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Measles outbreak on a cruise ship in the western Mediterranean, February 2014, preliminary report

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A measles outbreak occurred in February 2014 on a ship cruising the western Mediterranean Sea. Overall 27 cases were reported: 21 crew members, four passengers. For two cases the status crew or passenger was unknown. Genotype B3 was identified. Because of different nationalities of cases and persons on board, the event qualified as a cross-border health threat. The Italian Ministry of Health coordinated rapid response. Alerts were posted through the Early Warning and Response System.

The event

On 26 February 2014, a cruise line company informed the Unit for the Coordination of the Port, Airport and Border Health Offices (USMAF) of the Italian Ministry of Health (MoH) in Rome of a suspected measles outbreak among crew members aboard one of its ships cruising in the western Mediterranean Sea. The ship's medical staff requested assistance from the USMAF Unit of Civitavecchia, Maritime Health Office and on 27 February, when the ship arrived in Civitaveccia, USMAF staff conducted a thorough assessment of the medical situation on board. Consultants from the National Institute of Infectious Diseases (INMI) 'Lazzaro Spallanzani' undertook a physical examination of patients and the clinical presentation suggested measles. On the same day, the MoH received the notification of a laboratory-confirmed measles case in a 27-year-old female crew member, who had disembarked the same cruise ship in Genoa, Italy, on 22 February 2014, where she had been hospitalised because of respiratory symptoms, fever and rash.

The cruise ship in question regularly sails on seven-day cruises in the western Mediterranean with ports of call in Italy (Civitavecchia, La Spezia, Savona), France (Marseille) and Spain (Barcelona, Palma de Mallorca). It has a capacity of up to 3,750 passengers and about 1,000 crew members. Passengers and crew come from a wide range of countries and may embark and disembark at any of the ports listed above. The event thus qualified as a cross-border health threat [1].

After being informed of the outbreak, the MoH immediately alerted all regional health authorities in Italy by email to ensure timely reporting of measles cases, to request that information about recent travel on the involved cruise ship be collected from all reported cases and that biological samples be sent for genotyping to the national reference laboratory at the Istituto Superiore di Sanità (ISS) in Rome.

Here we report available epidemiological information about the outbreak as of 11 March 2014.

Epidemiological investigations

In the outbreak investigation we used a case definition based on the European Union (EU) 2012 case definition [2] for classification of measles cases: clinical criteria were restricted to fever and rash and an epidemiologic link was defined as having been aboard the cruise ship after 1 January 2014 or having been in contact with a case linked to the cruise ship outbreak. A probable case was defined as any person meeting the clinical criteria and having an epidemiologic link. A confirmed case was defined as any probable case with laboratory evidence of infection i.e. identification of viral RNA through polymerase chain reaction (PCR) and/or positive IgM serology test.

Information on cases was obtained from the national Italian integrated measles and rubella surveillance

system whereby physicians are required to report all suspected measles cases to the local health authorities within 12 hours. For each case, local health authorities carry out an epidemiological investigation and obtain specimens for laboratory confirmation. A standard measles notification form is sent to the regional health authorities within 24 hours of being informed by the physician. The regional health authorities, in turn, enter case-based data within 24 hours of receiving a report, into an electronic system developed by the Infectious Diseases Epidemiology Unit of the ISS. In some regions, local health authorities enter data directly into the web-based system [3].

We prepared a line list of cases with information on the presence of symptoms and complications, date of rash onset, hospitalisation, date of embarkation and disembarkation and vaccination status against measles. In addition, information on demographic characteristics, including nationality, of crew members and passengers on board the cruise ship during the week of 22 to 27 February 2014, was obtained from the cruise line company. Acute phase sera were collected from all cases and measles-specific IgG and IgM titers were measured at INMI Spallanzani. Viral detection and genotyping was performed on urine samples by the INMI Spallanzani and by the national reference laboratory using previously described methods [4,5].

Findings

Overall, 27 measles cases were identified: 24 through the Italian surveillance system (21 crew members, four passengers, two cases not specified whether crew or passenger); two cases were reported directly to the Italian MoH (both passengers) and one case (a passenger) was reported by the Austrian Ministry of Health via the EU's Early Warning and Response System (EWRS). Twenty-two of 27 cases were laboratory-confirmed, the remaining five were classified as probable cases. The age of cases ranged from one year to 42 years (median: 26 years); 21 were men. Information of the country of origin was available for 19 of 27 cases: Italy (6), India (5), Philippines (3), Honduras (2), Austria (1), Brazil (1), and Indonesia (1).

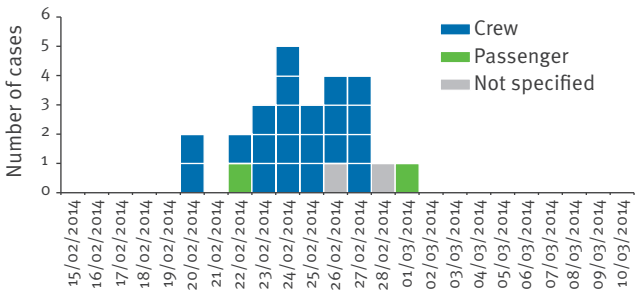
The vaccination status of the 24 cases reported through the Italian Surveillance system reported cases was as follows: unknown vaccination status (n=12), unvaccinated (n=9), vaccinated with 1 or 2 doses (n=2, n=1 respectively).

The earliest date of onset of rash was on 20 February and the most recent case developed rash on 1 March 2014. Figure 1 shows the distribution of cases by date of rash onset, among crew members and passengers.

On 27 February, 968 crew members aged 26-36 years (median: 30) were on board, 153 (16%) were women. They originated from four continents: the majority were from Asia (688; 71%) and Europe (206; 21%), 68 (7%) came from South America and the Caribbean and 5 (0,5%) from Africa. The median stay on board was 248 days (range 228 to 260 days).

Figure 1

Distribution of cases by date of rash onset[a] among crew members and passengers, measles outbreak on cruiseship, Italy, as of 11 March 2014 (n=25)



[a] For further two reported cases the date of onset of rash or fever was missing.

During the week of 22 to 27 February 2014, there were 3,352 passengers on board, of whom 2,891 (86%) were EU nationals. Over 60% of EU passengers were of either French or Italian nationals (1,101 and 939 respectively, 38% and 33%). Besides these, the six most frequently represented EU nationalities on board were German (279; 10%), British (124; 4%), Austrian (113; 4%) and Spanish (105; 4%) passengers. The median age of passengers was 41 years (range 6 months to 93 years).

Microbiological results

Samples for genotyping were collected from 22 cases and measles genotype B3 was identified in samples from 10 cases. Phylogenetic analysis demonstrated that identified sequences were 100% identical to each other, confirming a common origin, and to two British strains identified in February 2014 (MVs/Brighton.GBR/8.14/ and MVs/Tonbridge.GBR/7.14/, not shown).

Control measures

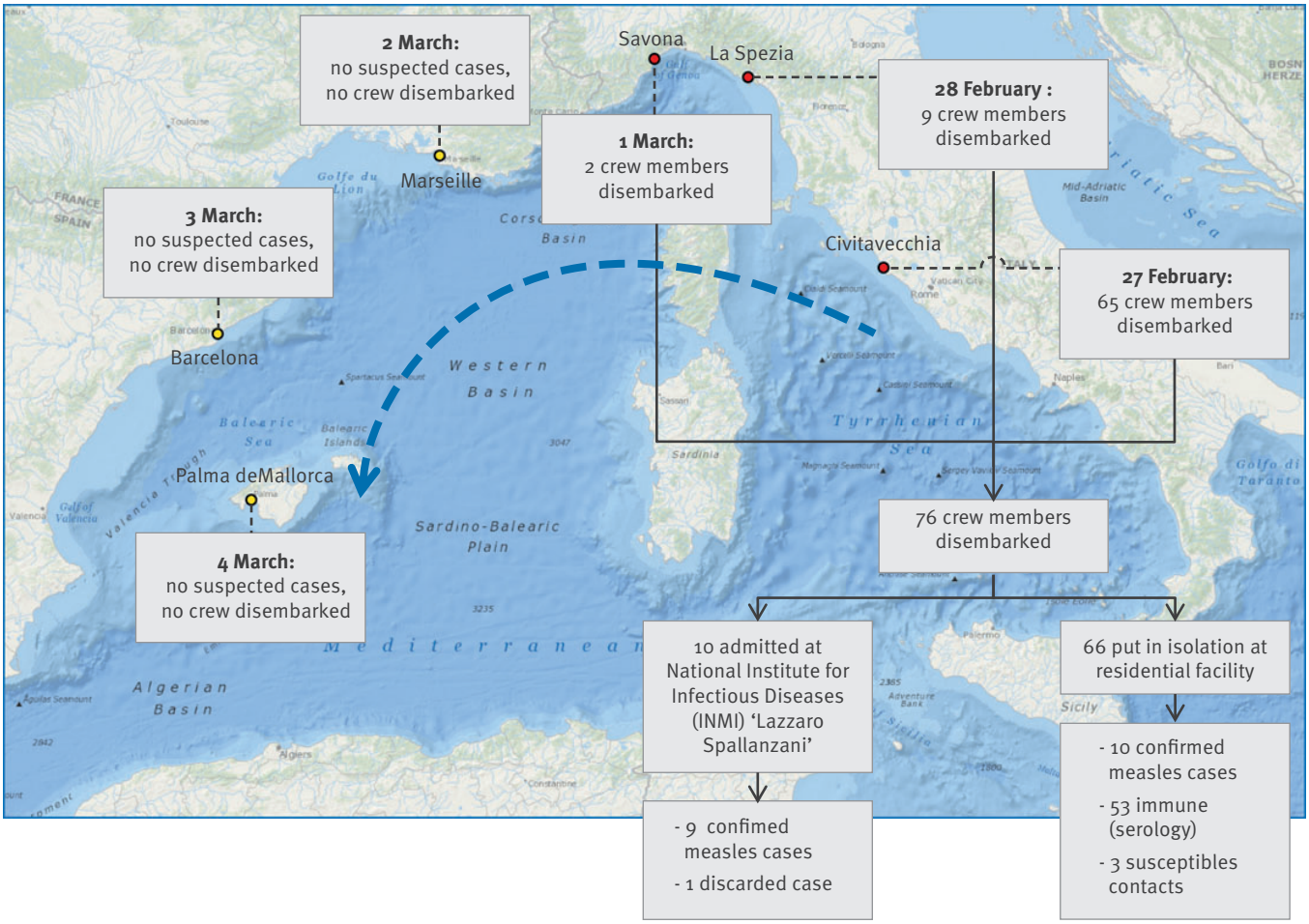
Case management and isolation

Upon the suspicion of measles cases, the ship medical team responded rapidly to the event by isolating suspected cases on board. A request for immediate support to the public health offices on shore followed when they realised that isolation of suspected cases and their contacts could not be managed on board. Symptomatic passengers and their close contacts were disembarked and either hospitalised or put in isolation to prevent further transmission.

On February 27, after inspection of the cruise ship by the Civitavecchia Maritime Health Offices, symptomatic crew members and their close contacts were disembarked. Nine crew members were hospitalised at the INMI Spallanzani and 56 were quarantined in

Figure 2

Flowchart of case management for symptomatic crew members and their close contacts, measles outbreak on cruise ship, Italy, 27 February–1 March 2014



a residential facility 100 km north of Civitavecchia. Between 28 February and 1 March, after inspections by the Maritime Health Offices in La Spezia and Savona, one additional crew member was hospitalised at the INMI Spallanzani and ten crew members were quarantined in the above mentioned residential facility (nine from La Spezia and two from Savona). Thus 76 crew members were either admitted at the INMI Spallanzani (10, of whom nine were confirmed as measles cases) or quarantined (66, of whom 10 were confirmed as measles cases). Among the 56 crew who were subsequently not diagnosed as measles cases, three were susceptible (anti-measles IgG and IgM negative) and 53 were immune (anti-measles IgG positive and IgM negative). All crew members who had developed symptoms while on board had been isolated prior to disembarking.

The cruise continued according to schedule and was investigated in Marseille on 1 March 2014, Barcelona on 2 March 2014 and Palma de Mallorca on 3 March 2014 by national maritime authorities. No additional measles cases were suspected on board (Figure 2).

Communication and international alerts

Since 28 February, disembarking passengers receive an information leaflet prepared by the cruise line company, in agreement with the Italian MoH, with a recommendation to contact local health services should they develop fever and rash, and to inform the health services that they have been on the cruise ship.

On 27 February, the Italian MoH communicated an alert through EWRS and the system was also used for communicating patient-related information to International Health Regulations focal points in countries where the exposed passengers originated from. Besides information on the outbreak, countries received lists with names and passport numbers of their citizens disembarked from the cruise. Unfortunately, for some of the EU Member States, in particular those with a high number of national citizens on board, the information provided by the cruise line company was not sufficient to track passengers.

Vaccination on board

On 27 February, the ship medical team initiated a vaccination campaign with measles-mumps-rubella (MMR) vaccine on board for crew and passengers with no

evidence of prior immunity. The cruise line company's medical team vaccinated 820 crew members who consented to be vaccinated and eight passengers who voluntarily accepted to be vaccinated. Among crew, 142 recalled previous vaccination and 108 recalled a history of measles. It should be noted that no efforts have been made to confirm serostatus of those who were vaccinated.

Vaccination is now also offered to newly embarked crew members when needed. A median of about 20 new crew members arrive per week on board.

Discussion

The spread of measles on board of a cruise ship represents a public health challenge [6,7] for several reasons. Firstly, because of the gathering of a large number of people with unknown vaccination status and having close contact in a closed setting, secondly because there is a constant flow of passengers in and out of the ship of passengers, with new susceptible passengers coming on board while potentially infected ones return to their different home countries with opportunity to further spread disease, and thirdly because on-board medical facilities to isolate and care for suspected measles cases are limited.

In the event described, a number of isolation and control measures were implemented on and off board including isolation of cases on board, request for immediate support to the relevant public health authorities at shore when realising that isolation of suspected cases and their contacts could not be managed on board, communication to crew and passengers (both incoming and leaving) and timely vaccination to susceptible crew and passengers. These measures seem to have been successful in controlling the circulation of the virus among the crew and this is indicated by the fact that since 27 February 2014, there have been no cases identified. Strict surveillance is nevertheless maintained among crew as some may have already been incubating the disease when vaccinated.

The limited number of cases reported among passengers to date, only four passengers are known to have developed measles after their stay on board, is likely to be due to underestimation. The average passenger time on board is about seven days which is usually not sufficient for a susceptible exposed patient infected to develop symptoms while still on board (range of 7 to 18 days from exposure to rash onset). However, a patient infected on board may develop symptoms upon return to their home country and infect others. Thus active follow-up of passengers at national and regional level would be desirable. Active contact tracing of passengers was done by some countries in the EU. However, some national authorities faced challenges when trying to contact their citizens due to the limited amount of information on passengers' contact details. Some countries issued press releases and put in place a telephone hotline. In Austria for example, this enabled the identification of one measles case.
Considering the highly contagious nature of measles [8], the cruise line company should continue informing new passengers boarding the ship about the risk of measles transmission on board for at least two incubation periods. To identify possible new cases among passengers after disembarking, the company should inform passengers leaving the ship to contact health services should they develop fever or rash, mentioning the recent cruise.

This outbreak is a further reminder that measles is still circulating in Europe and that actions at all levels are needed to meet the elimination goal. The lessons learnt and recommendations for preventing future occurrence of a measles outbreak on a cruise ship are as follows:

- International guidance for the management of a suspected measles outbreak on a cruise ship is needed and should include recommendations for suspected cases isolation, immediate notification to port authorities, the availability of vaccines on board and the minimal set of information required about passengers to allow for contact tracing.
- Anyone who travels should be reminded upon booking to check their vaccination records and ensure they are protected against measles prior to boarding according to national vaccine recommendations.

It is understood that these recommendations would fall within the wider activities implemented in Europe by health authorities in the frame of the measles elimination plan.

The cost and mobilisation of resources associated with this outbreak are considerable. Actions are now needed to prevent similar events from occurring in the future. Follow-up studies should to evaluate the full extent of this outbreak to learn additional lessons how to avoid the repetition of similar events in the future.

The central task force for the measles outbreak (in addition to the authors) consisted of: S Iannazzo, E Rizzuto (Ministry of Health, DG for Prevention, V Office), V Costanzo (Ministry of Health, DG for Prevention, III Office), S Declich, MC Rota, A Bella , S Salmaso (Istituto Superiore di Sanità, CNESPS), G Rezza (Istituto Superiore di Sanità, DMIPI), N Danielsson, L Pastore Celentano, D Plachouras, E Robesyn (ECDC), N Bevilacqua, L Bordi, F Carletti, E Lalle and E Nicastri (National Institute for Infectious Diseases (INMI) "Lazzaro Spallanzani").

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Conflict of interest

None declared.

Authors' contributions:

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Dengue virus serotype 4 and chikungunya virus coinfection in a traveller returning from Luanda, Angola, January 2014

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A concurrent dengue virus serotype 4 and chikungunya virus infection was detected in a woman in her early 50s returning to Portugal from Luanda, Angola, in January 2014. The clinical, laboratory and molecular findings, involving phylogenetic analyses of partial viral genomic sequences amplified by RT-PCR, are described. Although the circulation of both dengue and chikungunya viruses in Angola has been previously reported, to our knowledge this is the first time coinfection with both viruses has been detected there.

Detection of coinfection

Here we report the simultaneous detection of chikungunya virus (CHIKV) and dengue virus (DENV) genomes in the peripheral blood of a traveller who returned from Luanda, Angola, to Portugal in January 2014.

The traveller, a woman in her early 50s, was born and raised in Angola and has lived in Lisbon, Portugal, since the early 1990s. She stayed in Luanda from mid-December 2013 to early January 2014 at her family's place of residence. There were a large number of mosquitoes in the garden and the patient was repeatedly bitten during her stay.

The patient reported feeling unwell in early January, two days before her return to Portugal. Her condition worsened during the flight, and in the next few days she had high fever (up to 39.5 °C), severe arthralgia, myalgia, prostration and abdominal pain. Three days after her return, she went to the emergency department of a hospital: a malaria blood smear was negative and among a range of laboratory tests (including coagulation speed and levels of glucose, creatinine, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), sodium, potassium, chloride ions and C-reactive protein), the only abnormal findings were a mildly low platelet count (139 × 10^9/L; norm: 150–400 × 10^9/L) and mild leucopenia (2.9 × 10^9/L; norm: 4–10 × 10^9/L). The following day, she went to a hospital specialised in tropical diseases, where photophobia was detected. Further tests were carried out (described below). An arbovirus infection was suspected as the malaria blood smear was persistently negative.

Four days later, the fever had subsided and her condition improved progressively over the next two to three weeks. The patient did not have a rash, conjunctivitis or other clinical signs of a complicated dengue infection (DENV infection with haemorrhage); indeed, she had no other abnormal clinical signs at all during the course of her illness. To the best of her knowledge, none of her family or neighbours in Luanda experienced a similar illness.

Laboratory findings

Four days after her return from Luanda, DENV nonstructural (NS) protein 1 and anti-CHIKV IgM were detected (through the use of SD BIOLINE Dengue Duo NS1 Ag + Ab Combo and SD Bioline Chikungunya IgM), while DENV-specific IgM and IgG were not detected. Two days later, the same tests were performed: anti-CHIKV IgM and DENV-specific IgM and IgG were detected, but DENV NS1 was not. Using RNA extracted from the blood sample where NS1 had been found, detection of the viral genomes was carried out either by a nested RT-PCR as previously described [1,2] or by using primers that target the virus packaging sequence [3]. The sizes of the amplicons obtained were compatible with the presence of both DENV4 (approximately 390 bp, covering the C-prM region) and CHIKV (approximately 350 bp, in the NS2 coding region).

Additional molecular confirmation was obtained by performing phylogenetic analyses of the sequence of both amplicons (deposited in the GenBank/European Molecular Biology Laboratory (EMBL)/DNA DataBank of Japan (DDBJ) databases under accession numbers AB908053 and AB908054) using the using GTR+G+I model [4]. The DENV sequence obtained clearly clustered with DENV4 reference strains (Figure 1), while the CHIKV sequence segregated with those included in the Central/Eastern/Southern African genotype (Figure 2). Despite the presence of both viral genomes in the same blood sample, the viraemia dropped rapidly below the detection level, as both DENV and CHIKV RNA could not be detected in blood collected 48 hours later.

Background

Dengue has developed into a worldwide public health problem, especially over the last 50 years [5,6]. More recently, the impact of other arboviruses on human health has followed a similar trend [7]. This is true for CHIKV, which, since 2004, has been an emerging pathogen, causing large outbreaks in many islands in the Indian Ocean and in the Indian subcontinent, where, in 2005-2006 alone, well over a million cases of CHIKV infection were reported from different states [8].

The majority of DENV infections occur in the Asia–Pacific and Americas–Caribbean regions [5], while CHIKV is endemic to countries in Africa and Asia [9]. In Africa, the epidemiology and public health impact of both viruses is far from clear, but the wide geographical distribution of their primary vectors (*Aedes aegypti* and *Aedes albopictus*), rapid human population growth, unplanned urbanisation, and increased international travel make their transmission likely [10,11]. Moreover, as the clinical features of DENV and CHIKV are similar, CHIKV infections usually go undiagnosed in areas where DENV circulates [11]. Furthermore, where malaria is also endemic and the majority of febrile illnesses are diagnosed as such, often without laboratory confirmation, both viral infections may go undetected [12].

Although CHIKV/DENV coinfections were first reported in India in 1967 [13] and later confirmed in Sri Lanka (2008), Malaysia (2010) and Gabon (2007) [14-16], these coinfections are rarely notified.

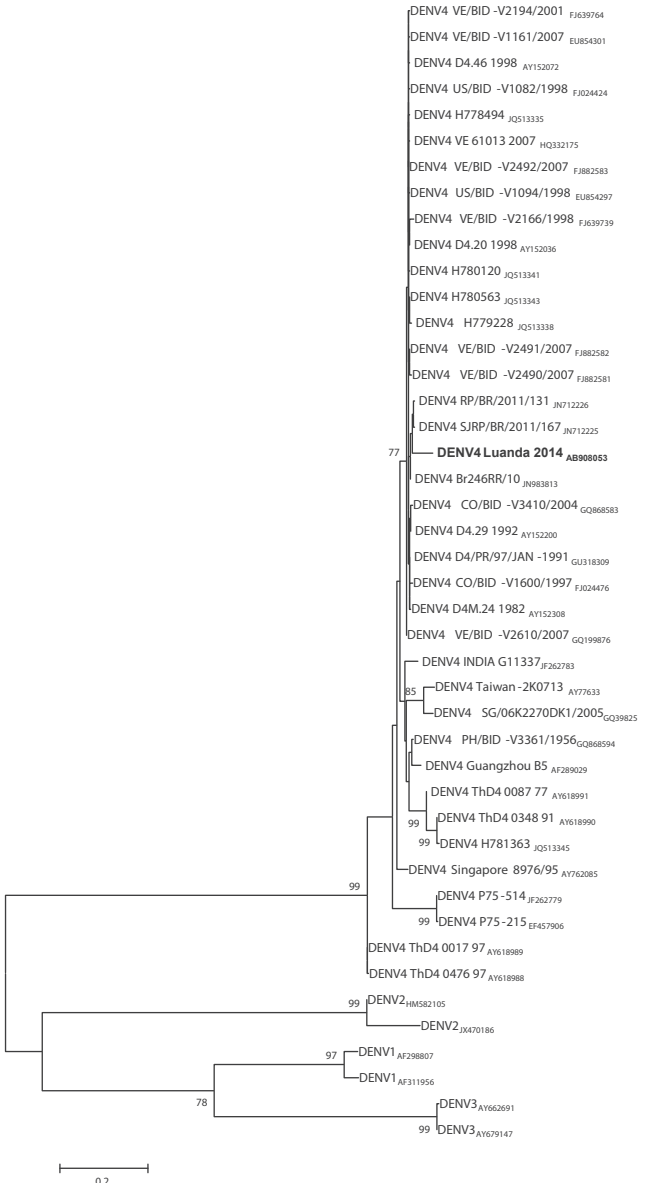
Discussion

Serological reports from the 1960s [17], the detection of DENV in travellers returning from Angola in the 1980s [10], and the detection of DENV1 and DENV2 in travellers in the 1980s and in 1999–2002 [10,18] suggest endemic DENV activity in Angola. As far as CHIKV is concerned, the situation is a lot less clear. However, serological studies from the 1960s not only identified the presence of anti-CHIKV neutralising antibodies in the north of the country, but also allowed the isolation of two strains from a viraemic individual and wild-caught mosquitoes during an outbreak of Kâtolu Tôlu (Kimbundu dialect for 'break-bone disease'), a dengue-like disease caused by the CHIKV, which occurred in Luanda in 1970 [19].

The detection of DENV4 in the recent traveller is of interest, given that on 1 April 2013, the Angolan health authorities reported a dengue outbreak in the country [20], which was later shown to have been caused by DENV1 [21], and the current description of DENV4 in

Figure 1

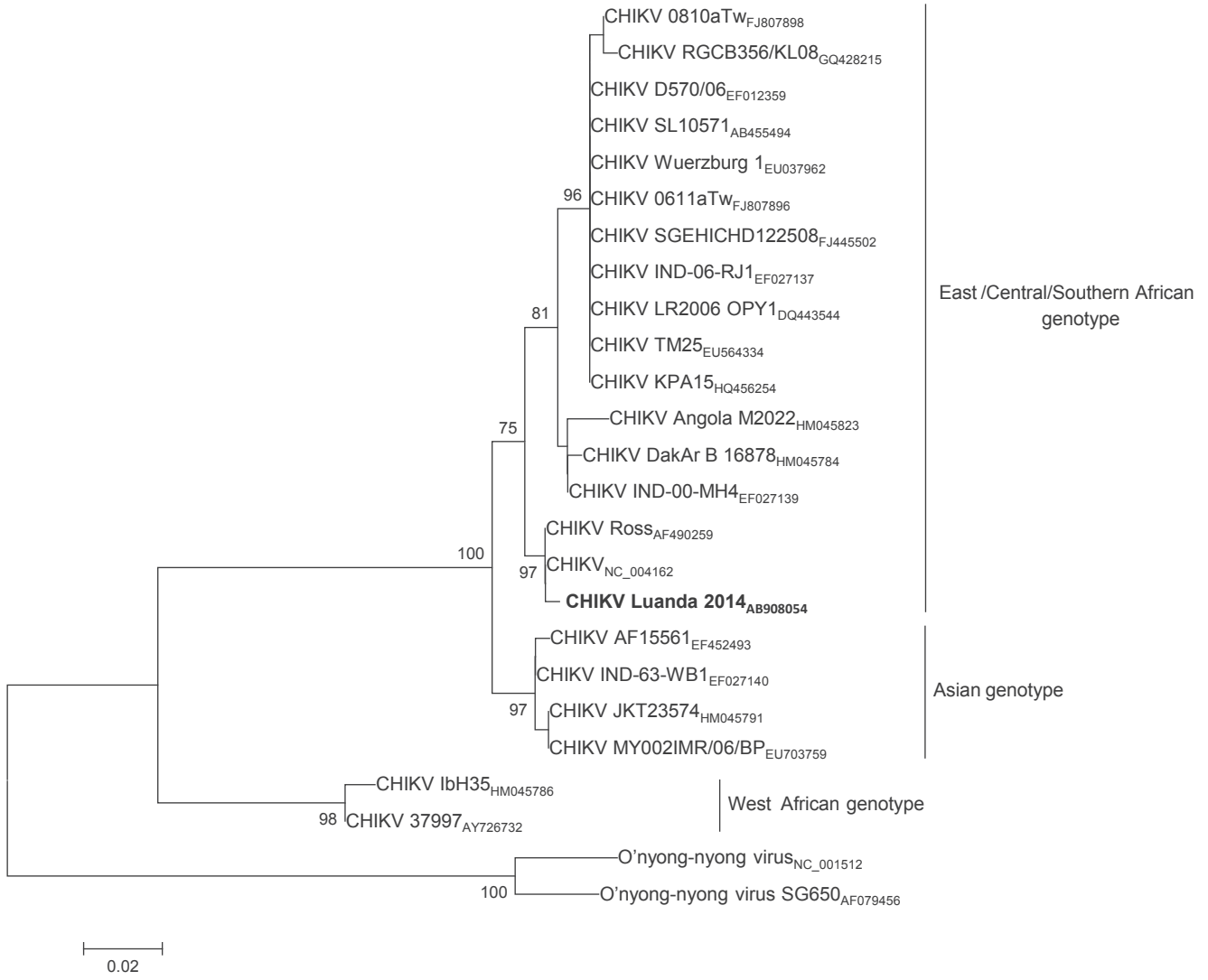
Maximum likelihood phylogenetic tree analysis of dengue virus (DENV) serotypes 1–4 C-prM sequences



The tree was constructed using the using the GTR+Γ+I model [4]. The amplicon isolated from the patient is shown in bold. Reference strains, downloaded from public databases, are identified by strain name and accession number (DENV4) or simply by viral serotype and accession number (DENV1–3). The numbers at specific branches indicate bootstrap values (only values ≥77% are indicated).

Figure 2

Maximum likelihood phylogenetic tree of chikungunya virus (CHIKV) partial nonstructural protein (NS) 2 sequences



The tree was constructed using the using the GTR+Γ+I model [4]. The amplicon isolated from the patient is shown in bold. Reference strains are indicated by strain name and accession number. The three CHIKV genotypes (East/Central/Southern African, West African and Asian) are indicated. The numbers at specific branches indicate bootstrap values (values ≥75% are indicated).Two strains of o'nyong nyong virus, the *Alphavirus* most closely related to CHIKV, have been used as an outgroup.

Luanda may indicate the circulation of multiple DENV subtypes in the country.

Although clinical examination of CHIKV/DENV coinfected patients has not yet allowed the identification of specific or severe symptoms, such observations should be interpreted with caution in view of the limited number of clinical and biological investigations reported. Our findings may add to the recognition of CHIKV/DENV coinfections and suggest that tests to detect the presence of both viruses should be carried out in individuals showing clinical signs of an infection with either CHIKV or DENV.

Conflict of interest

None declared.

Authors' contributions

Ricardo Parreira: molecular analyses and manuscript writing. Ângela Mendes: molecular analyses. Jaime Nina: clinical diagnosis and manuscript writing. Antónia Constantino: clinical diagnosis and manuscript writing. Sónia Centeno-Lima and Daniela Portugal Calisto: laboratory diagnosis and manuscript writing.

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Drug resistance among tuberculosis cases in the European Union and European Economic Area, 2007 to 2012

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The European Union and European Economic Area (EU/EEA) tuberculosis (TB) surveillance system collects detailed information on resistance to TB drugs. Using this information, we provide an overview of the current TB drug resistance situation and trends in the EU/EEA by performing a descriptive analysis, including analysis of treatment outcomes, of the TB cases reported between January 2007 and December 2012. The percentages of TB cases with different drug resistance patterns have been stable with about 90% of the new laboratory-confirmed cases pan-susceptible, 6% monodrug-resistant, 2% polydrug-resistant, 2% multidrug drug-resistant (MDR) TB – excluding extensively drug-resistant (XDR) TB –, and 0.2% XDR-TB. In previously treated laboratory-confirmed TB cases, the percentage with MDR-TB excluding XDR-TB declined until 2010 to 16% and remained stable thereafter. During the study period, the percentages of cases with monodrug- and polydrug-resistant TB remained constant at about 8% and 2% whereas the percentage of XDR-TB cases increased slightly to 2.6%. Treatment outcome results for all cases have been stable with overall 77.9% of the pan-susceptible cases, 69.6% of the monoresistant cases, 68.2% of the polyresistant cases, 32.2% of the MDR-TB cases (excluding XDR-TB), and 19.1% of the XDR-TB cases treated successfully. The treatment success rate target for new pulmonary culture-positive MDR-TB cases of 70% has not been reached. In addition, drug resistance surveillance can be improved by more complete reporting of drug susceptibility results and treatment outcome.

Introduction

Anti-tuberculosis (TB) drug resistance is a major public health challenge. Patients infected with *Mycobacterium tuberculosis* bacilli resistant to TB drugs often require longer, expensive treatment regimens, and show poorer treatment outcomes. In 2011, the global incidence of TB was estimated to be 125 cases per 100,000 population, with about 12 million prevalent TB cases [1]. Of the prevalent cases, 630,000 (5.3%) were estimated to have multidrug-resistant (MDR) TB [1]. The 53 countries of the World Health Organization (WHO) European Region notified 380,366 TB cases in 2011. For 127,936 (33.6%), drug susceptibility testing (DST) results were available, and 29,473 (19.0%) were diagnosed with MDR-TB. In the 29 reporting European Union (EU) and European Economic Area (EEA) countries the proportion of MDR-TB was 4.5%, while in non EU/EEA countries the proportion was 25.6% [2]. Although information on MDR-TB and extensively drug-resistant (XDR) TB is systematically collected and reported by the WHO [1], less information is available on the burden of mono- and polydrug resistance, or any drug resistance.

In the EU/EEA, many countries have based their system for surveillance of anti-tuberculosis drug resistance on recommendations of a WHO and International Union Against Tuberculosis and Lung Disease (IUATLD) working group [3]. Starting with the 2007 TB cohort, TB surveillance data from the country level are reported annually to the European Surveillance System (TESSy) database, operated by the European Centre for Disease Prevention and Control (ECDC). The ECDC previously published an analysis of MDR and XDR-TB in the EU/EEA using notification data from 2008 [4]. This analysis showed that MDR-TB remained a threat and that XDR-TB had been identified within the EU/EEA borders. In the annual European tuberculosis surveillance and monitoring reports, notifications on MDR-TB and XDR-TB are provided separately from data on resistance to isoniazid or rifampicin [2]. Although information on resistance to other TB drugs is collected in the TESSy database, this information is not routinely reported. Some EU countries or regions in EU countries have published detailed information on drug resistance to individual TB drugs [5-7]. For example, the United Kingdom reported an increase of the proportion of TB cases resistant to isoniazid from 1998 to 2005 [7]. In the same period, a study from Castilla y León, Spain, indicated that the incidence of primary drug resistance and monoresistance was low [8] and this information was used to establish a new standard anti-tuberculosis treatment. Systematic analysis of drug resistance data

helps to identify strengths and remaining challenges in TB control as well as to guide actions. It can also be used to assess whether the targets set in the EU/EEA, i.e. to test 100% of the culture-positive TB cases for resistance to first-line TB drugs, are achieved [9].

Analysis of characteristics of patients with drug resistance can help to identify populations most at risk. A study in London among individuals with isoniazid monoresistance showed that cases were more likely to be young adults, born in the United Kingdom and of white or black Caribbean ethnicity, imprisoned at the time of diagnosis, unemployed, drug dealers or sex workers [10]. Another study assessed risk factors for resistance to second-line anti-tuberculosis drugs in eight countries [11]. It showed that previous treatment with second-line drugs was the strongest risk factor, and resistance to fluoroquinolones and XDR-TB were more frequent in women than in men. In addition, unemployment, alcohol abuse, and smoking were associated with resistance to second-line injectable drugs. Although some risk factors for drug resistance are study-site-specific, others seem to be general.

To assess whether TB control programmes are able to provide adequate treatment and support, an analysis of TB treatment outcomes is useful. In general, cases infected with a TB strain that is resistant to TB drugs have a worse treatment outcome [12,13]. Other patient characteristics that are reported to be related to unsuccessful treatment outcomes are being male, older age, having pulmonary TB, alcohol dependence, homelessness, unemployment and diabetes [12,14,15]. In the TESSy database, only few patient characteristics are collected, which allows for a limited risk factor analysis.

An in-depth analysis of TB drug resistance in the EU/EEA has not been performed. Therefore, we aim to provide an overview of the current TB drug resistance situation in the EU/EEA and its trend, characteristics of drug-resistant cases, and their treatment outcomes.

Methods

Data source and collection

Data were extracted from the TESSy database on 4 October 2013. Data from 27 EU and EEA countries reporting DST results to ECDC were analysed. France, Italy, and Spain were not included as they are not reporting case-based drug resistance data to TESSy but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. DST data had been collected for the first line drugs ethambutol, isoniazid, rifampicin, and streptomycin, and for the second line drugs amikacin, capreomycin, ciprofloxacin, gatifloxacin, kanamycin, levofloxacin, moxifloxacin, and ofloxacin.

Data inclusion and surveillance definitions

Only confirmed TB cases according to the EU case definition [16] with data on drug susceptibility for at least isoniazid and rifampicin were analysed. Definitions and categories provided in the ECDC/WHO report on tuberculosis surveillance and monitoring in Europe 2013 were used [2]. 'Pan-susceptible' refers to a case susceptible to all drugs tested. 'Monodrug resistance' is defined as resistance to one anti-TB drug, while 'polydrug resistance' refers to resistance to two or more drugs, excluding MDR-TB. MDR-TB is defined as resistance to at least isoniazid and rifampicin. XDR-TB is a special form of MDR-TB defined as resistance to at least isoniazid and rifampicin with further resistance to a fluoroquinolone and a second-line injectable agent (amikacin, kanamycin or capreomycin). Any drug resistance refers to a case with resistance to at least one TB drug. The percentage tested for susceptibility to second-line drugs (injectable agents: amikacin, capreomycin, kanamycin; fluoroquinolones: ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin, and ofloxacin) was calculated for cases for whom DST results were reported for at least one fluoroquinolone and one injectable drug. 'Previously treated TB case' means that the case has received TB treatment before the current TB episode.

If data on previous treatment were not available, information on previous TB diagnosis was used. Treatment success is defined as a treatment outcome reported as 'cured' or 'completed' within 12 months after diagnosis in non-M(X)DR-TB cases, within 24 months in MDR-TB cases and within 36 months in XDR-TB cases.

Analysis

We performed a descriptive analysis of surveillance data to assess the burden and trends of drug resistance among TB cases in EU/EEA countries between January 2007 and December 2012. We described cases with available resistance data by previous TB treatment history and resistance type over the years covered. We analysed different resistance types by sex, age-group, origin, human immunodeficiency virus (HIV) status, site of disease and treatment success. Treatment outcome after 12 months was analysed for pan-susceptible, monoresistant, and polyresistant cases notified between 2007 and 2011, treatment outcome after 24 months for MDR-TB cases notified between 2007 and 2010 and treatment outcome after 36 months for XDR-TB cases notified between 2007 and 2009. Fisher's exact tests were used to compare categorical data. Chi-squared test for trends was used to analyse changes over time of categorical data using the ptrend command in STATA. A p value of <0.05 was considered significant. All data analyses were performed using STATA 12.1 (StataCorp LP, Texas, USA).

Table 1

Notified laboratory-confirmed tuberculosis cases with reported testing results for the first-line TB drugs isoniazid (H) and rifampicin (R), and multidrug-resistant TB cases with reported testing results for second-line TB drug resistance, EU/EEA, 2007–2012

Year	2007	2008	2009	2010	2011	2012
Number of countries reporting case-based data for drug susceptibility for the first-line TB drugs H and R	26[a,b]	25[a,b]	26[a,b]	26[a,b]	26[a,b]	25[a,b]
Number of notified laboratory-confirmed TB cases	41,943	39,628	40,220	37,401	37,577	35,279
Notified laboratory-confirmed TB cases with reported testing results for R and H resistance N (%)	26,622 (63.5)	27,688 (69.9)	28,356 (70.5)	27,831 (74.4)	28,985 (77.1)	27,694 (78.5)
Number of notified MDR-TB cases	1,511	1,556	1,499	1,382	1,421	1,310
Notified MDR-TB cases with reported testing results for resistance to second-line TB drugs N (%)	310 (20.5)	366 (23.5)	585 (39.0)	869 (62.9)	983 (69.2)	891 (68.0)
Number of notified XDR-TB cases	76	90	66	115	143	128

EU/EEA: European Union/European Economic Area; MDR: multidrug resistant; TB: tuberculosis; XDR: extensively drug resistant.

27 EU/EEA countries provided case-based data. France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform.

[a] Greece is not included in 2007, 2008 and 2012.

[b] Liechtenstein is not included after 2008.

Results

Completeness of drug resistance testing reporting

In 2007, 26 EU/EEA countries reported case-based rifampicin and isoniazid susceptibility testing results for 63.5% of all laboratory-confirmed TB cases, while 25 countries reported this data in 2012. Liechtenstein reported case-based data on DST for rifampicin and isoniazid only in 2007 and Greece only from 2009 to 2011 (Table 1). The percentage of laboratory-confirmed TB cases with testing results reported increased gradually from 63.5% (26,622/41,943) in 2007 to 78.5% (27,694/35,279) in 2012. Five countries reported results for rifampicin and isoniazid susceptibility testing for 100% of the laboratory-confirmed TB cases in 2012.

Reporting of DST results for first-line TB drugs for culture-positive non-MDR-TB increased between 2007 and 2012 (Table 2), both for new and previously treated TB cases.

Reporting of testing results of MDR-TB cases for resistance to second-line TB drugs increased steeply from 20.5% (320/1,511) of the notified MDR-TB cases in 2007 to 68.0% (891/1,310) in 2012 (Table 1). In 2012, ten countries reported DST results for more than 95% of all MDR-TB cases for second-line TB drugs, while the corresponding number was six countries in 2007. The testing percentage for second-line TB drugs was largely determined by the low percentage of MDR-TB cases for which a test result for second-line drugs was reported in Romania and the high number of MDR-TB cases reported in this country, i.e. 53.6% (284/530) in 2012. Germany (8/60), Ireland (1/5), and Poland (4/31) reported test results for second-line drugs for ‹50% of the MDR-TB cases. However, these countries reported a lower number of cases and thus affected the EU/EEA second-line drug susceptibility percentage to a lesser extent.

DST results to the first-line drugs ethambutol and streptomycin were reported in respectively 84.5% (1,107/1,310) and 82.9% (1,086/1,310) of the MDR-TB cases in 2012. This had increased from respectively 66.5% (1,005/1,511) and 64.2% (970/1,511) in 2007. For the most frequently tested second-line drugs, kanamycin and ofloxacin, results were reported in 22.0% (332/1,511) and 20.8% (315/1,511) of the MDR-TB cases in 2007 and in 64.6% (846/1,310) and 62.3% (816/1,310) in 2012. In 2012, 40.9% (536/1,310) of the MDR-TB cases were reported to be tested for amikacin resistance and 37.5% (491/1,310) for capreomycin resistance. Reporting on testing results for resistance to other second-line drugs was infrequent. DST results for the drugs gatifloxacin, levofloxacin, and moxifloxacin were only collected from 2013 onwards with updates for previous year data. None of the EU/EEA countries have reported susceptibility testing results for gatifloxacin or levofloxacin for the years 2007 to 2012.

Drug resistance notification and trends

In new laboratory-confirmed TB cases, the percentage of those with any resistance reported remained stable at around 10% (Figure 1A). Also, the percentages of new laboratory-confirmed TB cases with reported monoresistance, polyresistance, MDR-TB (excluding XDR-TB), and XDR-TB remained stable at about 6%, 2%, 2%, and 0.2% respectively.

Table 2

Resistance to first-line tuberculosis drugs in new and previously treated culture-positive non-MDR-TB cases by year, EU/EEA, 2007–2012

Year	All culture positive non-MDR-TB cases[a]	Isoniazid		Rifampicin		Streptomycin		Ethambutol	
		Tested N (%)	R N (%)	Tested N (%)	R N (%)	Tested N (%)	R N (%)	Tested N (%)	R N (%)
New culture-positive non-MDR-TB cases									
2007	38,904	20,407 (52.5)	1,166 (5.7)	20,407 (52.5)	115 (0.6)	12,933 (33.2)	784 (6.1)	18,378 (47.2)	135 (0.7)
2008	37,104	21,034 (56.7)	1,199 (5.7)	21,034 (56.7)	87 (0.4)	12,528 (33.8)	757 (6.0)	18,321 (49.4)	143 (0.8)
2009	36,826	21,689 (58.9)	1,128 (5.2)	21,689 (58.9)	91 (0.4)	13,183 (35.8)	681 (5.2)	18,250 (49.6)	84 (0.5)
2010	35,468	21,571 (60.8)	1,185 (5.5)	21,571 (60.8)	78 (0.4)	14,801 (41.7)	879 (5.9)	17,989 (50.7)	93 (0.5)
2011	35,441	22,955 (64.8)	1,250 (5.4)	22,955 (64.8)	77 (0.3)	16,190 (45.7)	929 (5.7)	19,030 (53.7)	97 (0.5)
2012	33,182	22,061 (66.5)	1,125 (5.1)	22,061 (66.5)	89 (0.4)	15,894 (47.9)	874 (5.5)	18,717 (56.4)	77 (0.4)
Previously treated culture-positive non-MDR-TB cases									
2007	7,119	3,129 (44.0)	370 (11.8)	3,129 (44.0)	78 (2.5)	1,570 (22.1)	140 (8.9)	1,926 (27.1)	39 (2.0)
2008	6,618	3,616 (54.6)	315 (8.7)	3,616 (54.6)	54 (1.5)	1,509 (22.8)	137 (9.1)	1,950 (29.5)	42 (2.2)
2009	6,714	3,872 (57.7)	352 (9.1)	3,872 (57.7)	66 (1.7)	1,642 (24.5)	140 (8.5)	2,083 (31.0)	41 (2.0)
2010	6,256	3,671 (58.7)	348 (9.5)	3,671 (58.7)	60 (1.6)	1,706 (27.3)	153 (9.0)	1,925 (30.8)	39 (2.0)
2011	5,695	3,469 (60.9)	315 (9.1)	3,469 (60.9)	54 (1.6)	1,657 (29.1)	155 (9.4)	1,811 (31.8)	35 (1.9)
2012	5,200	3,263 (62.8)	257 (7.9)	3,263 (62.8)	57 (1.7)	1,667 (32.1)	132 (7.9)	1,871 (36.0)	23 (1.2)

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

27 EU/EEA countries provided case-based data. France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. Liechtenstein reported case-based data on drug susceptibility testing for rifampicin and isoniazid only in 2007 and Greece only from 2009 to 2011.

[a] Only TB cases with information available on previous treatment are included.

In previously treated laboratory-confirmed TB cases, the percentage with any resistance and with MDR- (excluding XDR-TB) declined until 2010 (Figure 1B) and remained stable thereafter with about 30% of the previously treated cases showing any resistance. The percentage of cases with monoresistance and polyresistance did not change. The percentage of XDR-TB cases, increased from 1.4% in 2007 to 2.6% in 2012 (chi-squared (1) for trend=26.8, p<0.0001).
In 2012, the percentage of TB cases with any resistance varied considerably across EU/EEA countries with 3.3% (6/181) showing any resistance in Slovakia and 37.7% (90/239) in Estonia (Figure 2).

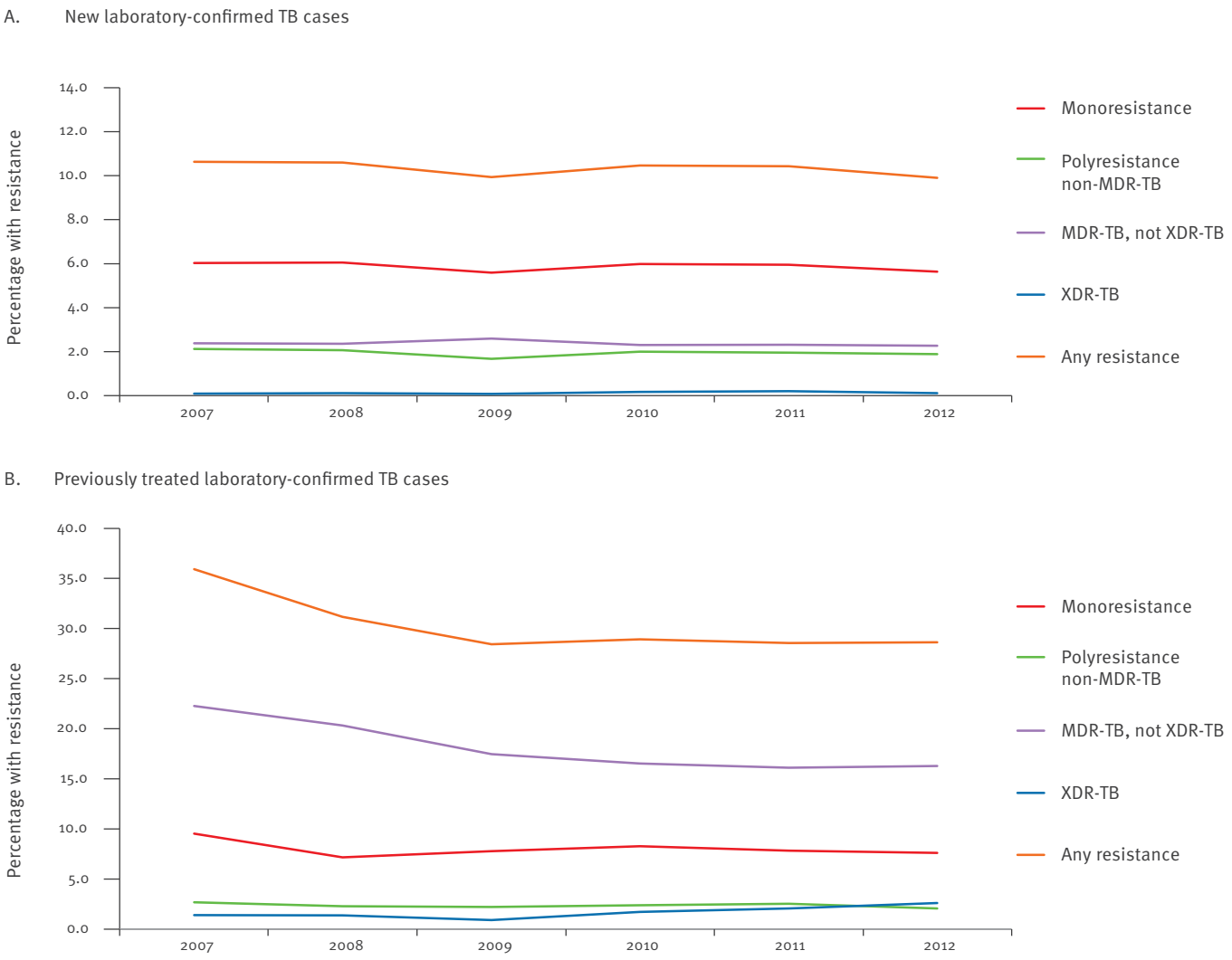
The percentage of TB cases with reported rifampicin monoresistance ranged between 0% and 1.3% in 2012 in the different EU/EEA countries (Table 3). For isoniazid monoresistance the range was 0% to 6.6% and for MDR-TB 0% to 25.5%.

The total number of notified MDR-TB (including XDR-TB) cases decreased from 1,511 in 2007 to 1,310 in 2012 (Table 1). Liechtenstein did not report to TESSy in 2012, but reported 0 MDR TB cases in 2007. The number of new cases with MDR-TB (including XDR-TB) remained stable over the years, 516 in 2007 and 542 in 2012, whereas the number of MDR-TB (including XDR-TB) cases that were previously treated decreased from 972 in 2007 to 727 in 2012 (Table 4). The percentage of new MDR-TB (including XDR-TB) cases of MDR-TB cases for which previous treatment was known, increased from 34.7% (516/1,488) in 2007 to (542/1,269) 42.7% in 2012 (chi-squared (1) test for trend=35.4, p<0.001).

Of the new MDR-TB (including XDR-TB) cases, ≥50% were reported to be resistant to ethambutol and >80% to streptomycin (Table 4). Among previously treated MDR-TB (including XDR-TB) cases, around 70% tested resistant to ethambutol, whereas resistance to streptomycin was comparable with the percentage in new MDR-TB cases. Resistance to kanamycin was reported in 40.7% of the new MDR-TB cases in 2007 and 23.5% in 2012. Ofloxacin resistance was less frequent in new MDR-TB cases with only 11.7% of the cases showing resistance in 2012. As expected, previously treated cases were more frequently resistant to both first-line and second-line TB drugs. Of all MDR-TB (including XDR-TB) cases tested for resistance to any of the second-line TB drugs between 2007 and 2012, 44.5% (1,782/4,004) were resistant to any of the second-line drugs.

Figure 1

Resistance pattern among new (a) and previously treated (b) laboratory-confirmed TB cases tested for at least isoniazid and rifampicin resistance by year, EU/EEA, 2007–2012



EU/EEA: European Union/European Economic Area; MDR-TB: multidrug-resistant tuberculosis; TB: tuberculosis; XDR-TB: extensively drug-resistant tuberculosis.

27 EU/EEA countries provided case-based data. France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. Of the 27 EU/EEA countries included in the study, Liechtenstein reported case-based data on drug susceptibility testing for rifampicin and isoniazid only in 2007 and Greece only from 2009 to 2011.

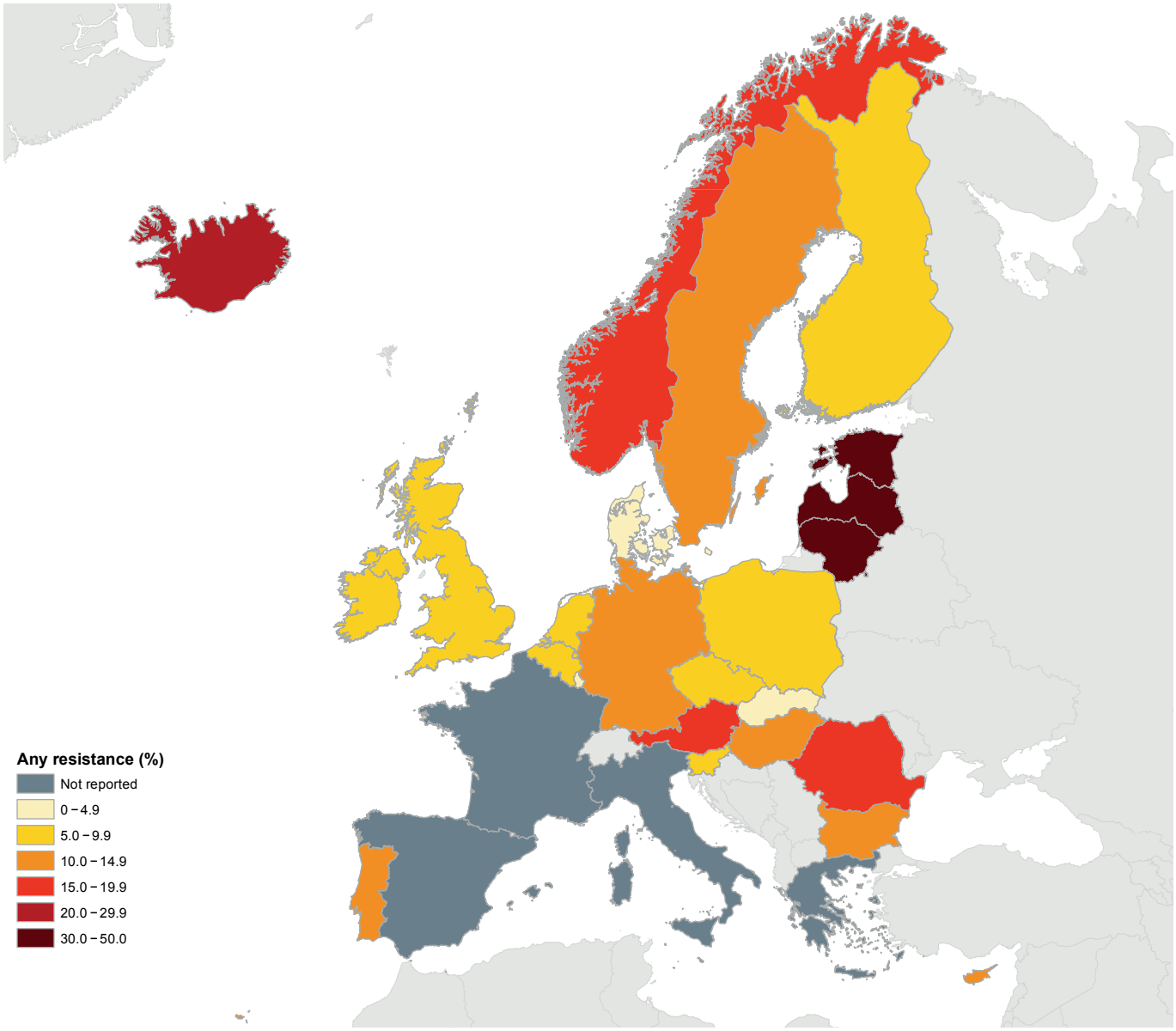
For 143 of the 183 (78.1%) MDR-TB cases (including XDR-TB) with ciprofloxacin resistance, no cross resistance with other fluoroquinolones was reported. Of the 815 MDR-TB cases (including XDR-TB) with ofloxacin resistance, for 727 (89.2%) no resistance to other fluoroquinolones was reported, and of the 55 cases resistant to moxifloxacin, for three (5.5%) no other resistance to fluoroquinolones was reported. Thirty-six MDR-TB cases (including XDR-TB) were reported with resistance to both ciprofloxacin and ofloxacin and 48 showed resistance to both ofloxacin and moxifloxacin. Only four MDR-TB (including XDR-TB) cases were reported with resistance to all three fluoroquinolones.

Characteristics of tuberculosis cases with drug resistance

In 2012, 32.6% of all laboratory-confirmed TB cases tested for at least isoniazid and rifampicin resistance were female (Table 5). In the groups with MDR-TB (excluding XDR-TB), and XDR-TB, females accounted for a lower proportion compared to the other groups, 25.6% and 21.9% of all cases, respectively ($p<0.001$ and $p=0.008$, respectively).

Figure 2

Percentage of laboratory-confirmed tuberculosis cases tested for at least isoniazid and rifampicin resistance with any drug resistance, EU/EEA, 2012[a]



EU/EEA: European Union/European Economic Area.

The 'Not reported' legend indicates that case-based drug susceptibility results are not reported to the European Surveillance System (TESSy).

[a] France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. Liechtenstein and Greece did not report to TESSy in 2012.

Most notified TB cases were between 25 and 64 years-old. The percentages of 25 to 64 year-olds were higher compared to those aged younger or older for XDR-TB (85.2%) and MDR-TB (excluding XDR-TB) (82.2%) cases when compared to pan-susceptible (70.1%) or monoresistant (74.4%) cases (comparison XDR-TB vs pan-susceptible, chi-squared (1)=13.8 p<0.001; comparison MDR-TB (excluding XDR-TB) vs pan-susceptible, chi-squared (1)=79.8 p<0.001; comparison XDR-TB vs monoresistant, chi-squared (1)=7.4 p=0.007; comparison MDR-TB (excluding XDR-TB) vs monoresistant, chi-squared (1)=24.4 p<0.001). Cases aged ≥65 years were significantly more frequent among pan-susceptible cases (17.3%, p<0.001) when compared to all other age groups (7.3–10.8%).

Of the pan-susceptible TB cases, 28.1% were recorded with a foreign origin. While monoresistant cases were more frequently of foreign origin, 34.6% (p<0.001), MDR-TB (excluding XDR-TB), and XDR-TB cases were

Table 3

Rifampicin monoresistance, isoniazid monoresistance, and multidrug-resistant TB in EU/EEA countries, 2012

Country	Number of TB cases with reported susceptibility testing results to at least isoniazid and rifampicin	Rifampicin monoresistant N (%)	Isoniazid monoresistant N (%)	MDR-TB N (%)
Austria	392	0 (0.0)	14 (3.6)	27 (6.9)
Belgium	735	5 (0.7)	37 (5.0)	20 (2.7)
Bulgaria	829	9 (1.1)	27 (3.3)	49 (5.9)
Cyprus	49	0 (0.0)	1 (2.0)	0 (0.0)
Czech Republic	397	1 (0.3)	4 (1.0)	4 (1.0)
Denmark	298	0 (0.0)	8 (2.7)	1 (0.3)
Estonia	239	3 (1.3)	3 (1.3)	61 (25.5)
Finland	222	0 (0.0)	8 (3.6)	3 (1.4)
France[a]	–	–	–	–
Germany	2,794	5 (0.2)	96 (3.4)	60 (2.1)
Greece[b]	–	–	–	–
Hungary	449	2 (0.4)	17 (3.8)	11 (2.4)
Iceland	5	0 (0.0)	0 (0.0)	1 (20.0)
Ireland	265	0 (0.0)	10 (3.8)	5 (1.9)
Italy[a]	–	–	–	–
Latvia	766	0 (0.0)	28 (3.7)	106 (13.8)
Liechtenstein[b]	–	–	–	–
Lithuania	1,368	10 (0.7)	56 (4.1)	271 (19.8)
Luxembourg	29	0 (0.0)	0 (0.0)	0 (0.0)
Malta	14	0 (0.0)	0 (0.0)	0 (0.0)
Netherlands	656	1 (0.2)	23 (3.5)	11 (1.7)
Norway	280	1 (0.4)	7 (2.5)	6 (2.1)
Poland	4,659	9 (0.2)	104 (2.2)	31 (0.7)
Portugal	1,321	1 (0.1)	21 (1.6)	17 (1.3)
Romania	5,966	75 (1.3)	232 (3.9)	530 (8.9)
Slovakia	181	0 (0.0)	4 (2.2)	1 (0.6)
Slovenia	126	0 (0.0)	2 (1.6)	0 (0.0)
Spain[a]	–	–	–	–
Sweden	503	1 (0.2)	33 (6.6)	14 (2.8)
United Kingdom	5,151	8 (0.2)	196 (3.8)	81 (1.6)
Total EU/EEA	27,694	131 (0.5)	931 (3.4)	1,310 (4.7)

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

[a] No case-based reporting to the European Surveillance System (TESSy) of drug susceptibility testing results but aggregated results were reported to the World Health Organization's Tuberculosis Monitoring and Evaluation platform.

[b] No reporting to TESSy in 2012.

less frequently of foreign origin, respectively 18.7% and 12.5% ($p<0.001$ for both comparisons).

Previous treatment was reported for 14.4% of all laboratory-confirmed cases, but it was much more common in cases with MDR-TB (excluding XDR-TB), and XDR-TB, accounting for 53.1% ($p<0.001$) and 77.3% ($p<0.001$), respectively compared to all other cases (pan-susceptible, monoresistant and polyresistant cases). Surprisingly, only 15.3% of the TB cases with a polyresistant pattern had previously received TB treatment. This is a bit more than the 11.9% ($p=0.022$) of the pan-susceptible that had previously received TB treatment, and comparable to the 18.0% ($p=0.168$) of monoresistant TB cases.

Fifteen countries reported case-based HIV testing results. Overall, only 28.1% of the TB cases had HIV status reported, but this percentage was much higher for MDR-TB (excluding XDR-TB), and XDR-TB cases, i.e. respectively 52.7% and 65.6%. Also, the percentage testing positive for HIV was higher for MDR-TB (excluding XDR-TB) cases (10.4%, $p<0.001$), and XDR-TB cases (13.1%, $p<0.001$), when compared to all other cases

Table 4

Anti-tuberculosis drug resistance in new and previously treated multidrug-resistant TB cases[a] (including extensively drug resistant tuberculosis) by year, EU/EEA, 2007–2012

New MDR-TB cases (including XDR-TB)												
Tuberculosis drugs[b]	2007 (N MDR-TB=516)		2008 (N MDR-TB=533)		2009 (N MDR-TB=596)		2010 (N MDR-TB=547)		2011 (N MDR-TB=593)		2012 (N MDR-TB=542)	
	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)
Ethambutol[c]	439	268 (61.0)	460	269 (58.5)	498	249 (50.0)	480	264 (55.0)	529	337 (63.7)	487	292 (60.0)
Streptomycin[c]	397	355 (89.4)	384	321 (83.6)	456	392 (86.0)	466	411 (88.2)	514	454 (88.3)	470	403 (85.7)
Amikacin[d]	124	21 (16.9)	169	47 (27.8)	219	48 (21.9)	245	57 (23.3)	308	55 (17.9)	293	56 (19.1)
Capreomycin[d]	140	34 (24.3)	163	48 (29.4)	219	51 (23.3)	251	63 (25.1)	292	58 (19.9)	278	51 (18.3)
Ciprofloxacin[d]	8	0 (0.0)	40	6 (15.0)	30	5 (16.7)	33	5 (15.2)	58	7 (12.1)	32	2 (6.3)
Kanamycin[d]	140	57 (40.7)	151	47 (31.1)	236	71 (30.1)	355	98 (27.6)	406	97 (23.9)	358	84 (23.5)
Moxifloxacin[d]	0	0 (–)	0	0 (–)	1	0 (0.0)	47	7 (14.9)	69	16 (23.2)	102	8 (7.8)
Ofloxacin[d]	151	23 (15.2)	197	31 (15.7)	247	32 (13.0)	373	60 (16.1)	410	77 (18.8)	375	44 (11.7)
Previously treated MDR-TB cases (including XDR-TB)												
Tuberculosis drugs[b]	2007 (N MDR-TB=972)		2008 (N MDR-TB=1,003)		2009 (N MDR-TB=873)		2010 (N MDR-TB=817)		2011 (N MDR-TB=792)		2012 (N MDR-TB=727)	
	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)	Tested N (%)	Resistant N (%)
Ethambutol[c]	543	400 (73.7)	652	472 (72.4)	606	396 (65.3)	545	342 (62.8)	604	416 (68.9)	580	411 (70.9)
Streptomycin[c]	561	523 (93.2)	616	478 (77.6)	607	539 (88.8)	559	498 (89.1)	614	534 (87.0)	582	507 (87.1)
Amikacin[d]	96	23 (24.0)	111	38 (34.2)	139	34 (24.5)	209	50 (23.9)	208	47 (22.6)	218	56 (25.7)
Capreomycin[d]	108	27 (25.0)	113	38 (33.6)	150	40 (26.7)	204	47 (23.0)	201	43 (21.4)	189	54 (28.6)
Ciprofloxacin[d]	11	8 (72.2)	159	68 (42.8)	103	16 (15.5)	106	18 (17.0)	136	27 (19.9)	101	21 (20.8)
Kanamycin[d]	192	128 (66.7)	172	112 (65.1)	319	133 (41.7)	470	170 (36.2)	526	193 (36.7)	480	208 (43.3)
Moxifloxacin[d]	0	0 (–)	0	0 (–)	1	0 (0.0)	11	3 (27.3)	15	6 (40.0)	56	15 (26.8)
Ofloxacin[d]	162	72 (44.4)	221	45 (20.4)	231	56 (24.4)	399	113 (28.3)	422	128 (30.3)	418	134 (32.1)

EU/EEA: European Union/European Economic Area; MDR-TB multidrug resistant tuberculosis; TB: tuberculosis; XDR-TB: extensively drug resistant tuberculosis.

27 EU/EEA countries provided case-based data. France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. Liechtenstein reported case-based data on drug susceptibility testing for rifampicin and isoniazid only in 2007 and Greece only from 2009 to 2011.

[a] MDR-TB cases (including XDR-TB) with information available on previous treatment are included.

[b] Drug susceptibility testing results for the drugs levofloxacin, gatifloxacin, and moxifloxacin were only collected from 2013 onwards with updates for previous year data. None of the EU/EEA countries has reported susceptibility testing results for levofloxacin or gatifloxacin.

[c] First line drugs.

[d] Second line drugs.

Table 5

Characteristics of all laboratory-confirmed TB cases tested for at least isoniazid and rifampicin resistance by drug resistance pattern, EU/EEA, 2012

Characteristics	Pan-susceptible N (%)	Monoresistant N (%)	Polyresistant non-MDR-TB N (%)	MDR-TB not XDR-TB N (%)	XDR-TB N (%)	Total N (%)
Total	**24,199 (100.0)**	**1,648 (100.0)**	**537 (100.0)**	**1,182 (100.0)**	**128 (100.0)**	**27,694 (100.0)**
Sex						
Female	7,968 (32.9)	536 (32.5)	180 (33.5)	303 (25.6)	28 (21.9)	9,015 (32.6)
Male	16,227 (67.1)	1,112 (67.5)	356 (66.3)	879 (74.4)	100 (78.1)	18,674 (67.4)
Unknown	4 (0.0)	0 (0.0)	1 (0.2)	0 (0.0)	0 (0.0)	5 (0.0)
Age groups (years)						
0–14	356 (1.5)	34 (2.1)	7 (1.3)	13 (1.1)	0 (0.0)	410 (1.5)
15–24	2,687 (11.1)	210 (12.7)	64 (11.9)	111 (9.4)	9 (7.0)	3,081 (11.1)
25–44	9,125 (37.7)	685 (41.6)	246 (45.8)	515 (43.6)	62 (48.4)	10,633 (38.4)
45–64	7,843 (32.4)	541 (32.8)	174 (32.4)	457 (38.7)	47 (36.7)	9,062 (32.7)
≥65	4,186 (17.3)	178 (10.8)	46 (8.6)	86 (7.3)	10 (7.8)	4,506 (16.3)
Unknown	2 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.0)
Origin						
Foreign	6,805 (28.1)	570 (34.6)	174 (32.4)	221 (18.7)	16 (12.5)	7,786 (28.1)
Native	17,060 (70.5)	1,057 (64.1)	356 (66.3)	959 (81.1)	112 (87.5)	19,544 (70.6)
Unknown	334 (1.4)	21 (1.3)	7 (1.3)	2 (0.2)	0 (0.0)	364 (1.3)
Previous treatment						
No	20,343 (84.1)	1,288 (78.2)	430 (80.1)	517 (43.7)	25 (19.5)	22,603 (81.6)
Yes	2,885 (11.9)	296 (18.0)	82 (15.3)	628 (53.1)	99 (77.3)	3,990 (14.4)
Unknown	971 (4.0)	64 (3.9)	25 (4.7)	37 (3.1)	4 (3.1)	1,101 (4.0)
HIV status						
HIV tested	6,431 (26.6)	470 (28.5)	173 (32.2)	623 (52.7)	84 (65.6)	7,781 (28.1)
HIV infected[a]	282 (4.4)	41 (8.7)	15 (8.7)	65 (10.4)	11 (13.1)	414 (5.3)
Unknown	17,768 (73.4)	1,178 (71.5)	364 (67.8)	559 (47.3)	44 (34.4)	19,913 (71.9)
Site of disease						
Pulmonary	20,450 (84.5)	1,368 (83.0)	461 (85.8)	1,126 (95.3)	127 (99.2)	23,532 (85.0)
Extra-pulmonary	3,699 (15.3)	280 (17.0)	75 (14.0)	55 (4.7)	1 (0.8)	4,110 (14.8)
Unknown	50 (0.2)	0 (0.0)	1 (0.2)	1 (0.1)	0 (0.0)	52 (0.2)

EU/EEA: European Union/European Economic Area; HIV: human immunodeficiency virus; MDR-TB: multidrug resistant tuberculosis; TB: tuberculosis; XDR-TB: extensively drug-resistant tuberculosis.

27 EU/EEA countries provided case-based data. France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. Liechtenstein and Greece did not report to TESSy in 2012.

[a] The denominator for the calculation of percentage of HIV infected was the number of HIV-tested cases.

(pan-susceptible, monoresistant and polyresistant cases).

Over 95% of the MDR-TB (excluding XDR-TB), and XDR-TB cases had pulmonary TB. For the other resistance patterns, around 85% were reported to have pulmonary TB.

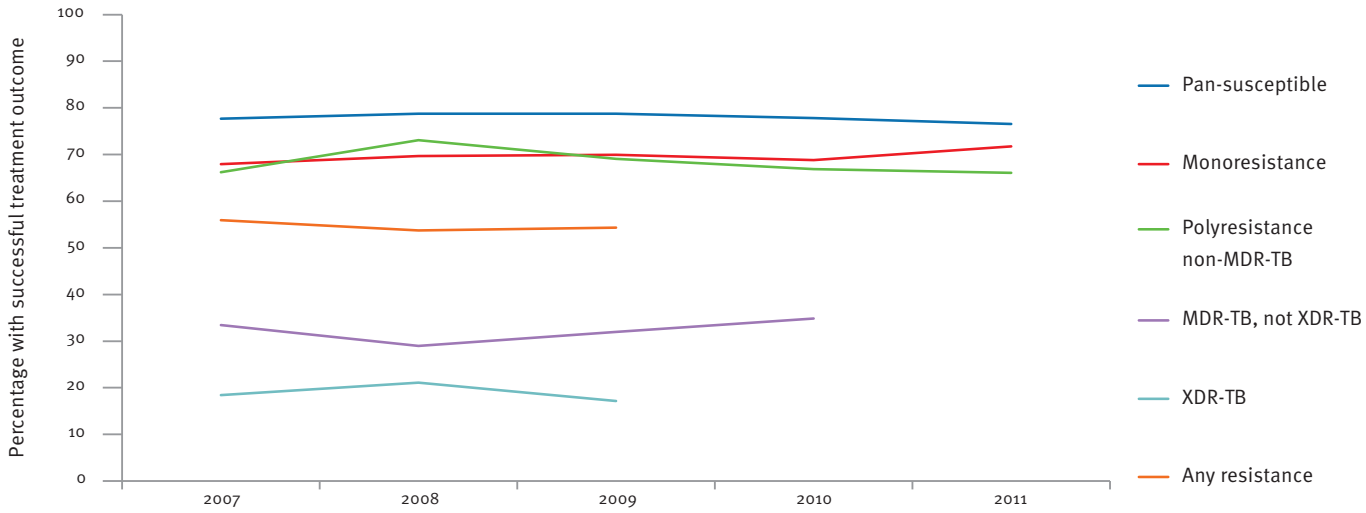
Treatment outcome of tuberculosis cases with drug resistance

In period from 2007 to 2011, 77.9% of the pan-susceptible laboratory-confirmed TB cases with a test result for at least isoniazid and rifampicin resistance had a successful treatment outcome. Cases with a monoresistant or polyresistant non-MDR-TB drug resistance pattern showed slightly lower (69.6% and 68.2%, respectively) treatment success rates. For MDR (excluding XDR-TB) reported in period from 2007 to 2010 and XDR-TB cases reported in period between 2007 and 2009, the treatment success rates were 32.2% and 19.1%, respectively (Figure 3). Trends in successful treatment outcome were relatively stable over the years.

The treatment success rate of new pulmonary MDR-TB (excluding XDR-TB) cases was 48.2% and 21.4% for

Figure 3

Successful treatment outcome of laboratory-confirmed tuberculosis (TB) cases tested for at least isoniazid and rifampicin resistance, by drug resistance pattern, EU/EEA, 2007–2011



EU/EEA: European Union/European Economic Area.

27 EU/EEA countries provided case-based data. France, Italy, and Spain do not report case-based drug susceptibility results to the European Surveillance System (TESSy) database but report aggregated results to the World Health Organization's Tuberculosis Monitoring and Evaluation platform. Of the 27 EU/EEA countries included in the study, Liechtenstein reported case-based data on drug susceptibility testing for rifampicin and isoniazid only in 2007 and Greece only from 2009 to 2011.

previously treated pulmonary MDR (excluding XDR-TB) cases in the period between 2007 and 2010. For new pulmonary XDR-TB cases the treatment success rate was 49.2% in the period from 2007 to 2009 and for previously treated pulmonary XDR-TB cases it was 13.3%.

Of all MDR-TB cases (including XDR-TB) diagnosed between 2007 and 2009, 32.4% had a successful treatment outcome. Of all MDR-TB cases (including XDR-TB) that had an unsuccessful treatment outcome, 21.4% died, 24.5% failed treatment and 19.8% defaulted from treatment, 0.6% transferred out and for 1.4% outcome was unknown (Figure 4). Treatment outcomes did not show any improvement in the years 2007 to 2009.

In period between 2007 and 2009, Romania reported a treatment success rate of 20.5% for 2,089 MDR-TB (including XDR-TB) cases. Since this significantly influences the overall picture for the EU/EEA, we provide treatment outcome results without the data reported by Romania. Without these data, 49.1% successfully finished treatment, 20.7% died, 7.5% failed treatment, 18.1% defaulted, 1.3% were transferred out, and 3.3% were reported as unknown.

Discussion

The percentage of laboratory-confirmed TB cases with different drug resistance patterns, i.e. pan-susceptible, mono-, and polydrug resistance, MDR-TB (excluding XDR-TB), and XDR-TB, has been stable for new cases during the period of the study, from 2007 to 2012. In new TB cases, drug resistance does not seem to be a significant problem with only 2% being diagnosed with MDR-TB. However, in previously treated TB cases, a much higher percentage, i.e. 16% is diagnosed with MDR-TB. The observed decline in the percentage of previously treated TB cases with MDR-TB from 22.3% in 2007 to 16.5% in 2010 can partly be explained by less selective testing. In 2007, 37.2% of all previously treated TB cases were tested for isoniazid and rifampicin resistance and in 2010 this had increased to 46.6%.

Our analysis also showed that treatment outcome results have been stable over the years with an acceptable treatment success rate in pan-susceptible TB cases, though below the target of 85% of the monitoring framework of the Framework Action Plan to Fight Tuberculosis in the European Union [9]. Measured against the set target of 70% in the monitoring framework of the Framework Action Plan to Fight Tuberculosis in the European Union [9], the treatment success rate of new pulmonary culture-positive MDR-TB was unacceptably low at only 48.2% in the period from 2007 to 2010.

Not all notified laboratory-confirmed TB cases had a result reported for rifampicin and isoniazid sensitivity testing. In 2010, the EU/EEA target for testing culture-confirmed TB cases for susceptibility to first-line TB drugs was set at 100% [9]. Even though a higher percentage of TB cases was tested in 2012 (78.5%)

compared to 2007 (63.5%), the target was far from being reached for the EU/EEA overall. However, five EU/EEA countries have reached and nine are close to reaching the target, reporting DST results including for rifampicin and isoniazid for 95% or more of the laboratory-confirmed TB cases. The low percentage of notified laboratory-confirmed TB cases with a result reported for rifampicin and isoniazid drug sensitivity testing is mainly explained by the low percentage of testing results reported by Romania, the country that reported the highest number of laboratory-confirmed TB cases in the EU/EEA, and reported rifampicin and isoniazid DST results for less than half of their cases. In addition, several EU countries do not report case-based DST results to TESSy but report aggregated results to the WHO's Tuberculosis Monitoring and Evaluation platform.

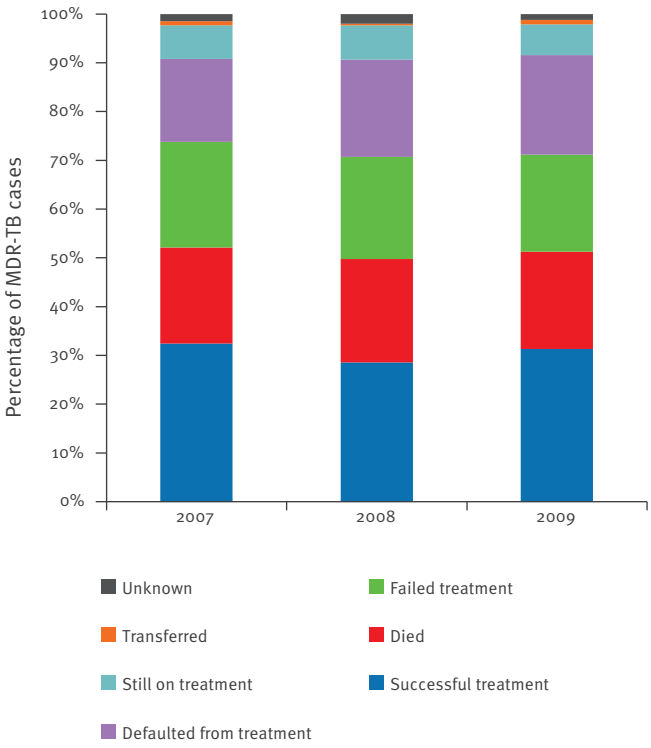
Information on the drug susceptibility pattern for second-line TB drugs is likely to be beneficial for the treatment outcome of MDR-TB patients as drug susceptibility results allow for adequate choice of treatment. The EU/EEA surveillance data showed that in 2012, for 68.0% of MDR-TB cases, a testing result for susceptibility to second-line TB drugs was reported and between 2008 and 2010 there was a sharp increase. ECDC started collection of drug susceptibility results for second line drugs in 2008. Few countries provided second line drug susceptibility data for the years before 2008. After 2008 the number of countries reporting second line drug susceptibility data increased and also the completeness of reporting improved.

TB patient characteristics differed across resistance patterns. Characteristics of cases with mono- and polydrug resistance were largely similar to those of pan-susceptible cases whereas MDR- and XDR-TB cases seemed to differ from cases with other resistance patterns. Of the limited number of risk factors that we could evaluate, we found that male sex, native origin, previous TB treatment, and HIV infection were more frequent in MDR-TB (excluding XDR-TB) and XDR-TB. Of note is the low percentage of TB cases for which HIV status was reported. Other studies have shown similar results [17-21]. Especially a history of previous treatment is frequently identified as a strong risk factor for MDR-TB [17, 18, 21]. Other reported risk factors are a history of imprisonment, alcohol abuse, smoking, and hospitalisation for more than 14 days [17,18,20].

Treatment outcome results of TB cases with a mono- or polydrug resistance (not MDR-TB) were only slightly less favourable compared to those of pan-susceptible TB cases. The reported treatment success rates for MDR-TB and XDR-TB cases were significantly below the EU/EEA target of 70% [9]. In a recently published meta-analysis, using individual MDR-TB patient data, the pooled treatment success rate was 54% and ranged between 11 and 89% in the different studies that were included [22]. Surveillance data reported in the Global Tuberculosis Report 2012 showed that MDR-TB

Figure 4

Treatment outcome of all MDR-TB cases (including XDR-TB) after 36 months, EU/EEA countries[a], 2007–2009



EU/EEA: European Union/European Economic Area; MDR-TB: multidrug resistant tuberculosis; XDR-TB: extensively drug resistant tuberculosis.

[a] Countries reporting treatment outcome for the different cohorts are provided in [27].

treatment success rates ranged between 44% and 58% for the 2009 treatment cohort in the different WHO regions [1]. Since 58.5% of the MDR-TB patients in the EU/EEA were notified by Romania between 2007 and 2009, the treatment outcomes achieved in this country have a considerable effect on the overall MDR-TB treatment outcomes in the EU/EEA.

In the analysis we included confirmed TB cases according to the EU case definition with available data on drug susceptibility for at least isoniazid and rifampicin. These inclusion criteria may potentially underestimate the percentage of TB cases with any resistance as well as polydrug resistance.

This study is based on the TB surveillance data submitted to ECDC by the EU/EEA countries. As listed above, this limits the information available on risk factors. Also, not all reported information is complete and the quality of the reported information is the responsibility of the individual country. However, the substantial amount of data available allows for obtaining a rather adequate picture of the drug resistance situation in the EU/EEA.

The EU/EEA TB surveillance system does not contain information on drug susceptibility data for the drug pyrazinamide. This is because of the technical complexity of achieving reliable and reproducible results. Also, there is no external quality assurance for pyrazinamide available [23]. Pyrazinamide is important in the treatment of tuberculosis and is included in many of the new TB regimens that are currently evaluated [24].

Conclusion and recommendations

The available data show that the number of TB cases with drug resistance is stable in the EU/EEA, but not declining. Patients having drug resistant TB need to sustain longer treatment with more drugs, and they have worse treatment outcomes, especially if diagnosed with MDR-TB or XDR-TB. Also, treatment costs of MDR-TB are at least five times higher compared to treatment costs of drug susceptible TB [25]. Health systems in EU/EEA countries should be prepared to adequately diagnose and treat drug-resistant TB, and test all TB cases for drug susceptibility in a quality-assured laboratory [26]. Monitoring of drug resistance data at national and EU/EEA level should be continued to support identification of risk groups and areas where improvement may be needed.

Conflict of interest

None declared.

Authors' contributions

Marieke J. van der Werf designed and drafted the manuscript and coordinated the input from the other authors. Csaba Ködmön performed the data extraction and analysis and provided input for specific parts of the manuscript, reviewed the draft manuscript, and gave final approval of the version to be published. Vahur Hollo checked the data provided in the manuscript, reviewed the draft manuscript, and gave final approval of the version to be published. Andreas Sandgren checked the data provided in the manuscript, performed the statistical testing, reviewed the draft manuscript, and gave final approval of the version to be published. Phillip Zucs provided input for specific parts of the manuscript, reviewed the draft manuscript, and gave final approval of the version to be published.

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Increased incidence of *Clostridium difficile* PCR ribotype 027 in Hesse, Germany, 2011 to 2013

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After the first outbreak of *Clostridium difficile* PCR ribotype (RT) 027 in Germany in 2007, no further outbreaks were reported until the recent re-emergence of RT 027 in Hesse, a federal state with 6 million inhabitants located in south-west Germany. We undertook a survey to determine the prevalence of RT 027 and other strains in a prospective study. From January 2011 to July 2013, we analysed 291 specimens from patients diagnosed with *C. difficile* infection (CDI) in 40 healthcare facilities in Hesse. The mean incidence of CDI in hospitals including at least 10 patients in the survey was 9.9 per 10,000 patient days (range 4.8-22.8) in November 2012. We obtained 214 toxigenic *C. difficile* isolates. RT 001 was the most prevalent (31.8%). RT 027, the second most common type (26.6%), was prevalent in all hospitals (n=14) from which at least seven isolates were available for typing, but its frequency varied considerably (range: 9.1–70%). The annual frequency of RT 027 increased from 21.4% in 2011 to 30.0% in 2013 (p=0.04). Our study indicates that infections with *C. difficile* RT 027 are now prevalent in Hesse. It underscores the need for surveillance programmes to analyse the molecular epidemiology of *C. difficile*.

Introduction

Clostridium difficile is the main cause of antibiotic-associated diarrhoea in hospitals in industrialised countries. It characteristically occurs in elderly patients with co-morbidity in whom the intestinal flora has been disrupted by previous use of antibiotics. Since early 2003, increasing rates of *C. difficile* infection (CDI) have been reported in Canada and the United States, with a larger proportion of severe and recurrent cases than previously reported [1,2]. The raised incidence and virulence of CDI have coincided with the spread of hypervirulent strains, particularly the NAP1/PCR-ribotype (RT) 027 strain [3]. Subsequently, epidemics of CDI caused by RT 027 have been recognised in hospitals in European countries, e.g. the United Kingdom, the Netherlands, Belgium, and Austria [4,5].

In Germany, the first outbreak of infection caused by the RT 027 strain was reported from Rhineland-Palatinate in 2007 [6]. Since then, sporadic cases of infection by RT 027 have been detected in other regions, but no further outbreak has been reported. In a study from Bavaria, south-east Germany, this strain accounted for 4.6% of *C. difficile* isolates collected in 2009 [7]. In a nationwide study by Zaiss et al., RT 027 was detected in 8% of isolates obtained from patients with severe CDI in 84 German hospitals in 2008 [8]. In a pan-European survey, RT 027 was not detected among 25 *C. difficile* isolates collected in German hospitals in 2008, although it accounted for 5% of isolates from different European countries [9].

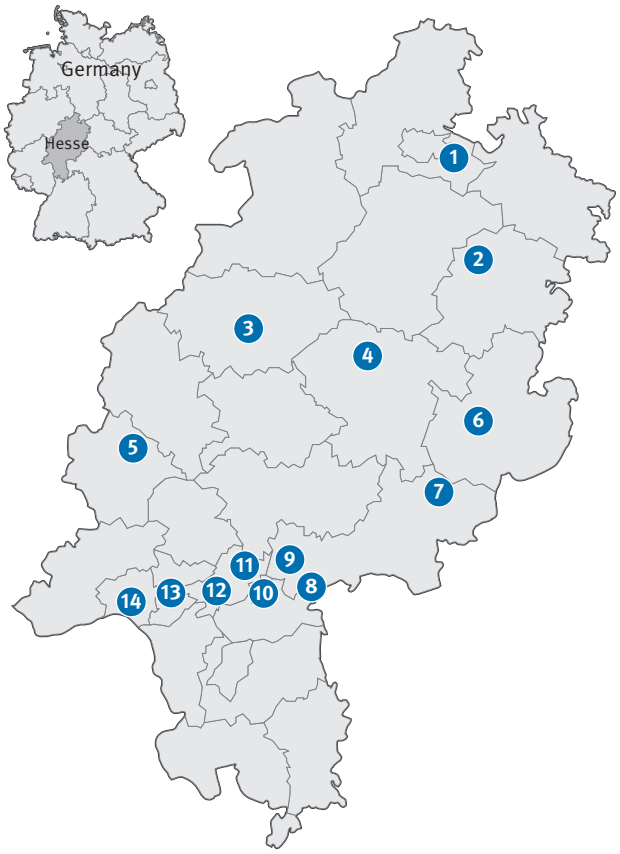
Mandatory reporting of severe CDI was introduced in Germany in 2007 and a case definition was developed by the Robert Koch Institute [10]. Although incidence rates and also prevalence of severe CDI increased in Germany after 2000, an association with particular strains remained unclear, since no microbiological characterisation of the isolates accompanied the nationwide surveillance [11-13]. Although we have previously reported on severe CDI due to RT 027 in Hesse, a federal state with six million inhabitants located in south-west Germany (Figure 1), our studies were mainly focussed on severe cases of CDI reported to the regional health authorities [14,15]. Similar to the national surveillance programme, our regional surveillance did not reveal the actual prevalence of RT 027 and other circulating types. The aim of the present study was to analyse the molecular epidemiology of a comprehensive sample of *C. difficile* isolates associated with any clinical manifestation of CDI. We here present the results of surveillance of CDI in over 40 hospitals and other healthcare facilities in Hesse from January 2011 through July 2013.

Methods

Hospitals, rehabilitation clinics, regional health authorities and physicians in private practices were approached to voluntarily participate in the programme '*C. difficile* surveillance in Hesse' via internet,

Figure 1

Distribution of hospitals that contributed to this study by submitting at least 10 specimens from patients with *Clostridium difficile* infection, Hesse, 2011 to 2013 (n=14)



email, information events and telephone calls. Patients older than two years, who were diagnosed with CDI in a hospital or other healthcare facility in Hesse, were eligible for participation. The hospitals participating in the study were requested to enrol between 10 and 20 patients with a positive toxin test of faeces. There was no selection of patients based on specific criteria such as disease severity, duration, recurrence, etc. Patients with epidemiological link (cluster of cases or outbreak) were excluded. Patients were tested on request of their physician in the local laboratory that provided diagnostic service for the respective hospital. Participation requirements were submission of a faecal sample to our laboratories and completing a questionnaire on clinical symptoms, previous and current antibiotic therapy, and previous laboratory testing results. The participating centres also provided data to determine the incidence rates of CDI. The medical ethical committee of the Hessian Medical Association approved the investigation; no patient agreement was necessary because the samples were collected for routine microbiological diagnostics.

Additional specimens were obtained from a group of patients who had been reported to the local health authorities as severe cases of CDI (mandatory reporting). Upon request of the local health authorities to the respective hospital or local laboratory, the local diagnostic laboratory forwarded the specimens to our institution for PCR ribotyping. The isolated *C. difficile* strains (n=12) were included in this study. Furthermore, some specimens from diarrhoeal patients residing in nursing homes or rehabilitation clinics were directly submitted to our laboratory for diagnostic tests for CDI. These isolates (n=14) were also included in the study.

Together, 291 samples collected in 40 healthcare facilities were enrolled from January 2011 through July 2013. *C. difficile* was detected by culture on *C. difficile*-selective agar containing cycloserine, cefoxitin and fructose (Oxoid, Wesel, Germany) with and without pre-treatment with ethanol as described previously [16]. Identification was performed by routine microbiological techniques and a latex agglutination test for *C. difficile* (Microgen, Cambereley, United Kingdom). All isolates were tested for in vitro production of *C. difficile* toxins A and B by ELISA (Biopharm, Darmstadt, Germany). *C. difficile* toxin A and B genes were detected by commercial PCR kits (Hyplex, Gießen, and Hain, Nehren, Germany). PCR ribotyping was performed in the laboratories of the Hesse State Health Office or in the Department of Medical Microbiology, Leiden University Medical Center, according to the protocol of Bidet et al. [16,17].

The incidence of CDI in the collaborating hospitals was evaluated using the data provided by the study coordinator of each facility. The incidence rate was calculated by dividing the number of patients with laboratory-confirmed CDI who were treated in the hospital in November 2012 by the total number of patient days in November 2012. This month was selected for assessing the incidence of CDI in our study in order to allow a better comparison with the results of a pan-European study that analysed the incidence in the participating hospitals in November 2008 [9].

Statistical analysis was performed with the linear regression model using Excel 2007. A p value of <0.05 was considered significant.

Results

Participating hospitals and patients' characteristics

Fourteen hospitals located in 12 cities in 11 different districts across Hesse participated in this study by submitting at least 10 faecal samples (Figure 1). Of these, 13 hospitals offered secondary or tertiary care, and one was specialised in geriatrics. Table 1 lists general information about these facilities. The samples from these hospitals accounted for 240 of 291 analysed specimens. The remaining 51 samples were obtained from 26 other healthcare facilities, including acute care hospitals, rehabilitation clinics, nursing homes, general practitioner or internist practises, and one prison.

Table 1

Clostridium difficile isolates submitted by collaborating hospitals, Hesse, January 2011–July 2013 (n=180)

Hospital number	Number of beds	Incidence per 10,000 patient days	Number of toxigenic isolates	Number and proportion of ribotype 027, n (%)
1[a]	90	17.3	7	1 (14.3)
2	570	4.8	9	1 (11.1)
3	1,200	7.9	16	2 (12.5)
4	190	5.1	11	1 (9.1)
5	180	7.3	12	4 (33.3)
6	930	NA	8	3 (37.5)
7	280	11.3	13	4 (30.8)
8[b]	270	12.6	10	7 (70.0)
9	750	5.4	17	2 (11.8)
10	790	11.5	14	2 (14.3)
11	1,190	5.9	20	9 (45.0)
12	1,030	11.2	16	4 (25.0)
13[b]	140	22.8	11	1 (9.1)
14	1,030	5.6	16	9 (56.3)

NA: not available.

[a] Geriatric hospital.

[b] Hospital with large geriatric unit.

All submitting hospitals were acute care hospitals located in 12 cities in 11 different districts in Hesse and submitted at least 10 specimens from patients with *Clostridium difficile* infection to this study (Figure 1).

Of 291 samples analysed, 229 samples which were collected from 219 patients contained *C. difficile*. From 219 patient-adjusted isolates, 214 (97.7%) were toxigenic. Together, 193 (90.2%) of 214 toxigenic isolates were from patients in acute care hospitals, nine (4.2%) from rehabilitation clinics, seven (3.3%) from outpatients, and five (2.3%) from nursing homes. Four of seven outpatients had been hospitalised within four weeks before onset of diarrhoea. These cases were considered as hospital-acquired.

The median age of patients with confirmed CDI (n=214) was 77 years (range: 2.5–98 years), and 107 patients (49.5%) were female. According to the questionnaire, 36 (16.7%) patients had previous CDI episodes in the three months before the current episode and 20 (9.3%) patients had died at the time the questionnaire was completed, i.e. within 30 day after collection of the faecal sample.

Characteristics of *Clostridium difficile* isolates

The 214 toxigenic isolates were further characterised. Altogether, 41 ribotypes were detected. Ribotype 001 was the most prevalent type, accounting for 68 (31.8%) of toxigenic isolates (Table 2). It was detected in 20 of 37 healthcare facilities that had submitted samples with positive culture results. Ribotype 027 represented the second most common strain, accounting for 57 (26.6%) of toxigenic isolates. It was detected in 19 healthcare facilities. One isolate was defined as a RT 027 variant because it displayed a slight banding difference compared to the epidemic RT 027 strain. In contrast to the epidemic RT 027, this isolate was sensitive to erythromycin and moxifloxacin. But similar to the epidemic RT 027 strain, this isolate also contained binary toxin genes and had a mutation in *tcdC* at positions 18 and 117. Ribotype 014 accounted for 21 (9.8%) of toxigenic isolates and was detected in 13 healthcare facilities. Ribotypes 078, 002, 029, 012, 017, and 005 were encountered in 2.8%, 2.8%, 2.8%, 1.9%, 1.9% and 1.4% of toxigenic isolates (Table 2). Other ribotypes were detected sporadically, i.e. once or twice, in this study. They included RT 003, 011, 013, 043, 045, 046, 049, 052, 062, 071, 081, 087, 126, 136, 150, 159, 181, 207, 209, 216, 235, 258, 268, 293, 476, and seven unknown ribotypes.

Prevalence of *Clostridium difficile* RT 027 in the collaborating hospitals

We next determined the frequency of isolation of RT 027 in those hospitals that had submitted at least 10 specimens to this study (Figure 1). The epidemic RT 027 strain was detected in all hospitals, but its prevalence varied markedly between different hospitals, ranging from 9.1 to 70% (Table 1).

Incidence of *Clostridium difficile* infection in the collaborating hospitals

We further evaluated the incidence of CDI in collaborating hospitals that had submitted at least 10 specimens from CDI patients to this study. Incidence data were collected for one month (November 2012). Data were available from 13 of 14 hospitals. The mean incidence

Table 2

Frequency of *Clostridium difficile* ribotypes among toxigenic isolates from 37 healthcare facilities in Hesse, January 2011 to July 2013 (n=214 isolates)

Ribotype	Number of isolates	Proportion of isolates (%)	Number of healthcare facilities
001	68	31.8	20
027	57	26.6	19
014	21	9.8	13
078	6	2.8	6
002	6	2.8	4
029	6	2.8	4
012	4	1.9	3
017	4	1.9	1
005	3	1.4	2

In total, 39 different ribotypes were detected. Ribotypes detected only once or twice in this study are not listed in the Table.

of CDI in these hospitals was 9.9 per 10,000 patient days. The incidence varied considerably between different hospitals, ranging from 4.8 to 22.8 per 10,000 patient days (Table 1). The highest incidence rates were reported from a geriatric clinic (Hospital 1: 17.3 per 10,000 patient days) and a hospital with a large geriatric unit (Hospital 13: 22.8 per 10,000 patient days).

Changing frequency of *Clostridium difficile* RT 027 over time

In order to monitor the spread of RT 027, we determined the annual frequency of isolation of RT 027, along with other prevalent ribotypes such as RT 001 and 014, over the study period. Ribotype 027 accounted for 21.4% of toxigenic isolates in 2011. Its frequency increased to 26.2% in 2012 and 30.0% in 2013 (Figure 2). The increase in the prevalence of RT 027 was statistically significant (correlation coefficient: 0.995; p=0.04).

Discussion

To our knowledge, this is the first report on increased incidence of *C. difficile* RT 027 in Germany. The epidemic RT 027 strain represented the second most prevalent type and accounted for 26.6% of isolates collected in 37 healthcare facilities in Hesse in 2011 to 2013. Previous studies have revealed a relatively low prevalence (0–8%) of RT 027 among isolates collected in different parts of Germany, including Hesse, in 2008 and 2009 [7-9]. RT 027 was not detected in a random collection of *C. difficile* isolates from German hospitals in 2008 [9]. More recent data from a representative nation-wide sample are not yet available. Our data suggest that the epidemiology of RT 027 has changed in Hesse and probably also in other parts of Germany.

Changing epidemiology of CDI has been recently reported from other European countries [5,18]. A substantial increase in the incidence of CDI and prevalence of RT 027 was observed in England and Wales in 2005 to 2007 [19,20]. The introduction of a bundle of infection control measures at national level led to a significant decrease in the incidence of CDI and prevalence of RT 027 [5,18]. In the Netherlands, RT 027 was associated with outbreaks in several hospitals in 2005 and 2006 [21]. Its prevalence decreased in the period from 2006 to 2009 and seemed to remain stable thereafter [22]. However, recent reports in 2013 suggest re-emergence of RT 027 associated with severe CDI and outbreaks in the Netherlands, especially in healthcare facilities associated with nursing homes [23,24].

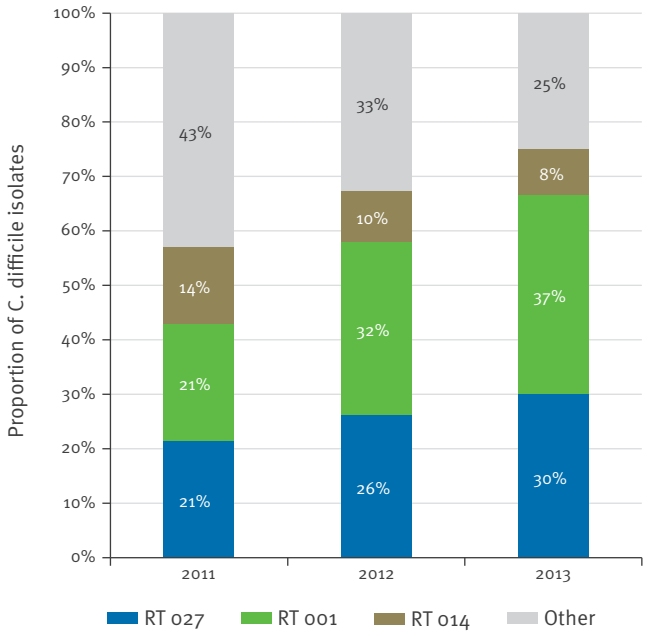
In our study, *C. difficile* RT 027 was prevalent in all hospitals from which a sufficient number of isolates were available for typing, but its frequency varied between hospitals. Higher prevalence rates were observed in the southern part of Hesse (Hospitals 5–14), which is characterised by a higher density of population and hospitals. However, we also observed striking differences between hospitals in the same city. For example, the prevalence of RT 027 was 70% in Hospital 8 and 11.8% in Hospital 9, which are located in the same city. Similarly, Hospitals 11 and 12 were located in the same city and had a different prevalence of RT 027 (45% versus 25%). Differences in patient characteristics, antimicrobial therapy regimens, diagnostic tests, awareness of the doctors, and infection prevention policy may have contributed to this variability.

None of the participating hospitals in this study had noticed or reported an outbreak of CDI at the time of sample collection. Nonetheless, the high prevalence of RT 027 in some hospitals indicates possible ongoing transmission. Further investigations to evaluate this hypothesis are in progress. Together, our data suggest that RT 027 has become endemic in hospitals in Hesse. Because RT 027 is associated with outbreaks, more severe diarrhoea, higher attributable mortality, and more recurrences than other ribotypes, our findings underscore the need for effective infection control measures to curb the spread of RT 027 and other hypervirulent strains in Germany.

The mean incidence of CDI in the collaborating hospitals was 9.9 per 10,000 patient days in our study. This is higher than the rate reported by the national surveillance system CDAD-KISS in 2012 (7.2 per 10,000 patient days) [25]. This difference may in part be due to methodological differences. Our incidence data were derived from one month (November 2012), whereas the KISS data include the whole year. It is also possible that the high prevalence of RT 027 in our region may have contributed to a higher incidence of CDI in our study. However, it is also plausible that differences in patients' characteristics and selection of participating hospitals could have contributed to this discrepancy. The participating hospitals in CDAD-KISS are mainly large academic hospitals. None of the collaborating hospitals in our study were participants of CDAD-KISS.

Figure 2

Frequency of *Clostridium difficile* ribotypes 027, 001, 014, and other ribotypes among toxigenic *C. difficile* isolates from Hesse,



RT: ribotype

The percentage of isolates assigned to each ribotype is shown in the columns.

Note: data for 2013 include only seven months.

Our study included several smaller hospitals with a large proportion of geriatric patients, who are generally at higher risk of developing CDI. It is important to note that the highest incidence rates in our study were observed in geriatric hospitals and those with a large geriatric unit.

In a pan-European survey, the incidence of hospital-associated CDI ranged from 0 to 19.1 per 10,000 patient days in different countries [9]. In comparison, the incidence of CDI in our study seems moderate. However, we believe that the incidence of CDI is generally underestimated in Germany, mainly because diagnostic tests are not routinely performed for all patients with healthcare-associated diarrhoea. Further educational programmes are necessary to increase the awareness of doctors and healthcare personnel for CDI.

The main limitation of our study is the small sample size, which can be explained by the voluntary nature of participation and limitations in time and resources in the collaborating hospitals. Since the study is being continued, we hope to overcome this restriction by increasing the number of the samples analysed.

In conclusion, *C. difficile* infections caused by RT 027 are now observed frequently in Hesse and their prevalence seems to be increasing. Our data underscore the need for surveillance programmes that include both microbiological and epidemiological data at regional, state, and national level in Germany and for intervention programmes to combat CDI and the spread of hypervirulent strains.

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Conflict of interest

None declared.

Authors' contributions

MA designed and conducted the survey

DV, GB-W, CH, EK, and MA performed laboratory investigation and data analysis

MA, wrote the manuscript

The C. difficile study group Hesse coordinated the study in the hospitals, enrolled patients, and collected data

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Nucleic acid-based detection of influenza A virus subtypes H7 and N9 with a special emphasis on the avian H7N9 virus

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In 2013, a novel influenza A virus of subtype H7N9 was transmitted from avian sources to humans in China, causing severe illness and substantial mortality. Rapid and sensitive diagnostic approaches are the basis of epidemiological studies and of utmost importance for the detection of infected humans and animals. We developed various quantitative reverse transcriptase PCR (RT-qPCR) assays for (i) the generic detection of the haemagglutinin (HA) gene of H7 viruses or the neuraminidase (NA) gene of N9 viruses, and (ii) the specific detection of HA and NA of the novel avian H7N9/2013 virus. The sensitivity of the newly developed assays was compared with previously published PCRs, and the specificity of all RT-qPCRs was examined using a panel of 42 different H7 and 16 different N9 isolates. Furthermore, we analysed the performance of the RT-qPCR assays with dilution series and diagnostic samples obtained from animal experiments. Our study provides a comprehensive set of RT-qPCR assays for the reliable detection of the novel avian H7N9 virus, with high sensitivity and improved and tailored specificity values compared with published assays. Finally, we also present data about the robustness of a duplex assay for the simultaneous detection of HA and NA of the avian influenza H7N9/2013 virus.

Introduction

Multiple reassortment events, trans-species transmissions, and viral adaptation of influenza A viruses (IAV) in non-human host species shaped the latest human pandemic influenza virus that emerged in 2009 [1]. Most recently, another animal influenza virus, this time of purely avian origin, was introduced into the human population in the east of China: influenza A subtype H7N9 [2], hereafter referred to as avian H7N9 virus. At least 354 people were infected, most probably after contact with infected poultry, other avian species, or contaminated environment [3,4]. A total of 113 deaths ensued (last revised on 18 February 2014, WHO) [5].

The avian reservoir of this virus has remained obscure. In contrast to highly pathogenic influenza A(H5N1) virus, avian H7N9 virus is of low pathogenicity in tested avian host species. Experimentally infected birds did not develop any overt clinical signs, and a natural infection with avian H7N9 virus did not induce disease [2,6]. This severely impedes syndromic surveillance as an early warning measure for the spread of this virus in poultry and wild birds. The risk of the disease spreading to Europe is considered low [7].

Experiments using the ferret model, demonstrated that avian H7N9 virus could easily be transmitted via close contact, while air-borne transmission between the ferrets was limited [8]. Avian H7N9 virus is transmitted between birds [9], and probably continues to circulate in poultry and/or wild birds in China. As a consequence, surveillance systems based on rapid, highly specific, and sensitive molecular-diagnostic approaches are mandatory for the verification of clinical cases in humans, but also for monitoring and surveillance of poultry and wild bird populations. For this purpose, we developed a set of real-time quantitative reverse transcriptase polymerase chain reactions (RT-qPCR), which target different fragments of the haemagglutinin (HA) and the neuraminidase (NA) genes of influenza A viruses, with special emphasis on the novel avian H7N9/2013 virus and with explicit advantages.

Methods

Viruses and RNA samples

The avian influenza A(H7N9) virus (A/Anhui/1/2013) used in this study was kindly provided by the World Health Organization (WHO) Collaborating Centre London, United Kingdom. For virus propagation, embryonated chicken eggs or Madin Darby canine kidney (MDCK) cell cultures were inoculated at different multiplicities of infection (MOIs) and incubated at 37 °C for five or three days, respectively. In addition to this H7N9 virus, RNA samples from 458 influenza virus strains representing 16 HA and nine NA subtypes from the German National Reference Laboratory for Avian Influenza at the Friedrich-Loeffler-Institute,

Insel Riems, were used for the analytical validation of the newly developed assays. This panel includes viruses of 42 different H7 and 16 different N9 subtypes. All viruses were of avian origin, except the H7 strain A/equi/Prague/1/56, which was of equine origin. Furthermore, a dilution series of avian H7N9 virus as well as swabs and tissue samples originating from animal experiments with chickens, pigeons and ferrets inoculated with influenza A/Anhui/1/2013 virus were used for the validation of the different assays. The animal trials gained governmental approval under the registration number LVL MV/TSD/7221.3-1.1-021/13.

RNA isolation

Viral RNA was extracted from supernatants of infected cell cultures or allantoic fluids of embryonated chicken eggs using the QIAamp viral RNA kit (Qiagen, Hilden, Germany). Swab samples as well as organ samples were extracted using the MagAttract Virus Mini M48 kit (Qiagen) on a Biosprint 96 platform (Qiagen).

Primers and probes

Primers and probe of the pan-influenza A IAV-M1.2 assay [10] were used to determine the quantification cycle of all samples tested. For comparative analyses, the recently published assays from Corman et al. [11] and Wong et al. [12] were tested in parallel, accompanied by a further assay (FLI-H7-CODA), which uses the so-called CODA primers from the Belgian National Reference Laboratory for Avian Influenza at the Veterinary and Agrochemical Research Centre (CODA-CERVA) in combination with the FLI probe (published by the OIE/FAO Network of expertise on animal influenza (OFFLU) on the website www.offlu.net) [13]. In addition, for the in silico identification of primers and probes specific for the avian H7N9 virus, consensus sequences for the HA gene as well as the NA gene were generated from sequences published within the Global Initiative on Sharing All Influenza Data (GISAID) database (www.gisaid.org). Assays for each segment were designed for broad detection of all published H7 (FLI-H7generic-2) and N9 (FLI-N9generic-11) sequences. In addition, assays for H7 (FLI-H7anhui-8) and for N9 (FLI-N9anhui-1) of avian H7N9 A/Anhui/1/2013 and related strains were designed. For the selection of the primers and probes of the generic H7 and N9 assays, we used 910 nearly complete H7 gene sequences as well as 181 nearly complete N9 gene sequences available at GenBank (www.ncbi.nlm.nih.gov/genbank). All oligonucleotides were synthesised by Metabion GmbH (Martinsried, Germany) and stored at -20 °C until use. Sequences of primer and probe sets used in this study are summarised in Table 1. A schematic of the relative location of the target regions of various sets of primer and probe along the HA and NA genes is given in the Figure. In silico analysis of primer and probe binding properties was carried out with the software Primer 3 [14].

Internal extraction control

For IAV screening investigations, the IAV-M1.2 assay was combined with an internal control system in a duplex assay [15]. Therefore, an in vitro transcript of the enhanced green fluorescent protein (EGFP) gene was used in a duplex PCR set-up and the specific fragment was detected using a HEX-labelled probe to exclude false negative results.

Real-time RT-PCR (RT-qPCR)

A one-step RT-qPCR protocol was chosen in order to minimise the risk of cross-contamination. The composition of a 12.5 µL total reaction using the RNA UltraSense One-Step qRT-PCR kit was as follows: 5.875 µL RNase-free water, 2.5 µL 5x RT-PCR buffer, 0.625 µL RT-PCR enzyme mix, and 1 µL primer–probe mix, and 2.5 µL RNA template. All RT-qPCR runs were performed on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) using the following temperature profile: 15 min at 50 °C, 2 min at 95 °C, 45 cycles of 15 sec at 95 °C, 15 sec at 60 °C and 30 sec at 72 °C. Fluorescence values (FAM, HEX) were collected during the annealing step. All analyses were done in triplicate (development and comparison of the RT-qPCR) or duplicate (animal trial), and mean values are presented. Reactions with a quantification cycle (Cq) value of less than 42 scored negative.

Generation of in vitro-transcribed standard RNA

The HA and the NA sequences of the H7N9 A/Anhui/1/2013 virus (accession numbers: EPI439507, EPI439509) were ordered as synthetic genes flanked by EcoRI and XhoI cloning sites in a pUC-derived plasmid backbone (GeneArt, Regensburg, Germany). Synthesis of RNA run-off transcripts from a T7 promoter site upstream of the 5' EcoRI cloning site was performed as recommended by the manufacturer (T7 RiboMAX, Promega, Germany). RNA copies were calculated according to the formula:

(X g/µL RNA / [transcript length in nucleotides x 340]) x 6.022 x 10^{23} = Y molecules/µL.

PCR reactions for quantification of the in vitro-transcribed standard RNA were performed using the RNA UltraSense One-Step qRT-PCR kit with its specific parameters.

Results

In order to develop primer and probes for the generic detection of the HA of H7 viruses (assay FLI-H7generic-2) or the NA of N9 viruses (assay FLI-N9generic-11), 910 nearly complete H7 sequences as well as 181 nearly complete N9 sequences were analysed in silico. Furthermore, primer and probes for the specific detection of the HA (assay FLI-H7anhui-8) or NA (assay FLI-N9anhui-1) of the novel avian H7N9/2013 virus were designed based on consensus sequences published on www.gisaid.org (Table 1). In the present study, we analysed the performance of the newly developed

Table 1

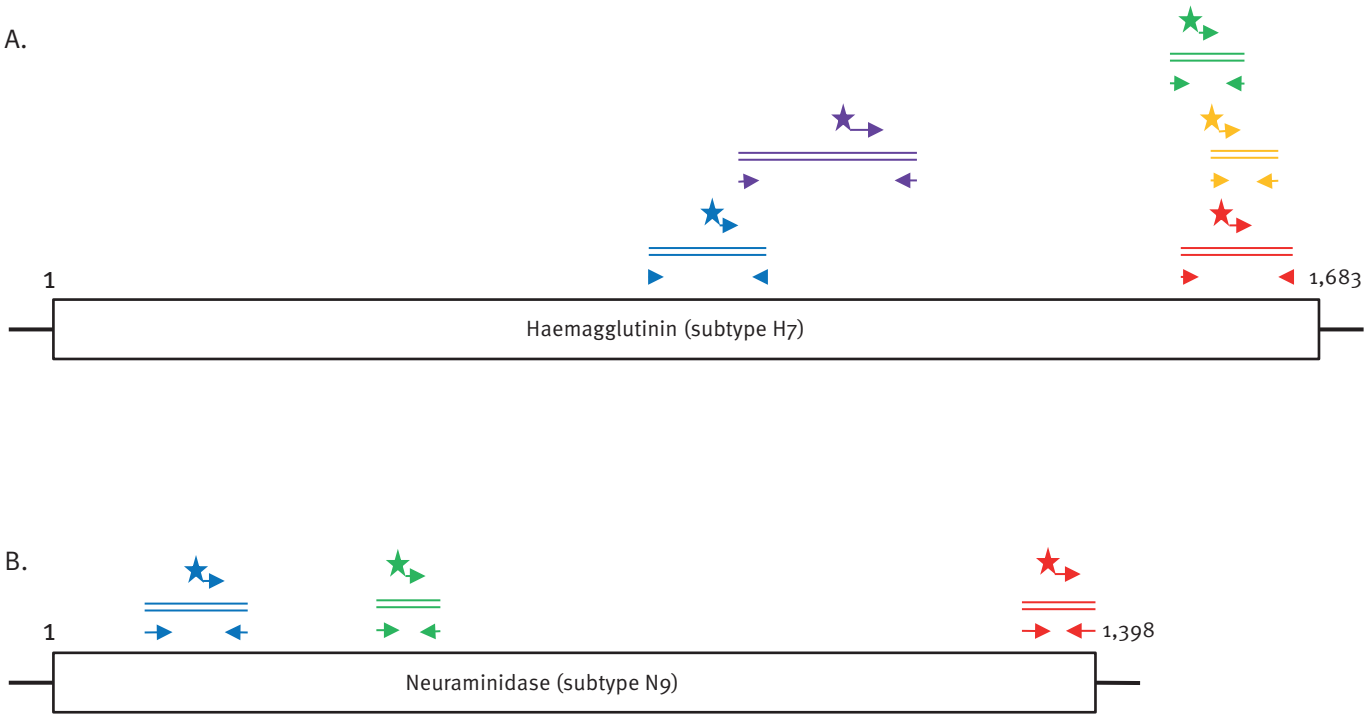
Primers and probes used in this study for nucleic acid-based detection of influenza A virus subtypes H7 and N9

Designation	Sequence 5'→3'	Genome position[a]	Concentration of primer and probes in the primer–probe mix
Pan-IAV assay			
IAV-M1.2 [10]			
IAV-M1-F	AGA TGA GTC TTC TAA CCG AGG TCG	1–24	20 µM
IAV-M1.1-R	TGC AAA AAC ATC TTC AAG TYT CTG	99–76	15 µM
IAV-M1.2-R	TGC AAA GAC ACT TTC CAG TCT CTG	99–76	15 µM
IAV-M1-FAM	FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1	49–68	2.5 µM
H7 assays			
FLI-H7generic-2 [This study]			
IAV-HA7-1593-F	AYA GAA TAC AGA TWG ACC CAG T	1,523–1,544	20 µM
IAV-HA7-1740-R	TAG TGC ACY GCA TGT TTC CA	1,653–1,672	20 µM
AIV-HA7-1649-FAM	FAM-TGG TTT AGC TTC GGG GCA TCA TG –BHQ1	1,579–1,601	2.5 µM
FLI-H7anhui-8 [This study]			
IVA-H7anhui-830F	TGA GAG GAA AAT CTA TGG GAA TC	806–828	15 µM
IVA-H7anhui-981R	CTT AAC ATA TCT CGG ACA TTT TCC A	933–957	15 µM
IVA-H7anhui-951FAM_as	FAM-CCT GCT ATC TAT GTT CTG AAA TGG CAA GT-BHQ1	899–927	5 µM
FLI-H7-CODA [13]			
IAV-HA7-CODA-F	GYA GYG GYT ACA AAG ATG TG	1,553–1,572	20 µM
IAV-HA7-CODA-R	GAA GAC AAG GCC CAT TGC AA	1,619–1,638	20 µM
IAV-HA7-CODA-FAM	FAM-TGG TTT AGC TTC GGG GCA TCA TG-BHQ1	1,579–1,595	2.5 µM
Wong-H7 [12]			
H7-anhui-916F	ATA GAT AGC AGG GCA GTT GG	916–935	5 µM
H7-anhui-1156R	GAT CAA TTG CCG ATT GAG TG	1,137–1,156	5 µM
H7-anhui-1096FAM	FAM-CCY TCY CCY TGT GCR TTY TG-BHQ1	1,096–11,15	5 µM
Corman-H7 [11]			
HA7_1_2013rtF	TAC AGG GAA GAG GCA ATG CA	1,501–1,520	10 µM
HA7_1_2013rtR	AAC ATG ATG CCC CGA AGC TA	1,584–1,603	10 µM
HA7_1-2013rtFAM	FAM-ACCCAGTCAAACTAAGCAGCGGCTA-TAMRA	1,538–1,562	5 µM
N9 assays			
FLI-N9generic-11 [This study]			
IVA-N9-1363F	AGY ATA GTA TCR ATG TGT TCC AG	1,315–1,337	20 µM
IVA-N9-1439R	AAG TAC TCT ATT TTA GCC CCA TC	1,369–1,391	20 µM
IVA-N9-1393FAM	FAM-TTC CTB GGA CAA TGG AAC TGG CC-BHQ1	1,345–1,367	5 µM
FLI-N9anhui-1 [This study]			
IVA-N9anhui-173F	AAC CTG AAA CAA CCA ACA CAA G	140–161	15 µM
IVA-N9anhui-299R	GTT AAG TTA TTG AAA TTC CTG CTT G	227–251	15 µM
IVA-N9anhui-227HEX	HEX-CAA ACA TCA CCA ACA TCC AAA TGG AAG AG-BHQ1	194–222	5 µM
Corman-N9 [11]			
NA9_2013rtF	CCAGTATCGCGCCCTGATA	447–465	10 µM
NA9_2013rtR	GCATTCCACCCTGCTGTTGT	497–516	10 µM
NA9_2013rtFAM	FAM-CTGGCCACTATCATCACCGCCCA-TAMRA	468–490	5 µM

[a] Genome position according to influenza A/Anhui/1/13 (H7N9); GISAID accession numbers: HA: EPI439507, NA: EPI439509, M: EPI439506.

Figure

Relative location of primer and probe sets for the detection of the haemagglutinin (A) or neuraminidase gene (B) of influenza A virus subtypes H7 and N9



A. red: FLI-H7generic-2; blue: FLI-H7anhui-8; orange: FLI-H7-CODA; violet: Wong-H7; green: Corman-H7.
B. red: FLI-N9generic-11; blue: FLI-N9anhui-1; green: Corman-N9.

RT-qPCRs as well as recently published assays with regards to the specific detection of the HA or NA of influenza A/Anhui/1/13 (H7N9), the broad generic detection of H7 or N9 viruses, and the diagnostic sensitivity for the detection of avian influenza A(H7N9) virus in samples from experimentally infected animals. All samples were tested by the pan-influenza IAV-M1.2-assay to verify the viral genome load by generic amplification of conserved parts of the M segment of all IAV [10]. Amplification plots and the calculation of the efficiency, linearity, and precision of the newly developed and recently published assays are available from authors upon request.

RT-qPCR systems for the haemagglutinin genes of influenza A(H7) viruses and the avian influenza A(H7N9) virus

We compared the sensitivity of the generic H7 assay FLI-H7generic-2 with the FLI-H7-CODA assay which used the primers previously developed by CODA-CERVA [16] and has been published by the World Organisation for Animal Health/Food and Agriculture Organization (OIE/FAO) Network of expertise on animal influenza (OFFLU) in combination with a FLI probe [13]. In addition, the newly developed avian H7N9-specific H7 assay FLI-H7anhui-8 was compared with the published assays Wong-H7 and Corman-H7 [11,12]. To this end, we tested a dilution series of viral RNA from influenza A/Anhui/1/13 (H7N9) as well as a dilution series of an RNA run-off transcript of the H7 gene of influenza A/Anhui/1/13 (H7N9) (see Tables 2 and 3).

All assays detected the HA gene of the avian influenza A (H7N9) virus with similar high analytical sensitivity (only the Wong-H7 assay reacted with slightly higher Cq-values; Tables 2 and 3). Based on the Cq-values of the dilution series, the two generic assays had a 10-fold lower detection limit compared with the three H7N9-specific assay, which performed nearly identically (Table 2). The generic assays, FLI-H7generic-2 and FLI-H7-CODA produced similar Cq-values, whereas the H7N9-specific assays FLI-H7anhui-8 and Corman-H7 produced slightly lower Cq-values. The Wong-H7 assay exhibited the highest Cq-values. However, based on the optimal setting of an in vitro transcript, a detection limit of less than 10 genome copies per PCR reaction could be observed for all three influenza A/Anhui/1/13 HA assays (Table 3).

In order to verify both the inclusiveness and the exclusiveness of the newly developed assays in comparison with previously published H7 assays, 42 available H7 IAV isolates were tested (Table 4). The FLI-H7generic-2 assay detected all 42 different H7-isolates, whereas

Table 2

Comparative analytical sensitivity for the detection of haemagglutinin sequences of avian influenza A(H7N9) based on RNA dilution series

Dilution series of A/Anhui/1/2013[a]	IAV-M1.2[b]	Generic H7 RT-qPCRs[b]		Avian H7N9-specific H7 RT-qPCRs[b]		
		FLI-H7generic-2	FLI-H7-CODA	FLI-H7anhui-8	Corman-H7	Wong-H7
H7N9_10-3	19.4	20.3	19.5	21.8	21.5	23.7
H7N9_10-4	23.3	23.7	22.9	25.2	25.0	27.6
H7N9_10-5	26.2	27.0	26.5	28.6	28.5	30.9
H7N9_10-6	29.5	30.5	29.7	31.9	31.3	34.1
H7N9_10-7	33.5	33.7	33.2	34.9	34.6	37.7
H7N9_10-8	No Cq	No Cq	No Cq	37.8	37.9	39.0
H7N9_10-9	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq

No Cq: no value obtained >42.

[a] RNA extracted from allantoic fluid of embryonated chicken eggs infected with the isolate A/Anhui/1/13 (H7N9) was used.

[b] Numerals in columns represent Cq values.

the FLI-H7-CODA protocol detected 37 samples. We also tested 416 non-H7 isolates with the FLI-H7generic-2 assay. Here, six of 31 subtype H10 isolates showed weak cross-reactivity (Cq-values of 20–25 for the IAV-M1.2 assay and Cq >35 with the FLI-H7generic-2 assay) and therefore a specificity of 98.6% (data not shown). Based on the alignments, the primer set designated for the FLI-H7-CODA assay is not optimised for the broad detection of H7-viruses, but performed very well for the detection of A/Anhui/1/13 H7N9. Both the Corman-H7 assay and the Wong-H7 assay were initially designed for the sensitive detection of avian H7N9 virus [11,12], but showed cross-reactivity with several other H7-strains. Therefore, both assays were not specific for the recent avian H7N9 virus. In contrast, despite high viral loads (low Cq values in the IAV-M1.2 assay), none of the analysed H7 isolates showed any reactivity with the FLI-H7anhui-8 assay, which is highly specific for the HA of the recently emerged Chinese lineage of the avian H7N9 virus.

Table 3

Comparative analytical sensitivity for the detection of haemagglutinin sequences of avian influenza A(H7N9) based on an in vitro transcript of the H7 segment

T7 in vitro transcript of the HA of A/Anhui/1/2013[a]	Generic H7 RT-qPCRs		FLI-H7anhui-8
	FLI H7generic-2	FLI-H7-CODA	
200,000	18.7[b]	19.9	19.9
20,000	22.2	23.6	23.4
2,000	25.6	26.9	26.8
200	29.0	30.3	30.2
20	31.9	33.7	33.7
2	36.6	37.1	36.9
0.2	No Cq	No Cq	No Cq

HA: haemagglutinin; No Cq: no value obtained >42.

[a] RNA copies per µL of template.

[b] Numerals in columns represent Cq values.

Viruses of the American lineages of subtypes H7 and N9 were underrepresented in our set of RNAs available for PCR validation. RNA from only one American strain was available (A/chicken/Jalisco/12283/12 (H7N3)). This Mexican strain was detected by the FLI-H7generic-2 assay, while all other H7 assays failed to detect it. In order to obtain an impression of the amplification potential for a larger set of American strains, an in silico analysis of primer and probe binding properties was carried out. This was based on an alignment of 548 H7 and 228 N9 sequences extracted from GenBank. The alignment of AIV strains from North and South America was then analysed with Primer3 [14]. Successful amplification was assumed, when not more than a single mismatch was detected in the respective sequence (one mismatch per primer/probe) (Table 5). When at least one primer or the probe harboured two or more mismatches, amplification was assumed to be unlikely. On the basis of this crude assessment, the newly developed FLI-N9generic-11 and FLI-H7generic-2 PCRs were expected to be able to amplify the majority of American H7 and N9 strains, since 164 of 199 N9 sequences and 448 of 547 H7 sequences revealed a perfect match with both primers and probe (Table 5). Nevertheless, a thorough wet-lab characterisation and validation is an indispensable prerequisite for use of these RTqPCRs on the American continent. Primers and probes of other PCRs listed in Table 5 analysed showed different grades of mismatches, and the generation of specific amplicons with cDNA of American H7 and N9 strains is expected to be less likely.

Table 4

Comparative analytical specificity for the detection of haemagglutinin sequences of H7 influenza A viruses (n=42)

Strain (influenza subtype, biotype)	IAV-M1.2[a]	Generic H7 RT-qPCRs[a]		Avian H7N9-specific H7 RT-qPCRs[a]		
		FLIH7generic-2	FLI-H7-CODA	FLI-H7anhui-8	Corman-H7	Wong-H7
A/equi/Prague/1/56 (H7N7, LP)	22.4	24.6	34.7	No Cq	No Cq	No Cq
A/Turkey/Ontario/18-1/2000 (H7N1, LP)	21.7	24.6	No Cq	No Cq	No Cq	No Cq
A/Teal/Föhr/Wv180/05 (H7N2, LP)	20.3	20.6	19.6	No Cq	21.5	No Cq
A/mallard/Alberta/8734/2007 (H7N3, LP)	23.1	24.6	No Cq	No Cq	No Cq	No Cq
A/swan/Germany/736/06 (H7N4, LP)	20.5	20.7	19.8	No Cq	21.5	No Cq
A/chicken/Italy/473/99 (H7N1, LP)	18.6	18.8	18.0	No Cq	19.9	39.4
A/duck/Alberta/48/76 (H7N3, LP)	22.6	23.4	No Cq	No Cq	No Cq	No Cq
A/swan/Potsdam/62/81 (H7N3, LP)	22.7	25.5	23.1	No Cq	No Cq	No Cq
A/swan/Potsdam/64/81 (H7N3, LP)	25.6	27.9	25.6	No Cq	No Cq	No Cq
A/duck/Potsdam/13/80 (H7N7, LP)	19.8	21.8	20.8	No Cq	No Cq	36.0
R87/99 (H7N7, LP)	17.5	23.6	17.2	No Cq	No Cq	No Cq
A/duck/Potsdam/15/80 (H7N7, LP)	18.9	22.5	19.8	No Cq	No Cq	36.5
A/Avian/R224/10 (H7N7, LP)	18.2	20.9	19.8	No Cq	No Cq	36.1
A/Mallard/NVP/41/04 (H7N1, LP)	21.1	21.3	20.3	No Cq	22.9	40.8
A/turkey/Italy/472/99 (H7N1, LP)	21.2	20.7	19.6	No Cq	21.9	No Cq
A/Alexandria tyrode/T145 (H7N1, LP)	18.4	23.6	17.4	No Cq	No Cq	41.8
A/turkey/Ireland/PV8/98 (H7N7, LP)	21.8	21.4	20.3	No Cq	29.7	No Cq
A/ch/Dgania/Israel/1980_R709/09 (H7N2, LP)	24.4	27.2	24.9	No Cq	29.3	35.8
A/turkey/Germany/R655-5/09 (H7N7, LP)	20.8	20.5	19.7	No Cq	21.7	No Cq
A/Mallard/Germany/R192/09 (H7N7, LP)	21.7	28.7	20.9	No Cq	22.8	No Cq
A/mallard/Sko212-219K/07 (H7N3, LP)	23.7	23.7	22.9	No Cq	25.1	No Cq
A/guinea fowl/Germany/R2495/07 (H7N3, LP)	25.1	25.4	24.5	No Cq	26.6	No Cq
A/ch/Ger/79 “Taucha” (H7N7, HP)	16.4	20.0	17.8	No Cq	No Cq	37.7
A/FPV/Rostock/45/36 (H7N1, HP)	18.8	30.7	18.4	No Cq	No Cq	29.7
A/FPV/Rostock/45/34 (H7N1, HP)	19.5	30.5	18.5	No Cq	No Cq	30.5
A/FPV/dutch/27 (H7N1, HP)	19.1	29.8	17.9	No Cq	No Cq	36.8
A/chicken/Brescia/19/02 (H7N1, HP)	19.2	30.3	18.1	No Cq	No Cq	37.4
A/hen/Italy/444/99 (H7N1, HP)	19.2	19.5	18.5	No Cq	20.7	No Cq
A/chicken/Britsh Columbia/CN-07/2004 (H7N3, HP)	21.2	20.7	No Cq	No Cq	No Cq	No Cq
A/broiler/Itlay/445/99 (H7N1, HP)	18.7	18.8	17.7	No Cq	19.7	39.5
A/chicken/Germany/R28/03 (H7N7, HP)	18.7	18.7	17.5	No Cq	21.0	39.9
A/Mallard/Germany/R756/06 (H7N4, LP)	22.5	22.3	20.9	No Cq	23.0	No Cq
A/Mallard/Germany/R721/06 (H7N7, LP)	28.5	23.6	21.8	No Cq	24.4	No Cq
A/Greylag goose/Germany/R752/06 (H7N7, LP)	26.5	21.2	20.0	No Cq	23.5	No Cq
A/Mute swan/Germany/R901/06 (H7N1, LP)	20.5	20.4	18.9	No Cq	21.2	No Cq
A/Mallard/Föhr/Wv190/05 (H7N7, LP)	24.3	23.4	22.5	No Cq	24.5	No Cq
A/Teal/Föhr/Wv177/05 (H7N7, LP)	22.4	27.6	26.8	No Cq	28.6	No Cq
A/sentinel-duck/Germany/SK207R/07 (H7N3, LP)	25.4	26.1	24.7	No Cq	27.5	No Cq
A/Mute swan/Germany/R57/06 (H7N7, LP)	24.4	24.7	23.4	No Cq	29.9	No Cq
A/duck/Italy/636/03 (H7N3, LP)	20.0	20.7	19.6	No Cq	21.6	No Cq
A/turkey/Italy/2043/03 (H7N3, LP)	21.3	22.6	20.7	No Cq	23.3	No Cq
A/chicken/Jalisco/12283/12 (H7N3, HP)	21.8	22.8	No Cq	No Cq	No Cq	No Cq

HP: highly pathogenic; LP: low pathogenic; No Cq: no value obtained >42.

[a] Numerals in columns represent Cq values.

Table 5

In silico analysis of primer and probe binding properties with avian influenza subtype H7 (n=548) and N9 (n=228) sequences of North and South American origin

Assay	Number of sequences tested	Primer/probe match 100%	Primer/probe single mismatch[a]	Primer/probe double mismatch[a]	Primer/probe more than two mismatches[b]	Expected detection
FLI-N9generic-11	199[c]	164	34	1	0	Yes
Corman-N9	228	0	0	154	74	Doubtful/poor
FLI-N9anhui-1	228	0	0	0	228	Unlikely
FLI-H7generic-2	547[c]	448	99	0	0	Yes
FLI-H7anhui-8	548	0	0	0	548	Unlikely
FLI-H7-CODA	548	0	0	0	548	Unlikely
Wong-H7	548	0	0	0	548	Unlikely
Corman-H7	548	0	0	0	548	Unlikely

[a] None of the mismatches were located within the last three nucleotides at the 3' end of the primers except for Corman-N9 (NA9_2013rtF) were one mismatch in the last two nucleotides were identified.

[b] At least one primer or the probe harboured more than two mismatches, rendering stable binding unlikely.

[c] 29 sequences of subtype N9 and one sequence of subtype H7 had to be excluded from analysis due to lack of sequence information for the specific site (sequences too short).

RT-qPCR systems for the neuraminidase genes of influenza A (N9) viruses and the avian influenza A (H7N9) virus

An RNA dilution series of viral RNA from the avian influenza A (H7N9) virus was analysed using two newly developed N9 assays (FLI-N9generic-11 and FLI-N9anhui-1) in comparison with the N9 assay specific for the avian H7N9 virus published by Corman et al. (Corman-N9, [11]) (Table 6). Furthermore, the results for the two newly developed N9 assays were independently confirmed by the use of RNA run-off transcripts of the N9 gene of influenza A/Anhui/1/13 (Table 7).

Based on the Cq-values, the performance of all NA assays was nearly identical. Analysing the dilution series of viral RNA, the FLI-N9generic-11 assay was more sensitive than the FLI-N9anhui-1 assay or the Corman-N9 assay, which exhibited identical detection limits. The analysis of the in vitro transcript revealed a lower detection limit for the FLI-N9generic-11 assay (less than 10 genome copies per reaction) than the FLI-N9anhui-1 assay (less than 100 genome copies per reaction).

Furthermore, we verified the analytical specificity of the three PCR systems by analysing 16 IAV isolates of subtype N9 (Table 8). Since all N9 strains were detected by the newly developed FLI-N9generic-11 assay, but none of 442 tested non-N9 subtypes (data not shown), this assay was confirmed to be suitable for the specific and sensitive generic detection of N9 viruses. Despite very high viral genome loads (low Cq values in the

Table 6

Comparative analytical sensitivity for the detection of neuraminidase sequences of avian influenza A (H7N9) based on RNA dilution series

Dilution series of A/Anhui/1/2013[a]	IAV-M1.2[b]	FLI-N9generic-11[b]	Avain H7N9-specific N9 RT-qPCRs[b]	
			FLI-N9anhui-1	Corman-N9
H7N9_10-3	19.4	20.9	22.0	20.6
H7N9_10-4	23.3	24.7	25.4	23.7
H7N9_10-5	26.2	27.8	28.7	26.9
H7N9_10-6	29.5	31.1	31.4	29.7
H7N9_10-7	33.5	34.1	35.1	33.1
H7N9_10-8	No Cq	36.0	No Cq	No Cq
H7N9_10-9	No Cq	No Cq	No Cq	No Cq

No Cq: no value obtained >42.

[a] RNA extracted from allantoic fluid of embryonated chicken eggs infected with the isolate A/Anhui/1/13 (H7N9) was used.

[b] Numerals in columns represent Cq values.

Table 7

Comparative analytical sensitivity for the detection of neuraminidase sequences of avian influenza A(H7N9) based on an in vitro transcript of the N9 segment

T7 in vitro transcript of the NA of A/Anhui/1/2013[a]	FLI-N9generic-11[b]	FLI-N9anhui-1[b]
200,000	19.8	19.9
20,000	23.3	23.6
2,000	26.7	26.8
200	30.1	29.9
20	33.1	32.5
2	35.7	No Cq
0.2	No Cq	No Cq

NA: neuraminidase; No Cq: no value obtained >42.

[a] RNA copies per µL of template

[b] Numerals in columns represent Cq values.

IAV-M1.2 assay), only three of the 16 tested N9 isolates showed positive cross-reactivity using the newly developed FLI-N9anhui-1 assay, indicating a good specificity of this assay for the specific detection of influenza A/Anhui/1/13 (H7N9). Interestingly, although designed to specifically detect the NA of this avian H7N9 virus, the Corman-N9 assay detected 15 of the 16 tested N9 strains. Therefore, compared to the Corman-N9 assay, the FLI-N9anhui-1 test is more specific.

Analysis of the combined influenza A(H7N9) RT-qPCR

The use of different fluorescent tags in the newly developed assays allowed us to specifically detect the HA (FLI-H7anhui-8, FAM) and NA (FLI-N9anhui-1, HEX) of the novel avian H7N9 virus simultaneously in a duplex approach (Tables 9 and 10). Based on the in vitro transcripts, the detection limit of the duplex RT-qPCR was less than 10 genome copies per reaction for both HA and NA, and therefore corresponds with the results of the uniplex FLI-H7anhui-8 and FLI-N9anhui-1 assays.

We also used the 42 different H7 and the 16 different N9 isolates of our IAV panel for determining the specificity of the duplex assay. Like the single RT-qPCRs of the FLI-H7anhui-8 assay, none of the tested H7 viruses was detected with the combined assay (data not shown). However, the N9 gene of influenza A/wigeon/Germany/R636/07 (H11N9) and A/Anas platyrhynchos/Germany/R2219/2006 (H11N9), which were shown to be detected by the FLI-N9anhui-1 assay (Table 8), were also detected with the duplex assay (data not shown). Taken together, the duplex assay allowed the sensitive and specific detection of HA and NA of avian H7N9 in a single PCR run.

Validation of the diagnostic sensitivity of the RT-qPCR systems for the haemagglutinin and neuraminidase genes of the avian influenza A(H7N9) virus

Finally, we analysed the diagnostic performance of the newly developed assays for the generic detection of H7

Table 8

Comparative analytical specificity for the detection of neuraminidase sequences of N9 viruses influenza A viruses (n=16)

Strain(influenza subtype)	IAV-M1.2[a]	FLI-N9generic-11[a]	Avain H7N9-specific N9 RT-qPCRs[a]	
			FLI-N9anhui-1	Corman-N9
A/wild duck/Germany/R3111/07 (H2N9)	21.1	21.9	No Cq	20.5
A/mallard/Alberta/329/2006 (H5N9)	20.3	21.4	No Cq	35.5
A/mallard/British Columbia/544/2005 (H5N9)	24.7	27.8	No Cq	37.1
A/Mallard duck/Germany/R2711/07 (H2N9)	24.0	24.5	No Cq	23.5
A/wigeon/Germany/R636/07 (H11N9)	20.9	20.8	26.3	20.7
A/shearwater/West Australia/2567/79 (H15N9)	19.1	23.0	No Cq	No Cq
A/Ostrich/Germany/R48/10 (H6N9)	24.9	25.5	No Cq	22.5
A/duck/Germany/R3349/09 (H11N9)	22.2	22.4	No Cq	21.8
A/Graylag goose/Germany/R1416/08 (H2N9)	25.8	26.3	No Cq	25.2
A/Graylag goose/Germany/R1485/08 (H2N9)	25.6	26.5	No Cq	24.6
A/Graylag goose/Germany/R1486/08 (H2N9)	20.5	20.6	No Cq	19.5
A/Graylag goose/Germany/R1487/08 (H2N9)	26.3	27.6	No Cq	24.4
A/mallard/Germany/R3108/07 (H11N9)	22.9	22.7	36.8	21.5
A/tk/Ontario/7732/66 (H5N9)	18.4	19.4	No Cq	32.0
A/Mallard/Föhr/Wv1499-1503/03 (H11N9)	19.4	19.6	No Cq	19.1
A/Anas platyrhynchos/Germany/R2219/2006 (H11N9)	23.5	23.7	29.9	22.6

No Cq: no value obtained >42.

[a] Numerals in columns represent Cq values.

Table 9

Analytical sensitivity of the combined primer and probe sets for avian influenza A(H7N9) viruses based on RNA dilution series

Diluion series of A/Anhui/1/2013[a]	IAV-M1.2[b]	FLI-H7N9-Combi-FAM[b,c]	FLI-H7N9-Combi-HEX[b,d]
H7N9_10-3	19.4	20.7	20.9
H7N9_10-4	23.3	24.2	24.7
H7N9_10-5	26.2	28.1	28.4
H7N9_10-6	29.5	31.0	31.2
H7N9_10-7	33.5	33.2	34.2
H7N9_10-8	No Cq	34.3	No Cq
H7N9_10-9	No Cq	No Cq	No Cq

No Cq: no value obtained >42.

[a] RNA extracted from allantoic fluid of embryonated chicken eggs infected with the isolate A/Anhui/1/13 (H7N9) was used.
[b] Numerals in columns represent Cq values.
[c] FAM channel: FLI-H7anhui-8.
[d] HEX channel: FLI-N9anhui-1.

and N9 viruses (FLI-H7generic-2; FLI-N9generic-11), and for the specific detection of avian influenza A(H7N9) (duplex assay FLI-H7anhui-8/FAM; FLI-N9anhui-1/HEX) in comparison with previously published assays (FLI-H7-CODA, Corman-H7 assay, Wong-H7 assay, Corman-N9 assay) [11-13] on sample material from an animal experiment (Table 11). Ten samples per species (ferrets, chickens and pigeons) were randomly chosen and included both different individuals and several time points after infection. The ferret samples included nasal washings and organ samples, whereas chicken samples represented pharyngeal and cloacal swabs, and organs. Pigeon samples included pharyngeal and cloacal swabs (Table 11). Generally, the newly developed assays detected viral RNA robustly and irrespective of the sample matrix (swab, tissue, etc.). Compared with the most sensitive assay, IAV-M1.2, the generic assays performed almost equally well. The test systems specific for avian influenza A(H7N9) demonstrated an at least 10-fold drop in sensitivity. Sample material exhibiting low viral RNA loads, such as the pigeon cloacal swab samples, was identified as positive less frequently with all assays. Therefore, all primer and probe sets represent useful assays for the sensitive and specific diagnosis of the avian influenza A(H7N9) virus, however, the FLI-H7-CODA assay demonstrated a qualified alternative with better overall results based on the Cq-values and the signal strength.

Discussion

With the availability of RT-PCR, numerous assays for the subtyping of influenza viruses have been developed, and multiplex approaches have frequently been proposed [17]. Continuous progress is achieved regarding the signal detection of the PCR products, e. g. by PCR-ELISAs [18,19] and in particular by RT-qPCR technologies. Due to a seemingly constant increase in the number of outbreaks of highly pathogenic avian influenza (HPAI) caused by infections with subtype H5 or H7 viruses in many countries, RT-PCR and RT-qPCRs were especially designed for the broad detection and differentiation of these HA genes [18,20-22] and their pathotypes on the basis of the HA cleavage site motif [23-25]. Often these assays are combined with a differentiation of the NA subtypes N1, N2, or N7 [26]. So far, only few studies have focused on the generic detection of the N9 gene [27] or the H7 gene [16,28]. In addition, a one-step H7-specific reverse transcription loop-mediated isothermal amplification (LAMP) assay has been described for the identification of H7 viruses and shown to be more sensitive than conventional RT-PCR systems [29]. Another recent study uses the LAMP approach together with hydroxynaphthol blue dye for a colorimetric detection of the novel avian influenza A(H7N9) virus [30].

Within a few months of the appearance of the novel avian influenza A(H7N9) virus, different RT-qPCR assays were designed and validated for its detection, as a rapid response to its emergence in humans. They are therefore preferentially tailored for use with human samples [11,12]. Nevertheless, generic H7 assays are useful and needed, especially in places where different lineages of subtype H7 may be circulating in avian reservoir hosts. In our study, the assay by Wong et al. [12] detected the H7 of avian influenza A(H7N9) viruses with good sensitivity, but also detected further H7 viruses with high Cq values. This cross-reactivity has been observed with the use of high-titre allantoic fluids and may not play a role with clinical samples of avian or human origin that generally bear lower viral loads. Nevertheless, we characterise the Wong H7 assay as not specific for the avian influenza A(H7N9) virus. A similar result was obtained for the assay reported by

Table 10

Analytical sensitivity of the combined primer and probe sets for avian influenza A(H7N9) viruses based on in vitro transcripts of the haemagglutinin and neuraminidase segments

T7 in vitro transcript of the HA of A/Anhui/1/2013[a]	FLI-H7N9-Combi-FAM[b,d]	FLI-H7N9-Combi-HEX[c,d]
200,000	18.8	20.4
20,000	22.0	24.2
2,000	24.9	27.2
200	28.7	30.5
20	32.1	33.6
2	35.9	37.2
0.2	No Cq	No Cq

HA: haemagglutinin; No Cq: no value obtained >42.
[a] RNA copies per µL of template.
[b] FAM channel: FLI-H7anhui-8.
[c] HEX channel: FLI-N9anhui-1.
[d] Numerals in columns represent Cq values.

Table 11

Comparative diagnostic sensitivity for the detection of haemagglutinin and neuraminidase sequences of avian influenza A(H7N9) in samples obtained during experimental animal infections

Sample[a]	IAV-M1.2[d]	Generic H7 RT qPCRs[d]		Avian H7N9-specific H7 RT-qPCRs[d]			Generic N9 RT-qPCR[d]	Avian H7N9-specific N9 RT-qPCRs[d]	
		FLI-H7 generic-2	FLI-H7-CODA	FLI-H7N9-Combi-FAM[b]	Corman-H7	Wong-H7	FLI-N9 generic-11	FLI H7N9 Combi-HEX[c]	Corman-N9
Ferret, n.w. A	17.0	18.4	16.6	21.9	22.0	23.7	17.9	20.0	20.9
Ferret, n.w. B	17.0	18.3	17.1	21.4	22.4	23.8	18.2	20.0	21.7
Ferret, n.w. C	18.3	20.8	18.7	24.4	24.3	26.4	19.7	22.0	22.6
Ferret, n.w. D	31.7	34.9	32.1	39.8	37.6	39.7e	33.9	38.3	37.8
Ferret, n.w. E	27.8	29.0	28.0	32.1	33.5	35.4	29.1	31.5	32.9
Ferret, organ A	23.3	25.5	23.8	29.2	29.7	31.6	24.3	27.6	27.9
Ferret, organ B	22.6	24.8	22.6	28.4	28.6	30.8	23.3	25.5	26.8
Ferret, organ C	22.0	23.5	22.4	26.6	27.9	29.1	23.0	25.9	27.0
Ferret, organ D	26.7	27.9	26.4	33.4	32.8	35.3	28.4	31.0	33.4
Ferret, organ E	27.3	29.0	27.1	35.3	33.2	36.9	28.7	32.0	33.1
Chicken, ph.s. A	20.3	21.7	20.7	26.3	26.2	27.8	21.8	24.5	25.0
Chicken, ph.s..B	23.3	25.0	23.7	28.7	29.3	31.7	35.1	27.7	27.6
Chicken, ph.s. C	23.1	24.3	22.9	28.1	28.4	29.8	24.2	27.0	28.0
Chicken, ph.s. D	22.9	24.7	22.7	29.2	28.6	31.0	23.5	28.0	27.0
Chicken, c.s. A	30.0	31.5	30.4	36.5	36.1	37.5	30.7	34.7	34.5
Chicken, c.s. B	24.2	26.2	25.5	29.7	30.6	33.2	25.6	28.7	29.0
Chicken, c.s. C	24.3	26.5	25.1	30.2	30.4	32.4	25.4	29.0	29.1
Chicken, c.s. D	23.3	24.2	23.1	28.1	28.8	29.8	24.2	27.0	28.1
Chicken, organ A	30.7	35.2	32.2	41.7[e]	38.9	No Cq	30.2	No Cq	36.7
Chicken, organ B	26.2	27.2	25.6	32.4	32.1	35.5	26.6	29.7	32.2
Pigeon, ph.s. A	29.4	31.2	29.5	36.6	35.2	38.0	30.0	33.0	33.5
Pigeon, ph.s. B	24.7	26.3	23.8	29.8	30.5	31.5	26.7	28.7	29.8
Pigeon, ph.s. C	30.7	32.3	30.2	37.2	36.9	38.3	31.4	34.7	35.1
Pigeon, ph.s. D	31.3	33.8	32.1	38.2	38.0	40.2	32.1	36.1	35.9
Pigeon, ph.s. E	32.3	35.0	33.6	37.9	38.9	39.6[g]	34.5	40.1[e]	37.0
Pigeon, c.s. A	33.2	36.3[e]	34.6	No Cq	39.7	No Cq	35.5	38.0[e]	38.9
Pigeon, c.s. B	33.7	36.1[e]	33.4	39.1[e]	38.8	39.5[e]	34.4	37.5[e]	37.7
Pigeon, c.s. C	No Cq	36.4[e]	35.8[e]	40.8[e]	No Cq	No Cq	35.6	No Cq	No Cq
Pigeon, c.s. D	36.3[e]	No Cq	33.7	No Cq	40.7	No Cq	34.3	37.5	39.8
Pigeon, c.s. E	36.4[e]	No Cq	No Cq	No Cq	40.6	No Cq	35.7	No Cq	39.2

No Cq: no value obtained >42.

[a] RNA extracted from samples of different origin (nasal washing (n.w.), organs, pharyngeal swabs (ph.s.), or cloacal swabs (c.s.)) obtained at different days post infection with the isolate A/Anhui/1/13 was used.

[b] FAM channel: FLI-H7anhui-8.

[c] HEX channel: FLI-N9anhui-1.

[d] Numerals in columns represent Cq values.

[e] Only one of the duplicates was positive.

Corman et al. [11]. Lack of analytical specificity of these assays is likely to be due to the limited range of lineages selected during design of the primer and probe sets. Previous validation studies of these PCRs were limited to exclude cross-reactions with other human IAV subtypes (and other respiratory viruses of human origin). In addition to the H7 assay, Corman et al. also provided a primer and probe set for the detection of N9. This assay has a high sensitivity for the avian influenza A(H7N9) virus, but also detects all except one of the other N9-viruses tested, and, therefore, should be considered as a generic rather than a lineage-specific assay.

In order to improve the specificity for diagnostic purposes, focusing on animal (i.e. avian) swabs, we intended to define primer and probe sets for (i) the generic detection of the HA of H7 viruses, (ii) the

specific detection of the HA of the avian influenza A(H7N9) virus, (iii) the generic detection of the NA of N9 viruses, (iv) the specific detection of NA of the avian influenza A(H7N9) virus, and (v) the simultaneous detection of HA and NA of the avian influenza A(H7N9) virus. These assays proved to offer excellent sensitivities and specificities on the tested sample preparations. The definition of the new oligonucleotides was facilitated by the fact that a considerable number of new sequence records had accumulated in the EpiFlu database. The HA and NA sequences of the novel avian influenza A(H7N9) isolates form distinct phylogenetic lineages which can be discerned from other Eurasian H7 and N9 sequences [31]. The emerging zoonotic avian influenza A(H7N9) viruses in China, which display low pathogenicity in poultry, but still cause severe clinical disease in humans, pose, in contrast to the HPAI H5N1 epizootic, a particular problem for monitoring in poultry and other avian populations. Since no clinical symptoms are induced in infected poultry, the virus can be transmitted and spread by healthy poultry. According to results of experimental infections, chickens and quails are suspected to be particularly efficient transmitters because these species excreted the largest amounts of virus. The occurrence of a second wave of human infections with A(H7N9) since October 2013 [32] confirmed that pockets of presumably avian reservoirs of this virus continue to exist. This situation emphasises the relevance of surveillance programmes that are based on molecular diagnostic tools for rapid and sensitive but also highly discriminatory detection of influenza A(H7N9) viruses.

Although the reverse primer of the FLI-H7-CODA assay contained a mismatch to the HA sequence of influenza A/Anhui/1/13 (H7N9) virus, it performed well on the detection of the novel avian influenza A(H7N9) virus. A slightly reduced sensitivity could be observed for the combined HA and NA duplex assay, which is due to limitations in the primer design in favour of a high specificity.

In routine settings of diagnostic laboratories, qualitative determination is of primary importance. RT-qPCRs provide the added advantage of a semiquantitative impression, assuming that, as in our assays, inhibitory effects are excluded. Full quantitative analysis based on RNA copy number would be reserved for research purposes, in cases when the amount of excreted virus needs to be analysed comparatively. The newly developed assays would be suitable for each of these fields.

A further advantage of the newly developed RT-qPCRs is that all newly developed assays (FLI-H7generic-2, FLI-N9generic-11, FLI-H7anhui-8, FLI-N9anhui-1, and the FLI-H7anhui-8/FLI-N9anhui-1 combination), but also the previously published assays (IAV-M1.2, FLI-H7-CODA, Corman-H7, Wong-H7, Corman-N9) can be run with the same chemistry and the same amplification protocol, allowing easy handling in diagnostic laboratories.

Previously established assays such as the FLI-H7-CODA and Corman-H7 assay displayed a high sensitivity for the avian influenza A(H7N9) virus, but failed to detect several other isolates of subtype H7. When a panel of 459 viruses (including the avian H7N9 virus) was tested with the FLI-H7generic-2 assay, all 39 tested strains of Eurasian origin, and in addition even one H7 strains of the North American lineage as well as the avian H7N9 virus, were detected. A weak cross reaction was observed with only six viruses of non-H7 subtypes, all six belonging to the closely related subtype H10. The FLI-N9generic-11 showed a high sensitivity for the avian influenza A(H7N9) virus, detected all tested isolates of subtype N9, but none of the 442 strains of the other eight non-N9-NA subtypes.

While this manuscript was in preparation, Hackett et al. published three RT-qPCR assays for the specific detection of the influenza A(H7N9) virus based on the conserved M gene, that can be adopted into established protocols for the detection of human influenza viruses [33]. Furthermore, Li et al. developed an assay for the simultaneous detection of the HA and NA genes of influenza A(H7N9) viruses, but again focussing on samples of human origin and the diagnostic differentiation from other human respiratory viruses [34].

In light of the high mutation rate of IAV, we recommend different combinations of the introduced assays to be used in a diagnostic cascade system: If poultry is screened for the presence of IAV of subtype H7, the IAV-M1.2 assay should be applied first, and in case of a positive result, samples should subsequently be examined with the FLI-H7generic-2 assay, as this assay showed the broadest diagnostic inclusiveness of H7 viruses. Screening, especially of human samples, for IAV of subtype H7N9 should also start with the IAV-M1.2 assay. Positive samples should then be examined by either the FLI-H7-CODA or the Corman-H7 assay, as well as the FLI-N9generic-11 test. Finally, for a direct confirmation of avian influenza A(H7N9) virus in samples which are positive in the generic pan-IAV assays, the combination of the FLI-H7anhui-8/FLI-N9anhui-1 RT-qPCRs in a duplex assay is recommended. Notably, in our study, the newly developed assays have not been validated on human samples. Therefore, a validation of the performance of these assays should be performed on human clinical samples before they are implemented in diagnostic laboratories.

Taken together, suitable RT-qPCR assays with high sensitivity and considerably improved specificity for the generic detection of H7 and N9 subtypes, and for the specific detection of the avian influenzaA (H7N9) virus are provided in this study. Notably, the robustness of a specific duplex assay to confirm the avian influenza A(H7N9) virus was proven.

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Conflict of interest

None declared.

Authors' contributions

Designed the study: MB, BH, TH. Collected and synthesized the data: DK, TH, CG, AP, BH. Interpreted the data: DK, JB, TH, CG, AP, MB, BH. Prepared the first draft of this manuscript: DK, JB. All authors reviewed and revised the first and final drafts of this manuscript.

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