *nadA* gene as it was not found in their genomes (Table 2). A total of 1,102 of 1,344 (82.0%) isolates contained exact peptide sequence matches to components of any of the vaccines investigated (Figure 1). Isolates belonging to the two most prevalent clonal complexes (cc) in the MRF-MGL, ST-41/44cc and ST-269cc, had most sequence matches (302 and 181 instances, respectively). Exact matches to NonaMen components were found in 20 different clonal complexes. In total there were 1,077 of 1,344 isolates (80.1%) with genomes that encoded the PorA VR1 or VR2 antigen variants included in the NonaMen formulation. ST-41/44cc isolates comprised 294 of these 1,077 (27.3%), a consequence of their association with PorA subtype P1.7–2,4.

Unlike the other vaccine formulations, exact matches to Trumenba vaccine components were found in a single clonal complex (ST-213cc). Exact amino acid sequence matches to at least one of the four Bexsero vaccine antigen variants were present in 378 of 1,344 (28.1%) isolates, which belonged to 10 clonal complexes. Again, ST-41/44cc contained the majority of matches (276 isolates) due to its association with NHBA peptide 2 and PorA VR2 P1.4, but only two isolates of ST-269cc contained an exact match to any Bexsero antigen peptide variant. None of the major hyperinvasive clonal complexes, except for a single genogroup A ST-5cc isolate, contained an exact match to NadA subvariant 8, which was found in 13 ST-174cc isolates. However, 65.7% of ST-32cc isolates contained Bexsero fHbp variant family 1 peptide 1, consistent with derivation of this component from MC58, an isolate of the same complex [21].

In total, 37.0% of genogroup B isolates contained at least one exact match to any Bexsero vaccine component. For the other vaccines, the proportion of isolates with at least one exact match to vaccine antigen variants were NonaMen: 82.7%, MeNZB: 21.8%, MenBvac: 7.5% and VA-MENGOC-BC: 7.3%. When cross-protection among NadA peptide alleles belonging to variants NadA-1 and NadA-2/3 and among fHbp peptide alleles belonging to family 1 was assumed, the overall number of isolates containing an exact match to Bexsero antigen variants more than doubled to 847 isolates, or 63.0% of the MRF-MGL.

Vaccine antigen distribution by age group

Vaccine peptide diversity and distribution in the MRF-MGL were assessed by age group (Table 2, Figure 2). For all antigens, peptide diversity was lowest among meningococci isolated from patients younger than one year or 65 years and older. Significant differences in peptide diversity, those where the 95% CIs did not overlap, were seen. For example, fHbp diversity ($D = 0.93$; 0.91–0.95) was significantly greater in isolates from patients aged 25 to 29 years than in isolates from patients 65 years and older ($D = 0.85$; 0.81–0.89). Apart from isolates from patients 65 years and older, where PorA P1.7–2 predominated, and those from patients 40 to 64 years of age, where NHBA and PorA P1.7–2 were equally prevalent (15 occurrences), NHBA-2 was the most frequently identified Bexsero antigen peptide in all age categories (data not shown). PorA P1.7–2 was the second most prevalent vaccine subvariant in age groups 25 to 39 years and younger. The fHbp 1.1 subvariant was found at the lowest proportions in patients younger than one year and in those 65 years and older. NadA-3.8 was absent from isolates from patients aged one to four years and 15 to 24 years, and was identified in less than 2% of isolates from all other age groups except the group 65 years and older, where it was present in 3.9% of isolates. Thus, isolates possessing at least one of the four Bexsero antigen peptide variants were found at the highest proportions in patients aged one to four and five to 14 years (114/315 (36.2%) and 34/99 (34.3%) isolates, respectively), and at the lowest proportions in patients aged 65 years and older (37/207 (17.9%) isolates). The greatest proportion of genogroup B isolates with at least one exact amino-acid sequence match was identified in patients aged 15 to 24 years (48/107 (44.9%) genogroup B isolates), and the least was in patients younger than one year (88/277 (31.8%) genogroup B isolates). The association of ST-41/44cc and less common lineages with patients younger than one year meant that 30.3% of all isolates contained at least one exact peptide match. Isolates possessing at least NadA-1 or NadA-2/3, fHbp variant family 1, PorA P1.4, or NHBA-2 allele were found at the highest proportion in patients aged five to 14 years (72/99 (72.7%) isolates). Of the vaccine formulations

Table 1

Characteristics of the PubMLST global *Neisseria meningitidis* collection, 1937–2014 (n = 1,717)

Sample source	Number of isolates
Disease	926
Carriage	112
Unknown	679

Serogroup	Number of isolates
Unknown	596
W	413
B	278
C	256
A	82
Y	46
NG	40
E	2
X	2
Z	2

Continent	Number of isolates
Europe	1,373
Africa	234
North America	49
Asia	30
Unknown	15
South America	10
Oceania	6

Country	Number of isolates
United Kingdom	1,143
South Africa	178
Czech Republic	53
Greece	47
Norway	36
United States	25
Canada	22
Ireland	20
Unknown	15
Spain	15
Niger	13
Burkina Faso	12
Germany	12
The Netherlands	12
France	11
China	11
Cameroon	8
Malta	7
Turkey	6
Brazil	5
Chile	4
Denmark	4
New Zealand	4
Algeria	3
Chad	3
Gambia	3
Italy	3
Mali	3
Morocco	3
Portugal	3
Russia	3
Senegal	3
Australia	2
Cuba	2
Finland	2
Ghana	2
Iceland	2
India	2
Japan	2
Philippines	2
Argentina	1
Austria	1
Djibouti	1
Israel	1
Ivory Coast	1
Pakistan	1
Poland	1
Saudi Arabia	1
Sudan	1
Switzerland	1
Thailand	1

Year of isolation	Number of isolates
Unknown	319
1937	1
1940	1
1941	1
1947	1
1962	1
1963	5
1964	3
1965	1
1966	5
1967	3
1968	2
1969	3
1970	8
1971	1
1973	1
1974	2
1975	9
1976	6
1977	4
1978	4
1979	5
1980	4
1981	1
1982	1
1983	3
1984	6
1985	20
1986	10
1987	9
1988	19
1989	6
1990	5
1991	4
1992	5
1993	58
1994	7
1995	1
1996	39
1997	35
1998	62
1999	51
2000	100
2001	101
2002	34
2003	52
2004	56
2005	38
2006	36
2007	41
2008	29
2009	42
2010	33
2011	168
2012	167
2013	69
2014	19

Clonal complex	Number of isolates
ST-11cc	677
ST-41/44cc	154
ST-269cc	102
ST-32cc	88
ST-23cc	79
ST-213cc	52
ST-1cc	47
ST-22cc	46
ST-60cc	44
ST-53cc	35
ST-1157cc	28
ST-35cc	22
ST-5cc	21
ST-174cc	19
ST-8cc	19
ST-167cc	18
ST-162cc	15
ST-4cc	14
ST-198cc	10
ST-865cc	10
ST-18cc	9
ST-254cc	8
ST-103cc	6
ST-4821cc	5
ST-1136cc	4
ST-1117cc	3
ST-178cc	3
ST-334cc	3
ST-364cc	3
ST-461cc	3
ST-181cc	2
ST-282cc	2
ST-37cc	2
ST-613cc (lactamica)	2
ST-175cc	1
ST-212cc	1
ST-231cc	1
ST-4240/6688cc	1
ST-750cc	1
Unassigned STs	157

cc: clonal complex.

Table 2

Allelic diversity of Bexsero vaccine antigens per patient age group, MRF-MGL invasive meningococcal disease isolate genomes, England and Wales, 2010/11–2012/13 (n = 1,344)

Age (years)	NadA D (95% CI)	NHBA D (95% CI)	FHBP D (95% CI)	PorA VR1 D (95% CI)	PorA VR2 D (95% CI)
<1 (n = 300)	0.31 (0.24–0.38)	0.88 (0.86–0.91)	0.89 (0.87–0.91)	0.82 (0.78–0.85)	0.88 (0.86–0.90)
1–4 (n = 315)	0.39 (0.33–0.46)	0.88 (0.86–0.90)	0.90 (0.88–0.92)	0.84 (0.81–0.87)	0.92 (0.91–0.94)
5–14 (n = 99)	0.33 (0.21–0.44)	0.91 (0.88–0.94)	0.91 (0.88–0.94)	0.88 (0.85–0.92)	0.91 (0.89–0.94)
15–24 (n = 168)	0.42 (0.33–0.52)	0.90 (0.88–0.92)	0.90 (0.88–0.92)	0.88 (0.85–0.90)	0.93 (0.92–0.95)
25–39 (n = 73)	0.50 (0.36–0.64)	0.92 (0.90–0.95)	0.93 (0.91–0.95)	0.84 (0.79–0.90)	0.93 (0.91–0.96)
40–64 (n = 173)	0.42 (0.33–0.51)	0.91 (0.89–0.93)	0.90 (0.88–0.92)	0.87 (0.84–0.90)	0.92 (0.91–0.94)
≥ 65 (n = 207)	0.41 (0.32–0.49)	0.87 (0.84–0.90)	0.85 (0.81–0.89)	0.82 (0.78–0.86)	0.91 (0.89–0.92)
Total (n = 1,344)	**0.39 (0.36–0.42)**	**0.91 (0.91–0.92)**	**0.91 (0.91–0.92)**	**0.87 (0.86–0.88)**	**0.92 (0.92–0.93)**

CI: confidence interval; MRF-MGL: Meningitis Research Foundation Meningococcus Genome Library.
Diversity determined by Simpson's Diversity Index

assessed, NonaMen antigen peptide variants were the most frequently identified in isolates of all age groups (Figure 2), ranging from 76.3% of patients aged 40 to 64 years to 82.1% of patients 65 years and older. A total of 81.7% of all isolates and 82.3% of genogroup B isolates from patients younger than one year possessed at least one NonaMen PorA variant. The Trumenba vaccine antigen peptide subvariants fHbp 3.45 and fHbp 1.55 were not found in isolates from patients aged five to 14 years (n = 99).

Vaccine candidate antigen variants in PubMLST/neisseria database

There were 38 isolates in the PubMLST *Neisseria* collection with the fHbp 3.45 subvariant and none with the 1.55 subvariant (Table 3). A total of 36 of the 38 isolates that possessed the 3.45 subvariant were ST-213cc and were all from the UK. Those which had a date of isolation recorded in the database were from between 1999 and 2012. The Bexsero subvariant fHbp 1.1 was found in 73 isolates in the dataset. Of these, 66 were ST-32cc and 47 of 73 were serogroup B. They were found over a time span of 40 years from 1969 to 2009 and on all continents. NadA-3.8 was found in 41 isolates. Of those, 17 were ST-174cc and 16 were ST-5cc; 16 were serogroup Y, 16 serogroup A and five were serogroup B. They were found across 49 years from 1963 to 2012 and on all continents except Oceania. NHBA variant 2 was found in 121 isolates and 98 of these were ST-41/44cc. Of the 121 isolates for which serogroup data were recorded, 66 were serogroup B, 12 were serogroup C and 11 were non-groupable. NHBA variant 2 was found on all continents except Asia and Africa and across 37 years from 1976 to 2013.

Cross-protection among fHbp variant family 1 and NadA-1, NadA-2 and NadA-3 variant family members has been described [22,23], prompting an analysis of their distribution. There were 873 fHbp variant family 1 peptide variants and these were found across the whole time span of the collection (77 years) from 1937 to 2014 and on all continents. Of these, 349 (40.0%) were ST-11cc, 98 (11.2%) were ST-41/44cc, and unassigned sequence types (STs) accounted for 92 (7.4%) of the isolates. Of the 873, 241 were serogroup W, 159 were serogroup B, 113 were serogroup C and 76 were serogroup A. There were 709 isolates that were NadA-1, 2 and 3 variant family members. These spanned 51 years of the collection from 1963 to 2014 and were found on all continents. The majority were ST-11cc (n = 568; 80.1%), 368 were serogroup W and 135 were serogroup C.

Discussion

Since its introduction in the 1990s, sequence-based molecular typing has established a role in the clinical microbiology laboratory, replacing or complementing existing phenotypic typing methods. WGS is the latest sequencing technology and, as costs continue to decrease, will become more commonplace in clinical and reference laboratories [24-26]. WGS provides definitive sequence-level resolution with widespread applications including molecular epidemiology, surveillance, vaccine design and vaccine implementation monitoring [27]. To be useable by physicians and public health specialists, databases will need to use uniform nomenclature and be interoperable and compatible with other databases such as those that contain phenotypic information [28].

As WGS databases such as PubMLST are generic and scalable, they enable detailed deduction of potential coverage and preliminary assessment of the impact of given vaccine formulations on the meningococcal population, thus informing further work such as phenotypic assays [29]. This requires the assembly of representative collections of meningococcal isolate genomes. The MRF-MGL is an exemplar of a representative, contemporary, curated, publicly accessible database containing many hundreds of genomes and was expressly established as a resource for the meningococcal research and public health communities. It is embedded within the PubMLST database, which has been running for many years as a community resource

Table 3

Characteristics of Bexsero and Trumenba antigens in PubMLST *Neisseria* database

Antigen	Number of isolates	Dominant clonal complex(es) n (%)	Dominant serogroup(s) n (%)	Minimum lifespan (years)	Observed time period	Continents found
fHbp subvariant 3.45 (A05)[a]	38	ST-213: 36 (94.7)	Unassigned: 36 (94.7)	13	1999–2012	Europe
fHbp subvariant 1.55 (B01)[a]	0	NA	NA	NA	NA	NA
fHbp variant family 1/ subfamily B	873	ST-11: 349 (40.0), ST-41/44: 98 (11.2), Unassigned: 92 (7.4)	W: 241 (27.6), B: 159 (18.2), C: 113 (12.9), A: 76 (8.7)	77	1937–2014	Africa, Asia, Europe, North America, Oceania, South America
fHbp subvariant 1.1	73	ST-32: 66 (90.4)	B: 47 (64.4)	40	1969–2009	Africa, Asia, Europe, North America, Oceania, South America
NadA-3.8 subvariant	41	ST-174: 17 (41.5), ST-5: 16 (39.0)	Y: 16 (39.0), A: 16 (39.0), B: 5 (12.2)	49	1963–2012	Africa, Asia, Europe, North America, South America
NadA-1,2,3 variants	709	ST-11: 568 (80.1)	W: 368 (51.9), C: 135 (19.0)	51	1963–2014	Africa, Asia, Europe, North America, Oceania, South America
NHBA variant 2	121	ST-41/44: 98 (80.9)	B: 66 (54.6), C: 12 (9.9), NG: 11 (9.1)	37	1976–2013	Europe, North America, Oceania, South America

NA: not available; NG: non-groupable.

[a] A05, B01: Pfizer nomenclature.

and repository for isolate and characterisation data. The MRF-MGL is the most comprehensive epidemiological sample of meningococcal WGS currently available, allowing an assessment of vaccine antigen distribution among disease cases.

Molecular epidemiology using phenotypic or genotypic data has been used to inform vaccine design: tailor-made OMV vaccines were designed to contain the respective outbreak strain PorA variant [2-4]; and the broad-spectrum multivalent Hexamen/Nonamen formulation was based on the most prevalent PorA serosubtypes documented in the Netherlands at the time of its development [30]. One of the earliest uses of genome data in vaccine design was in the discovery of novel meningococcal vaccine candidates by 'reverse vaccinology' based on a single isolate genome [31]. Several of these genome-derived antigens are components of vaccines at various stages of clinical development and deployment at the time of writing [8,9].

The temporal and geographic spread of antigen variants and combination of variants, with some existing for long time periods and across several continents, demonstrated the stability of antigen clonal complex relationships and therefore the potential longevity of appropriate vaccine formulations [32,33]. Previous work using the PubMLST database collection [32] demonstrated the longevity of PorA VR types and their association with clonal complex which we here extend to other antigens available from WGS. For example, the most frequent strain type (PorA:FetA:cc) P1.5,2:F3–6:cc11 had a minimum lifespan of 49 years and was found on three continents (Europe, North America and South America) [32]. In the present analysis, multicomponent vaccines exhibited more potential to protect against isolates represented in the MRF-MGL than vaccine formulations containing one or a few components, although this did not take into account any potential cross-protection that may be offered by any particular vaccine antigen. Europe and North America are experiencing low rates of meningococcal disease at present [5,6], and in the absence of a dominant epidemic clone accounting for disease, a multicomponent vaccine formulation would be required to cover most disease [34,35]. Since a differential distribution of clonal complex and antigens has been demonstrated among different age groups and those at highest risk (the under one year-olds) are less at risk from lineages that affect the next peak of disease incidence, late adolescents, multicomponent vaccines are likely to be most appropriate, especially in a period of low incidence [36]. Therefore, comprehensive molecular epidemiology and surveillance is required in order to maximise the coverage of a given vaccine formulation. Continuous surveillance will be required to track changes in epidemiology that may need vaccine reformulation.

While genotypic data can provide valuable information on the potential utility of vaccines, the evaluation of antigen expression and potential cross-reactivity is fundamental to gauging the actual success of a given

formulation. Assays have been developed and expression studies carried out that attempt to predict the coverage of various meningococcal vaccine antigens in the population [14,37]. One assay, the ELISA-based meningococcal antigen typing system (MATS) was developed to predict the strain coverage of the Bexsero vaccine [37]. Based on a panel of invasive serogroup B-associated meningococcal isolates from several European countries it was estimated that it could protect against 78% of serogroup B cases and against a panel of serogroup B invasive isolates from Greece up to 90% [38,39]. One of the features of the PubMLST database is that phenotypic information such as MATS data can be added to isolate or allele data so that phenotypic and genotypic information may be associated allowing further analyses.

Conclusion

Highly variable pathogens require detailed characterisation to appropriately tailor clinical and public health responses such as treatment, immunisation, outbreak control, and novel vaccine design. This is especially true for organisms such as meningococci, in particular those that express the serogroup B polysaccharide, given that a universal capsular vaccine is unavailable. Well-characterised isolate collections can easily be investigated for any number of vaccine formulations and vaccine candidates when they are housed within databases embedded with analysis tools which can handle phenotype and genotype data including WGS. This high level of characterisation and molecular epidemiology provides a foundation for further phenotypic analyses so that a fuller picture of potential vaccine effectiveness can be seen. Detailed characterisation and monitoring is particularly relevant in periods of low incidence, such as experienced in high-income regions at present, as multivalent vaccines may be most appropriate and also most adaptable should changes in the meningococcal population occur. This rationale for vaccine formulation using molecular epidemiology may be applied to any pathogen and will become more readily applicable as well characterised datasets like the MRF-MGL and PubMLST become increasingly available. A combination of detailed genotypic characterisation and phenotypic investigation offer the best hope of producing vaccines with the widest possible coverage.

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Conflict of interest

DH was supported by the Meningitis Research Foundation. RB does contract research on behalf of his employer, Public Health England, for manufacturers of meningococcal vaccines including Baxter Biosciences, GlaxoSmithKline, Novartis Vaccines, Pfizer, and Sanofi Pasteur. MM has received grants and personal fees from Novartis outside the submitted work. The other authors declare no competing interests.

Authors' contributions

CB and DH undertook the data analysis and interpretation and prepared the figures and tables. CB wrote the first draft of the manuscript and DH, JL, RB and MM contributed to discussions of results, interpretation of data and contributed to writing the manuscript.

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