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Rapid communications

FIRST IDENTIFICATION OF TICK-BORNE ENCEPHALITIS IN DENMARK OUTSIDE OF BORNHOLM, AUGUST 2009

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The incidence of tick-borne encephalitis (TBE) in Scandinavia is increasing and spreading geographically. Following two clinical cases of TBE hospitalised after tick bites in northern Zealand, Denmark, specific IgM and IgG antibodies against tick-borne encephalitis virus (TBEV) were demonstrated in acute serum samples of these patients. TBEV was identified by RT-PCR in ticks collected from the same location. This is the first report of TBEV in *Ixodes ricinus* leading to clinical cases in Denmark outside of Bornholm island.

Background

Tick-borne encephalitis (TBE) is caused by TBE virus (TBEV), a member of genus *flavivirus*, family *Flaviviridae*. The incidence of TBE has been increasing in neighbouring countries of Denmark (Sweden and Germany) over the past years and mirrors the geographic spread and increased number of ticks [1,2]. The vector for the European subtype, TBEV-Eu, is *Ixodes ricinus* (the Common Tick) that is seen in most of Europe and is the dominant tick species in Denmark (>90%). In Denmark, TBE is endemic only on the island Bornholm in the Baltic sea, with a stable annual incidence of 4 per 100,000 inhabitants [3]. Three serum samples from roe deer from Zealand examined during the 2002-2003 hunting season were found to be antibody-positive for the TBE-complex of viruses [4]. However, a cross-reaction with Louping ill virus could not be excluded. Importantly, no clinical cases of TBE have been reported or TBEV detected outside Bornholm.

Clinical case and virological analysis

In July 2009, a man in his 40s developed fever and other influenza-like symptoms as well as arthritis about one week after receiving four tick bites in his own garden. The patient is a forest worker living in a house in the forest. After about four days of recovery he was hospitalised two weeks after the bites with symptoms of meningoencephalitis and mononuclear cells in the spinal fluid. Serum samples from the time of admission to hospital at the beginning of the encephalitis were negative for *Borrelia* but had positive IgM (optical density (OD_{450nm}) 1,190, cut-off 224) and IgG (OD_{450nm} 695, cut-off 224) titres to TBEV as measured by a validated ELISA (Enzygnost, Siemens) [5]. Spinal fluid was negative in the PCR for herpes simplex virus, enterovirus, varicella zoster virus and TBEV. At the time of publication of this report, the patient was recovering but continued to feel dizzy.

The patient reported about a man in his 30s who was working in a kindergarten in the same forest about 500 meters away, who had a similar unidentified viral meningoencephalitis after tick bites the year before (October 2008). When re-examining this second patients' acute serum from 2008, the antibody test was positive for anti-TBEV IgM (OD 609, cut-off 224) and IgG (OD 1,109, cut-off 243), and a recent follow-up convalescent serum (taken approximately one year later) was still IgG-positive (OD 942, cut-off 243) but IgM-negative for TBEV [5]. He was therefore rediagnosed as a TBE patient.

A TBEV antibody plaque neutralisation test (kindly performed by Dr. Matthias Niedrig, Robert Koch-Institute, Berlin) using TBEV K23 according to Reinhardt *et al.* [6] was positive on the convalescent serum but not on the acute serum drawn during the encephalitis from both patients.

Environmental analysis

Ticks were collected by "flagging" (dragging of a 1x1 m cloth through the grass) at the edge of the forest surrounding the forest worker's garden, identified by species and sorted into three pools of approximately 50 nymphs, 30 adult females and 25 adult males, respectively. Ticks were also collected (nine pools containing a total of 219 larvae and 62 nymphs and adults) at three different sites in an adjacent forest with the highest density of deer in Denmark. RNA was extracted from the ticks using MagNA Pure total NA kit (ABI), and a real-time RT-PCR was run in a quality-controlled routine PCR diagnostic laboratory using specific primers and probes as described in [7]. The PCR is specific for viruses of the TBEV complex as validated by the European Network for Diagnostic of Imported Viral Diseases, ENIVD (www.enivd.de). Only the pool of nymphs from the patient's garden was strongly positive (RT-PCR cycle threshold value of 22).

Discussion

These are the first two cases of TBE in Denmark outside Bornholm that are confirmed by identification of viruses of the TBEV complex in *I. ricinus* nymphs collected at the same location and same time of transmission to a patient. Both cases had a typical biphasic disease starting with influenza-like symptoms, easily misdiagnosed during the present influenza A(H1N1)v pandemic, and with some neurological sequelae (dizziness, fatigue) after the meningoencephalitis. Both patients were TBE IgM- and IgG-positive in the acute serum. For the patient from 2008, we had the

opportunity to obtain convalescent serum approximately one year later, which, as expected, was TBE IgG-positive and IgM-negative, and positive in the neutralisation test confirming the qualitative ELISA test. It takes time for neutralising antibodies to develop and they are normally not present during the acute illness [5].

It was expected that the distribution of TBE would expand in Europe [1,2,8], and spread in Denmark has been suggested based on serology in roe deer [4]. However, the investigation of roe deer serum antibodies has in itself limited relevance to human medicine, partly because of the uncertainty of the serology method used. So far the distribution on Zealand and the rest of Denmark is not known and could be either very local or very wide. The finding of two confirmed human cases in 2008 and 2009, respectively, suggests that TBEV has been present but unnoticed for a longer time.

According to the Danish legislation, TBE is not a notifiable disease. However, diagnostic tests for TBEV are only performed at the Department of Virology at Statens Serum Institut, and we have not seen any cases of TBE in Denmark outside Bornholm before these cases. We have begun a systematic collection of ticks in Denmark and have so far identified TBEV only in one of two likely locations in north Zealand. The PCR is specific for the TBEV complex, but in addition, we are in the process of culturing or otherwise amplifying the viruses isolated from the collected ticks in collaboration with ENIVD in order to obtain sequences for confirmation and molecular epidemiology. Further sampling, molecular characterisation of the Zealand TBEV, increased clinical awareness and continued monitoring should confirm and clarify the spread of TBE in Denmark.

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Rapid communications

IMPORTED HUMAN AFRICAN TRYPANOSOMIASIS IN EUROPE, 2005-2009

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Physicians in Europe are likely to see more African trypanosomiasis cases because of the increasing popularity of travel to Africa. In this paper the literature on imported cases in Europe, since 2005 is reviewed. Because of the high mortality risk associated with acute Rhodesian trypanosomiasis, travellers should be informed about preventive measures and the early disease manifestations.

Introduction

Human African trypanosomiasis (HAT) is endemic in sub-Saharan Africa. *Trypanosoma brucei rhodesiense* (East Africa) and *T. b. gambiense* (West Africa) are transmitted to humans by tsetse flies of the *Glossina morsitans* group (*T. b. rhodesiense*) and of the *G. palpalis* group (*T. b. gambiense*) which are found only in Africa. West African sleeping sickness has almost exclusively a human reservoir, while East African trypanosomiasis is a zoonosis involving antelopes, cattle and humans. Infections by both *T. b. gambiense* and *T. b. rhodesiense* are generally under-reported in humans due to acuteness and lack of specific symptoms at the onset of disease as well as its rural distribution. *T. b. rhodesiense* is focally endemic in many eastern and southern African countries. It tends to occur in form of epidemic outbursts. Human infections have been reported mainly from Malawi, south-east and central Uganda and Tanzania, and sporadically from Kenya, Mozambique, Rwanda, Zambia and Zimbabwe. *T. b. gambiense*, the parasite causing West African sleeping sickness is focally endemic in Angola, Democratic Republic of the Congo, Central African Republic, Chad, Republic of the Congo, Côte d'Ivoire, Guinea, southern Sudan and north-west Uganda. Cases have been sporadically reported from Burkina Faso, Cameroon, Equatorial Guinea, Gabon, Nigeria, Benin, Ghana and Mali [1]. All countries listed so far have a surveillance system for HAT, however, there is no dedicated structure for surveillance in Burundi, Ethiopia, Gambia, Guinea-Bissau, Liberia, Niger,

Senegal and Sierra Leone, where under-reporting may be likely [2]. A new HAT atlas initiative for sub-Saharan Africa has led to the creation of a geographic database to store and regularly update HAT epidemiological data. The resulting detailed, high quality regional level maps allow the geo-location of autochthonous cases that have been detected through active and passive surveillance [3].

HAT has always been an exceptional travel-associated disease. It is a rare cause of fever [4] cutaneous lesions and/or neurological signs in travellers returning from endemic areas. Although it has been estimated that about 50 cases are reported yearly outside Africa [5], no recent estimate is available. In Europe, the largest published data on imported HAT included 109 cases registered between 1904 and 1963 [6]. Over the last decades, 26 cases (including 24 West African HAT) seen in France between 1980 and 2004, were reviewed [7]. In addition, imported cases were reported in Italy [8,9], Spain [10], the United Kingdom [11-13], Germany [14], the Netherlands [15-19], Belgium [20], Norway and Sweden [21,22], Switzerland [23], Poland [24] and France [25-26].

We present the clinical and epidemiological characteristics of published HAT cases imported in Europe since 2005 (Table).

Diagnosis

T. b. gambiense represents more than 90% of all reported cases of HAT worldwide (autochthonous and imported cases) but *T. b. rhodesiense* accounts for 60% of imported cases. *T. b. rhodesiense* infection in humans is characterised by high grade fever, an inoculation chancre and substantial parasitaemia in its acute stage. Incubation period is about 6 to 10 days, but may be as short as three days. Gambian HAT may follow an indolent course with a very low or absent parasitaemia. It may remain

unrecognised for years [5]. *T. b. gambiense* is better adapted to its human host, allowing humans to be infective for extended periods thereby sustaining its endemicity. In active infection, *T. b. gambiense* and *T. b. rhodesiense* specific IgG and IgM antibodies are present in high concentration and can be detected by ELISA or immunofluorescence from about three to four weeks after infection. Parasite detection using blood concentration techniques should be done to confirm the infection. Furthermore, in 60% of infections with *T. b. gambiense*, parasites can be detected in lymph aspirate from enlarged cervical nodes. Cerebrospinal fluid examination is always required to evaluate neurological involvement which determines the choice of therapy [5].

Implications for travellers

Whereas imported HAT due to *T. b. gambiense* is more often seen in migrants and expatriates residing in rural endemic areas, HAT due to *T. b. rhodesiense* is more likely to be seen in travellers to East African game parks where the ungulate wildlife serves as a reservoir for the pathogen. In recent years almost all reported cases have been infected in northern Tanzania (Serengeti, Tarangire) or in Uganda (Queen Elizabeth National Park) [17,18,22,24,28]. Some emerging tourist destinations (Malawi: Kasungu National Park, Waza Game Reserve; Rwanda: Akagera National Park; Zambia: South Luangwa National Reserve; Tanzania: Moyowosi Game Reserve) are known foci of *T. b. rhodesiense*, and may pose a risk for travellers.

In travellers infected with *T. b. rhodesiense*, an evolving chancre on the bite site precedes the onset of high grade fever, and usually persists for a few days thereafter. This is an important clinical sign not to be missed by the attending physician. Fulminant disease progression has been reported in a German tourist in her forties with a history of tsetse bites during a visit to the Serengeti National Park. She died only six days after fever onset (13 days following tsetse bites), in Nairobi Hospital, after air ambulance evacuation from a private clinic in Dar es Salaam where the HAT diagnosis was made. She had two typical chancres that were missed when she first presented with fever in another clinic seven days after the tsetse bites, and a malaria diagnosis was alleged [29]. A history of tsetse fly bites in patients with clinical symptoms has to be considered a medical emergency. Early treatment with suramin (Germanin®, Bayer 205) or in case of non-availability, with pentamidine is essential to prevent severe complications and death. All available drugs for HAT treatment, including suramin can be obtained through the World Health Organization (WHO) trypanosomiasis control and surveillance unit, by contacting Dr. Simarro (simaropp@who.int) and Dr. Franco (francoj@who.int). A small stock of HAT drugs should be made available at one tropical medicine/travel medicine centre per country, in order to enable early treatment when required.

TABLE

Imported cases of African trypanosomiasis in Europe, since 2005, by date of publication

Sex	Age	Nationality	Clinical features (time before first symptoms and diagnosis)	Sub-species	Country of exposure (reason for travel)	Treatment	Reference (year)
M	44	Italian	Fever, headache, fatigue, weight loss, paresthesia, day-time somnolence, insomnia, hepatosplenomegaly, lymph nodes, ataxia (6 months)	<i>gambiense</i>	Gabon (expatriate)	Eflornithine	9 (2005)
F	54	Italian	Fever, headache, fatigue, splenomegaly, insomnia, hyperesthesia (3 months)	<i>gambiense</i>	Central African Republic (expatriate)	Eflornithine	9 (2005)
F	52	Dutch	Fever, headache, vomiting, diarrhea, confusion, depression, hallucinations, sleepiness. One relapse episode. Death. (4 days)	<i>rhodesiense</i>	Serengeti national park of Tanzania (tourist)	Suramin, Melarsoprol	17 (2006)
M	37	French	Fever, fatigue, anorexia, headache, arthralgia, insomnia, rash, pruritus, paresthesia, lymph nodes, weight loss (8 months)	<i>gambiense</i>	Gabon, Cameroun, Guinea (expatriate)	Pentamidine	26 (2007)
M	72	French	Fever, fatigue, pruritus, lymph nodes, weight loss (5 months)	<i>gambiense</i>	Gabon (expatriate)	Pentamidine	26 (2007)
M	26	British	Fever, insomnia, lethargia, vomiting chancre, erythema, jaundice (5 days)	<i>rhodesiense</i>	Malawi (military)	Suramin	12 (2007)
M	38	British	Fatigue, somnolence, headache, fever, lymph nodes, hepatomegaly, myalgia. One relapse episode. (4 months)	<i>rhodesiense</i>	Namibia, Mozambique, Malawi (unknown reason, travel for 2.5 years)	Suramin, Melarsoprol	13 (2007)
F	25	Dutch	Fever, headache, cellulitis, red papule, lymphangitis (4 days)	<i>rhodesiense</i>	Serengeti National park of Tanzania (tourist)	Suramin	18 (2009)
M	61	Polish	Fever, jaundice, respiratory distress, bleeding (disseminated intravascular coagulation - DIC), oliguria, skin rash, hepatosplenomegaly (8 days)	<i>rhodesiense</i>	Queen Elizabeth national park of Uganda (tourist)	Pentamidine	24 (2009)
M	50	French	Fatigue, fever, double skin ulceration, lymph nodes (7 days)	<i>gambiense</i>	Gabon (expatriate)	Pentamidine	27 (2009)
F	27	Dutch (immigrant from Angola)	Fatigue, apathy, sleepiness, loss of appetite, depression, coma (32 months)	<i>gambiense</i>	Angola (immigrant)	Eflornithine	19 (2009)

Conclusions

Physicians in Europe are likely to see more HAT cases because of the increasing popularity of travel to Africa, the only region that has recorded a growing number (3%) of tourist arrivals in 2009 according to the United Nations World Tourism Organization (UNWTO, www.unwto.org). The average annual growth of tourism in some sub-Saharan countries such as Tanzania and Uganda has ranged between 10 and 20% with a focus on safari travel. Because of the high mortality risk associated with acute Rhodesian trypanosomiasis, European travellers to destinations where the disease is endemic, particularly game parks and safari areas in eastern and southern Africa, should be informed about the early disease manifestations and advised to report tsetse bites to their physician upon return, when presenting symptoms. Although thousands of travellers are bitten by tsetse flies each year, the majority will not develop HAT. Nevertheless, caution is recommended. Preventive measures against tsetse fly bites are helpful. The tsetse fly is active during the daytime and is particularly attracted by motion and dark colours, with a marked preference for blue. Bites are painful and can be prevented by wearing wrist- and ankle-length clothing of thick material and avoiding bright or contrasting coloured clothing. Because the tsetse fly is able to bite through thinly woven fabric, the impregnation of clothing with permethrin is recommended together with the application of a skin repellent [30].

At present, imported HAT cases are not systematically reported through the existing channels to signal emerging infections (ProMED) or in the accessible medical literature. To harmonise reporting, we would recommend the creation of an electronic reporting form. This would allow for the evaluation of long term trends in imported HAT, and contribute to identifying risk factors and risk areas.

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Research articles

DETECTION OF INFLUENZA A(H1N1)v VIRUS BY REAL-TIME RT-PCR

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Influenza A(H1N1)v virus was first identified in April 2009. A novel real-time RT-PCR for influenza A(H1N1)v virus was set up *ad hoc* and validated following industry-standard criteria. The lower limit of detection of the assay was 384 copies of viral RNA per ml of viral transport medium (95% confidence interval: 273-876 RNA copies/ml). Specificity was 100% as assessed on a panel of reference samples including seasonal human influenza A virus H1N1 and H3N2, highly pathogenic avian influenza A virus H5N1 and porcine influenza A virus H1N1, H1N2 and H3N2 samples. The real-time RT-PCR assay for the influenza A matrix gene recommended in 2007 by the World Health Organization was modified to work under the same reaction conditions as the influenza A(H1N1)v virus-specific test. Both assays were equally sensitive. Clinical applicability of both assays was demonstrated by screening of almost 2,000 suspected influenza (H1N1)v specimens, which included samples from the first cases of pandemic H1N1 influenza imported to Germany. Measuring influenza A(H1N1)v virus concentrations in 144 laboratory-confirmed samples yielded a median of 4.6 log RNA copies/ml. The new methodology proved its principle and might assist public health laboratories in the upcoming influenza pandemic.

Introduction

Influenza A(H1N1)v virus was identified in humans in Mexico and the United States (US) in April 2009 [1] and has since spread worldwide [2]. The World Health Organization (WHO) declared pandemic alert stage 6 on 11 June 2009, indicating an ongoing influenza pandemic [3]. The transmissibility of the influenza A(H1N1)v virus was estimated to be higher than that of seasonal influenza viruses [4]. Influenza A(H1N1)v infections have been primarily seen among young and previously healthy adults suggesting that they are most vulnerable to infection. It remains speculative whether older people might have some level of cross-protection from pre-existing antibodies [4]. Clinical presentation and severity remains unclear, but with the exception of cases in Mexico, most confirmed cases have been characterised by mild influenza-like illness [5]. However, a considerable proportion of

patients reported vomiting or diarrhoea which is unusual in seasonal influenza [5]. To limit community or hospital transmission, as well as to initiate antiviral therapy in time as recommended by the WHO, the rapid detection of the virus in suspected cases remains crucial [6].

After the emergence of the H1N1 influenza pandemic no specific or well-validated diagnostic test was available. Rapid antigen-based tests for seasonal influenza seem to be compatible with pandemic H1N1 influenza, even though anecdotal reports exist on false-negative test results [1]. In the clinical diagnosis of influenza, nucleic acid testing by RT-PCR has widely replaced traditional virus culture due to shorter turnaround times and increased sensitivity [7]. Broadly reactive RT-PCR assays are indeed capable of detecting influenza A(H1N1)v virus [1], but they may lack sensitivity and cannot differentiate between contemporary influenza A viruses and influenza A(H1N1)v virus [8].

Immediately after the recognition of the new virus, sequence information was made publicly available by the Global Initiative on Sharing Avian Influenza Data (GISAID) [9]. We used this information to design and distribute a real-time RT-PCR assay specific for influenza A(H1N1)v [10,11]. In parallel, a published screening assay was evaluated for its ability to detect both influenza A(H1N1)v and seasonal influenza A virus [12]. This second assay served as a confirmatory test for pandemic H1N1 influenza, as well as for discriminating seasonal influenza from influenza A(H1N1)v infection. Pre-validated and quality-confirmed sets of oligonucleotides for both assays were centrally distributed within a large network of laboratories within Germany, covering most university hospitals and many public health institutions [13,14].

On 27 April 2009, samples from the first imported case of pandemic H1N1 influenza in Germany were received before specific assays for pandemic H1N1 influenza became available. The diagnosis was therefore confirmed overnight by sequencing of initial amplification products from an assay not specific for

pandemic H1N1 influenza [8]. The second imported case in Germany occurred on the evening of 28 April, the day the assay was first distributed. This case was diagnosed primarily with the new assays within three hours of receipt of the specimen. Here we report technical and clinical performance of the novel set of diagnostic tests on a large panel of samples.

Methods

Patient samples from the H1N1 influenza pandemic

At the beginning of the pandemic, 106 samples from 106 individual patients with acute onset of respiratory symptoms accompanied by fever and a recent travel history to countries with sustained human-to-human transmission of pandemic H1N1 influenza were analysed with the novel pandemic H1N1 influenza real-time PCR assay as well as the general influenza A (matrix gene) screening assay. These samples were collected and analysed in Bonn, Freiburg, Hamburg, Marburg and Regensburg. One of these samples was from an imported laboratory-confirmed case of influenza A(H1N1)v infection (Patient 1), and one from the first patient with hospital-acquired influenza A(H1N1)v infection in Germany (Patient 2). Patient 1 was diagnosed in Hamburg. Patient 2 was from Regensburg and had been infected by the first imported case to Germany [8].

A further 1,838 suspected cases were analysed at Bonn University Medical Centre later in the pandemic, until 30 July 2009. Of those, 211 cases were laboratory-confirmed pandemic H1N1 influenza. A selection of 144 pandemic H1N1 influenza-positive samples were used to determine virus concentrations in respiratory samples.

Specimens included nasopharyngeal swabs in viral transport medium, sputum, broncho-alveolar lavage fluid, throat washes, as well as cell culture medium containing reference virus strains. Viral nucleic acid was extracted using the Viral RNA mini kit (Qiagen).

Pandemic H1N1 influenza-specific real-time RT-PCR assays

Real-time RT-PCR oligonucleotides for influenza A(H1N1)v virus targeting the haemagglutinin (HA) gene were designed with Primer Express version 2 (Applied Biosystems) software. GenBank Accession number FJ966082 served as the template sequence. Several primer-and-probe combinations were evaluated experimentally to determine the most efficient combination.

A PCR reaction (One-step RT-PCR kit, Qiagen) of 25 µl for the pandemic H1N1 influenza specific assay contained: 5 µl of RNA extract, 1x reaction buffer, 400 µM of each dNTP, 40 ng/µl bovine serum albumine, 400 nM of primer H1SWS (CATTTGAAAGGTTTGAGATATTTCCC; GenBank Accession Number FJ966082, nt 380-404), 400 nM of primer H1SWAS (ATGCTGCCGTTACACCTTTGT; nt 457-437), 200 nM of probe H1SWP (FAM-ACAAGTTCATGGCCCAATCATGACTCG-BBQ; nt 409-435) and 1 µl of Enzyme Mix. All primers and probe were synthesised by TIB Molbiol, Berlin, Germany. Thermal cycling was done on a LightCycler 2.0 (Roche Diagnostics) instrument under the following conditions: 30 min at 50 °C; 15 min at 95 °C; 45 cycles of 15 s at 94 °C; and 30 s at 60 °C.

The same protocol can be run on a Lightcycler 480 system (Roche Diagnostics) without loss of sensitivity (data not shown). No other commercial test kits were evaluated in this study.

General influenza A (MA) real-time RT-PCR

Published real-time PCR primers targeting the matrix (MA) gene of influenza A virus were used [12]. A PCR reaction (One-step RT-PCR kit, Qiagen) of 25 µl for the matrix assay contained 5 µl of RNA extract, 1x reaction buffer, 400 µM of each dNTP, 40 ng/µl bovine serum albumine, 400 nM of primer M_InfA F (AAGACCAATCCTGTACCTCTGA; GenBank Accession number CY038773; nt 175-197), 400 nM of primer M_InfA R (CAAAGCGTCTACGCTGCAGTCC; nt 269-248), 200 nM of probe M_InfA TM (FAM-TTTGTGTTACGCTCACCCT-BBQ; nt 215-234) and 1 µl of Enzyme Mix. Thermal cycling was done on a LightCycler 2.0 (Roche Diagnostics) instrument under the following conditions: 30 min at 50 °C; 15 min at 95 °C; 45 cycles of 15 s at 94 °C; and 30 s at 60 °C.

As above, the same protocol can be run on a Lightcycler 480 system (Roche Diagnostics) without loss of sensitivity (data not shown).

Construction of in vitro-transcribed RNA controls

A partial HA gene fragment from the virus isolated from Patient 1 was amplified using primers HA_InfA_CaF1 (CAACAGACACTGTAGACACAG; GenBank Accession number FJ966082; nt 86-106) and HA_InfA_CaR1 (TTCCATTGCGAATGCATATCTCGG; nt 825-802) and cloned into a pJET12 plasmid vector (Fermentas). The complete MA gene from the same virus was amplified using primers Matrix_CaL_F (TAACCGAGGTGCGAAACGTACG; GenBank FJ969513; nt 11-31) and Matrix_CaL_R (TACTCTAGCTCTATGTTGAC; nt 982-902) and ligated and cloned as described above. Plasmids were transcribed into RNA by means of a MEGAScript T7 *in vitro* transcription kit (Ambion) as described [15]. RNA *in vitro* transcripts were purified and quantified spectrophotometrically. Sequence integrity was checked by sequencing on an ABI 3100 automated sequencer (Applied Biosystems).

Determination of lower limit of detection

Initial experiments were done with RNA extracted from nasopharyngeal specimens of Patient 1. To exactly determine the lower limit of detection (LOD) of both real-time RT-PCR assays, different concentrations of HA RNA transcript as well as MA RNA transcript were spiked into viral transport medium, and RNA was extracted using the viral RNA mini kit (Qiagen). Influenza-negative swabs to account for patient derived matrix effects were not used since possible PCR inhibitors will most likely be efficiently diluted by the viral transport medium. Five replicates of each concentration were processed and analysed by the pandemic H1N1 influenza (HA RNA transcript) and the screening (MA RNA transcript) real-time RT-PCR, respectively. Fractions of positive results for each concentration were subjected to probit regression analysis using the Statgraphics software package (Manugistics).

Quantitive results for pandemic H1N1 influenza

Nasal and throat swabs from a selection of 144 cases of pandemic H1N1 influenza (see above) were used to determine influenza A(H1N1)v virus RNA concentrations in the H1N1 influenza-specific (HA) real-time assay using *in vitro*-transcribed RNA as described [15]. An external curve was generated and cycle threshold values were transformed into log RNA copies/ml.

Results

Pandemic H1N1 influenza-specific (HA) real-time RT-PCR assays

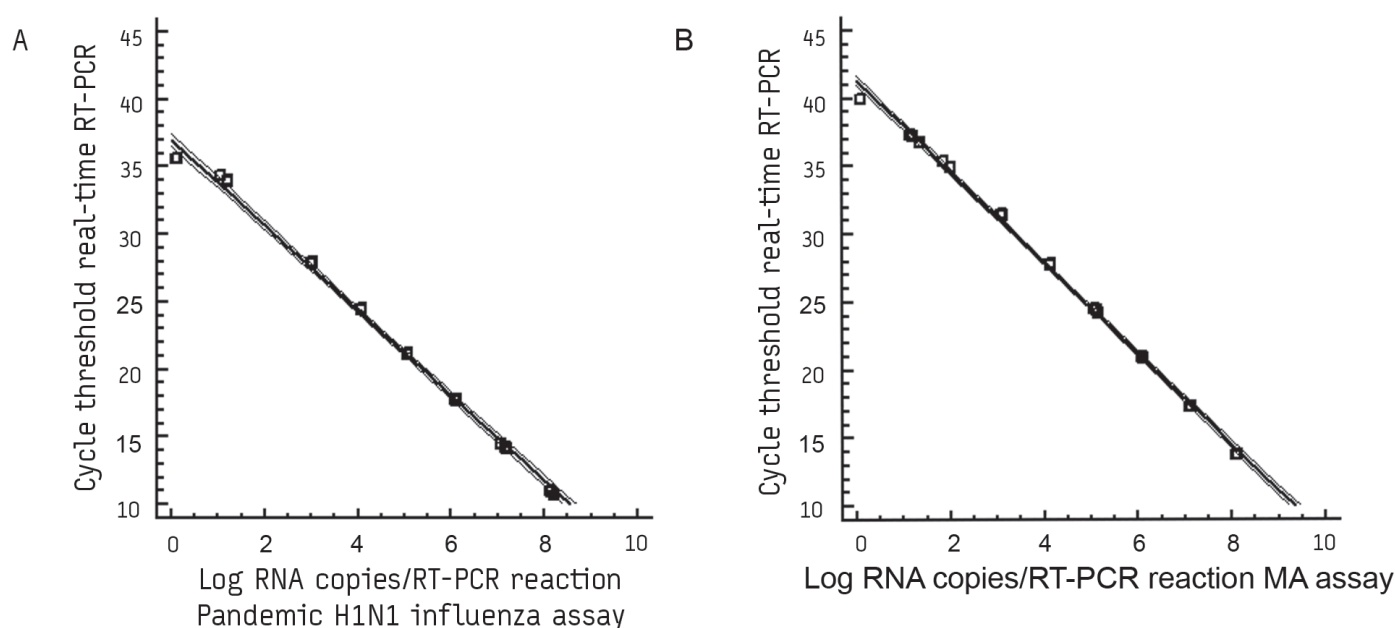
Tenfold serial dilution series of *in vitro*-transcribed HA RNA were tested in duplicates in the pandemic H1N1 influenza (HA)

RT-PCR (Figure 1A) and the general influenza A (MA) screening RT-PCR (Figure 1B), in order to determine the linear range of both real-time RT-PCR assays. The resulting end-points of detection in

the pandemic H1N1 influenza (HA) RT-PCR were 1 and 5 RNA copies/ μ l in different experiments. A linear correlation between the

FIGURE 1

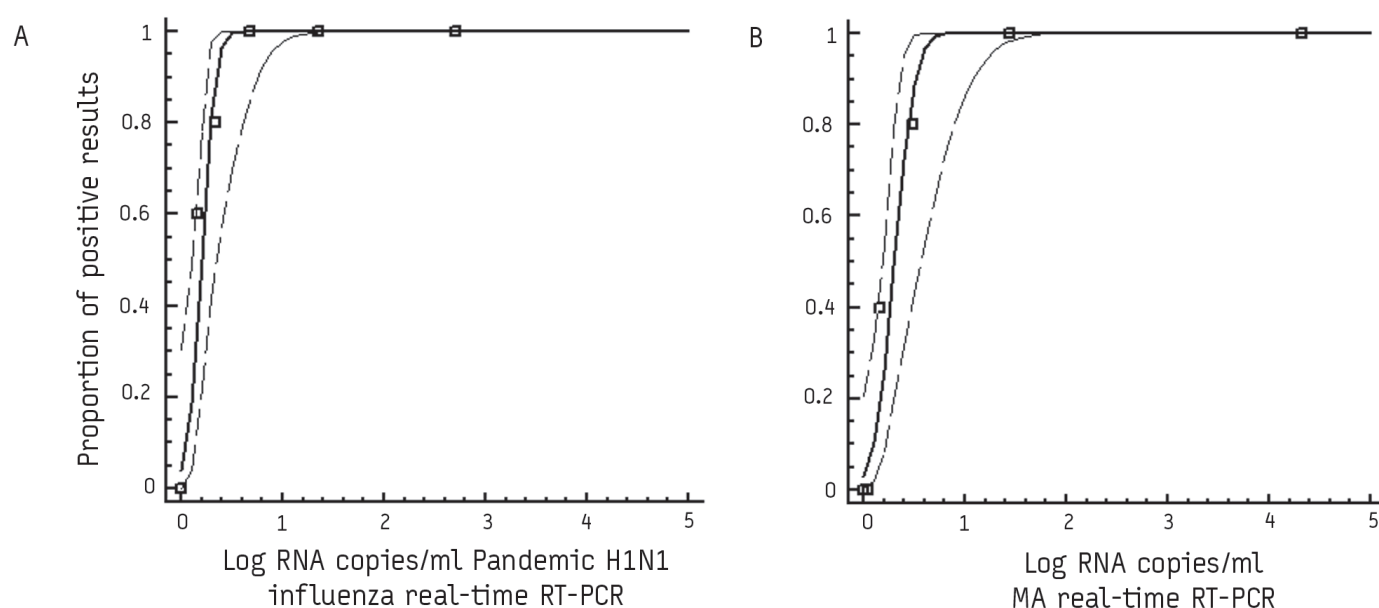
Linear range of pandemic H1N1 influenza (HA) real-time RT-PCR and general influenza A (MA) screening real-time RT-PCR



HA: haemagglutinin gene; MA: matrix gene. Observed cycle thresholds are plotted against log RNA concentration (square points). Thick centre lines represent the prediction line, thin lines the 95% confidence intervals.

FIGURE 2

Probit analysis of pandemic H1N1 influenza (HA) real-time RT-PCR and general influenza A (MA) screening real-time RT-PCR



HA: haemagglutinin gene; MA: matrix gene. Depicted are the observed proportion of positive test results in parallel experiments (square data points), as well as the derived predicted proportion of positive results (line) at a given input concentration of RNA. The centre line denotes the prediction, thin broken border lines are 95% confidence intervals.

log starting copy number and threshold cycle was achieved from 2.67 x 10² RNA copies/μl to at least 2.67 x 10⁸ RNA copies/μl.

TABLE 1

Serial tenfold dilutions of RNA extracted from Patient 1 tested by the MA broad-range assay as well as the pandemic H1N1 influenza-specific real-time RT-PCR

	Log ₁₀ dilution series RNA, Patient 1					
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
MA assay	+	+	+	+	-	-
Pandemic H1N1 influenza assay	+	+	+	+	-	-

MA: matrix protein gene.
+ indicates a positive result, - denotes a negative result.

The slope was calculated as 3.15 for the pandemic H1N1 influenza (HA) assay. Based on the slope value, PCR efficiency was calculated to be 1.0 (according to the PCR amplification formula $E=10^{(1/\text{slope})}-1$; E being the PCR efficiency), indicating 100% efficient PCR amplification.

The 95% LOD was determined next. This common technical specification indicates the concentration down to which an assay will detect the analyte with at least 95% probability. To generate defined reference material that mimics clinical samples, different concentrations of *in vitro*-transcribed RNA were spiked into viral transport medium in which swabs are routinely received in the laboratory. Each analyte concentration was tested in five replicate reactions in each RT-PCR assay and yielded a 95% LOD of 384 RNA copies/ml (95% CI: 273-876 RNA copies/ml) for the pandemic H1N1 influenza (HA) assay (Figure 2A).

TABLE 2

Specificity testing of influenza specimens of porcine and human origin by MA broad-range assay and pandemic H1N1 influenza-specific assay

Influenza virus specimen		Real-time RT-PCR result	
		MA assay	Pandemic H1N1 influenza assay
1	A/Swine/Hannover/1/81(H1N1)	+	-
2	A/Swine/Germany/2/81 (H1N1)	+	-
3	A/Swine/Italy/21599-3/03 (H1N1)	+	-
4	A/Swine/Borkum/1832/00 (H1N2)	+	-
5	A/Swine/Italy/30019-2/07 (H1N2)	+	-
6	A/Swine/Italy/65260-11/06 (H3N2)	+	-
7	A/Bayern/7/95 (H1N1)	+	-
8	A/Beijing/262/95 (H1N1)	+	-
9	A/Brazil/11/78 (H1N1)	+	-
10	A/Moscow/10/99 (H3N2)	+	-
11	Influenza A clinical samples 2008-9 (n=120)*	+	-
	Influenza quality assessment samples		
12	A/Fukushima/141/06 (H1N1)	+	-
13	A/New Caledonia/20/99 (H1N1)	+	-
14	A/Brisbane/10/07 (H3N2)	+	-
15	A/Brisbane/59/07 (H3N2)	+	-
16	A/California/7/04 (H3N2)	+	-
17	A/Wisconsin/67/06 (H3N2)	+	-
18	A/duck/Vietnam/TG24-01/05 (H5N1)	+	-
19	A/whooper swan/Germany/R65-2/06 (H5N1)	+	-
20	B/Florida/4/06**	-	-
21	B/Malaysia/2506/04**	-	-
22	B/Shanghai/361/02**	-	-

MA: matrix protein gene.
+ depicts a positive, - a negative result.
*All 120 samples tested positive in the MA and negative in the pandemic H1N1 influenza assay.
**All influenza B samples tested positive in an influenza B- specific RT-PCR (data not shown).

General influenza A (MA) real-time RT-PCR

For confirmation of influenza A virus detection, oligonucleotides from several published and evaluated RT-PCR assays were checked against the genome sequence of influenza A(H1N1)v virus. One assay developed by Ward et al. [12], was targeted against the MA gene. This assay had been recommended by the WHO for general influenza A virus screening, including avian influenza A virus (H5N1), matched the influenza A(H1N1)v virus sequence except for one base pair mismatch (C-T) 13 nucleotides from the 3'-end of the plus strand primer [16]. This mismatch was considered uncritical. The assay was optimised in order to comply with the same cycling conditions as the pandemic H1N1 influenza (HA) assay, so that both assays could be run in parallel in one LightCycler instrument. Serial dilution series of patient RNA were tested with both assays. As shown in Table 1, both assays were equally sensitive.

Using *in vitro*-transcribed MA RNA, the MA assay yielded an end-point dilution sensitivity of 13 RNA copies per μ l. The linear range extended from 1.28x10² RNA copies/ μ l to at least 1.28x10⁸ RNA copies/ μ l. The slope was calculated as 3.35, and PCR efficiency was 0.99 (Figure 1). In a probit analysis as described above, the MA-based broad range assay showed a 95% LOD of 570 RNA copies/ml (95% CI: 397-1,232 RNA copies/ml) (Figure 2).

Specificity of the pandemic H1N1 influenza (HA) assay

Specificity of the H1-based pandemic H1N1 influenza assay was confirmed on a panel of 21 stored clinical samples containing adenovirus (n=1), respiratory syncytial virus (RSV)-A (n=5), RSV-B (n=2), human coronavirus OC43 (n=1), human coronavirus 229E (n=3), human coronavirus NL63 (n=1), human metapneumovirus (n=1), parainfluenzavirus 3 (n=1) and entero-/rhinovirus (n=6) as assessed by xTAG Respiratory Viral Panel (Luminex; authors' unpublished data). As expected, none of these pathogens reacted with the pandemic H1N1 influenza real-time RT-PCR indicating its high specificity. The MA-based broad range assay was not evaluated on this panel but demonstrated its specificity as described. [12].

Because of the porcine origin of pandemic H1N1 influenza [17], the assay was also tested on cell culture supernatants containing porcine influenza A virus reference samples (Table 2, rows 1-6).

To exclude cross-reactivity with human influenza viruses, we tested cell culture supernatants of human influenza virus strains (Table 2, rows 7-10) as well as 120 original clinical samples from patients with seasonal influenza A virus infection from the 2008-9 season, including H1N1 and H3N2 viruses (Table 2, row 11). All of these were negative in the pandemic H1N1 influenza (HA) assay and positive in the MA-based broad-range assay (shown in Table 2).

In addition, 30 stored samples from recent quality assessment tests for influenza virus detection were evaluated (Table 2, rows 12-22). None of these materials, which included various dilutions of contemporary human influenza A(H1N1, H3N2) as well as avian influenza A(H5N1) and influenza B virus samples yielded a positive result with the pandemic H1N1 influenza (HA) assay. All influenza A samples were positive in the general influenza A MA-based assay.

Clinical evaluation

A preliminary clinical evaluation was done in five public health and university laboratories in Germany. By mid-May 2009 samples from 106 individual patients suspected on clinical and epidemiological grounds to have acquired influenza A(H1N1)v infection had been analysed with the new assays. Of these 106 samples, 102 gave negative results in both assays. Three of the four

remaining samples tested positive in the MA-based assay, but were negative in the HA-based pandemic H1N1 influenza assay. After to further confirmatory testing, these three samples turned out to be human seasonal influenza A virus infections (data not shown).

The last sample was positive in both assays. This patient (Patient 1) was preliminary classified as having acute influenza A(H1N1)v infection. She had a recent travel history to Mexico and sought medical treatment for fever and acute respiratory symptoms in Hamburg on 28 April 2009. Influenza A(H1N1)v infection was confirmed by the National Influenza Reference Centre at the Robert-Koch Institute (RKI), Berlin.

Material from another confirmed case (Patient 2) was provided retrospectively for testing with both assays. This patient had not reported a recent travel history but shared a hospital room with the first imported case of pandemic H1N1 influenza in Germany [8]. The patient had only very mild symptoms. Both assays reacted clearly positive.

Later in the pandemic, further samples of suspected influenza A(H1N1)v infection were analysed with the new assay at Bonn University Medical Center, so far 1,838 samples. Among those, 221 confirmed cases of pandemic H1N1 influenza have been identified as of 30 July.

Quantitative results for pandemic H1N1 influenza

Viral RNA concentrations were measured in samples from 144 laboratory-confirmed cases of pandemic H1N1 influenza for whom RNA preparations were available at Bonn University Medical Center. A median of 4.6 influenza A(H1N1)v virus log RNA copies per ml of viral transport medium was determined in the pandemic H1N1 influenza-specific (HA) assay (range 2.1-7.9 log RNA copies/ml), indicating rather low virus concentrations.

Discussion

A real-time RT-PCR specific for influenza A(H1N1)v virus was set up immediately after first sequence information became available, and evaluated thoroughly from a technical and clinical point of view.

In the currently evolving influenza pandemic, rapid and reliable case identification remains crucial to limit extensive transmission and to initiate therapy [18]. The performance of antigen-based tests for pandemic H1N1 influenza has not been extensively evaluated so far, but anecdotal reports do exist of false negative test results in confirmed cases of pandemic H1N1 influenza [19]. A further issue with antigen-based tests is the fact that they do not discriminate between seasonal influenza A virus strains and influenza A(H1N1)v virus strains. The concurrence of the first wave of the pandemic H1N1 influenza and regular seasonal influenza in the southern hemisphere poses a risk of intra-human reassortation, making it highly relevant to discriminate between viruses by laboratory testing [20].

Real-time RT-PCR has proven highly effective in the detection of seasonal human influenza [21]. First reports on clinical cases in which influenza A(H1N1)v virus was detected by real-time RT-PCR have become available, but the assays used so far had not been validated thoroughly from a technical point of view [19,22,23]. Our study presents the first fully validated real-time RT-PCR for pandemic H1N1 influenza. Its LOD at 384 RNA copies/ml (95% LOD, probit analysis) is comparable to that of commercial test kits [21,24]. Specificity was proven on a comprehensive panel of

120 clinical samples containing contemporary human influenza A and B viruses, on reference specimens from an external quality assurance study, and on a panel of selected swine influenza viruses. Clinical applicability was demonstrated on the first imported cases of pandemic H1N1 influenza in Germany, and by testing more than 200 confirmed cases later in the pandemic without any false positive or negative results. Interestingly rather low virus concentrations were measured by the pandemic H1N1 influenza-specific real-time RT-PCR, compared to viral loads seen in seasonal influenza [25,26]. It remains unclear if this is related to the host or propensity of the virus itself.

We have also shown in this study that a broad-range influenza A assay recommended by WHO and based on the MA gene had the same high sensitivity as the HA-based pandemic H1N1 influenza-specific assay and can be used for simultaneous detection of influenza A(H1N1)v virus and seasonal strains [12]. It can thus serve as a confirmatory test and for discriminating influenza A(H1N1)v virus from seasonal strains. Both assays were developed to allow for parallel testing on a single real-time PCR instrument, reducing the time of turnover significantly [27]. Use of the combined assays facilitates decentralised testing in clinical laboratories, which is necessary when the demands for testing will exceed the capacities of reference laboratories during the upcoming pandemic [20,28]. In this respect, it is important to mention that the validation data presented in this report have been generated by five different laboratories that had obtained the assays in form of protocols and pre-evaluated oligonucleotides. Recent experiences during the epidemics of severe acute respiratory syndrome (SARS) and chikungunya disease have demonstrated that rapid provision of pre-formulated diagnostic assays can facilitate immediate diagnostic capacity building [13,14,29].

To conclude, we could demonstrate that the testing algorithm proposed here is a feasible approach and might assist public health laboratories in the upcoming influenza season.

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Surveillance and outbreak reports

TUBERCULOSIS IN A CHILD – SEARCH FOR THE INFECTED ADULT NEARBY; CASE REPORT, PORTUGAL, 2007

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Tuberculosis (TB) transmission in a non-household setting is difficult to detect, because contact with the source case is often not obvious. Here, we report on a case of a four-year-old child who got infected through sporadic non-household exposure at a coffee shop. The source case was a woman who had suffered from weight loss, productive cough and fatigue for two months before being diagnosed with TB. Screening the child's contacts revealed two active TB cases within its family. Overall 148 contacts were screened for both cases and 18 cases of latent TB infection detected. The connection between the child and the source case, who were not aware of their contact, was confirmed by molecular fingerprinting. Our case report illustrates the difficulty in detecting non-household transmission between individuals that do not have significant contact, and draws attention to the need to look for the infected adult whenever a child falls ill with TB. This report is a reminder of the importance to consider possibly neglected ways of TB transmission and highlights once again the need of early diagnosis of TB.

Introduction

Childhood tuberculosis (TB) is an indicator of public health programme performance and all cases in children can be considered signals of missed opportunities. The most important missed opportunities are:

- failure to detect the infectious case;
- delayed reporting of an adult source case;
- failure to identify contacts of infectious cases with children;
- delayed or incomplete evaluation of children exposed to TB; and
- inadequate treatment of latent TB infection (LTBI) [1].

TB in young children (under five years) is often transmitted from a family or household member [2]. Therefore, screening procedures in cases of childhood TB should always start with the search for the source case within the family/household [3]. The transmission of TB to children through non-household exposure is difficult to ascertain. In many instances, public health authorities experience difficulties in establishing the contact with the source case as most of the affected are not aware of the contact which leads to the infection.

This paper describes a case of a four-year-old child who became infected through exposure at a coffee shop, which she visited

sporadically in the company of a family member. It illustrates the difficulty in detecting non-household transmission between individuals that did not have significant contact. Moreover, it demonstrates the need to look for the infected adult whenever a child is diagnosed with TB.

Case reports

Case 1 (child)

In September 2007, a four-year-old child came to our practice after having had a temperature around 37, 5-38°C for 15 days (Figure 1).

Chest radiography showed a right hilar enlargement. TB was confirmed by bronchoalveolar lavage analysis, which was smear and culture positive. The *Mycobacterium tuberculosis* (MTB) isolate was susceptible to all first-line drugs. The clinical strain was genotyped using mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing [4] and IS6110 restriction fragment-length polymorphism (IS6110-RFLP) typing [5]. The patient was treated with isoniazid, rifampicin and pyrazinamide by means of directly observed treatment (DOT).

Contact tracing after diagnosis

Upon diagnosis, the parents were interviewed and asked to describe the daily activities and routines of the child at home, at school and in its social environment. The parents were not aware of any contact with TB patients. Initially we considered three relatives (the parents and grandmother) and later on an additional 11 close members of the family as well as 30 pupils and nine staff from the school as contacts of the child. All identified contacts were offered a screening programme which included a symptom questionnaire, a tuberculin skin test (PPD RT23 SSI) and chest radiography. The tuberculin skin test was considered positive if after 72 hours an induration >10 mm was visible, and an interferon gamma assay was performed in all positive cases. One case of LTBI was identified among the contacts, a school staff who was subsequently treated with isoniazid for 6 months.

Case 2 (adult source case)

In October 2007, a woman in her early forties presented at our clinic with progressive weakness, weight loss and a persistent productive cough for the past two months (Figure 1). Chest

radiography showed bilateral pulmonary infiltrates and cavities. The sputum was smear and culture positive for *M. tuberculosis* and the isolate was susceptible to all first-line drugs. The strain was genotyped using MIRU-VNTR [4] and IS6110-RFLP. The patient began treatment with isoniazid (INH), rifampicin (RIF) and pyrazinamide (Z) and ethambutol (E) in form of DOT.

Contact tracing

Eight close non-household contacts were identified and offered screening as described above. Two of them had LTBI and were treated with isoniazid for six months. Afterwards, contact screening was broadened to include casual contacts defined as exposure/contact for less than eight cumulative hours during the symptomatic period from August 2007. An additional 20 persons were identified and screened. LTBI was diagnosed in five contacts and active pulmonary TB was diagnosed in two.

Case 1 and case 2 were apparently unrelated. However, it was noticed that both patients lived in the same geographic area. Further questioning identified a coffee shop as a place where both individuals would spend time occasionally. At first, it seemed there was little chance of a connection between them. The visits to the coffee shop were rare and short. In order to clarify the situation and to enable other public health measures, clinical isolates of both patients were genotyped. Strain typing was done through the use of MIRU-VNTR and IS6110-RFLP standard methods [4, 5]. Both fingerprinting results showed that the two clinical isolates had

identical fingerprinting (Table 1 and Figure 2), and therefore were epidemiologically related.

Lane 1: Molecular marker (1kb marker): 10.000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3500 bp, 3000 bp, 2000bp
Lane 2: *M. tuberculosis* isolate from case 1 (child)
Lane 3: *M. tuberculosis* isolate from case 2 (adult source case)

Contact tracing after results from genotyping

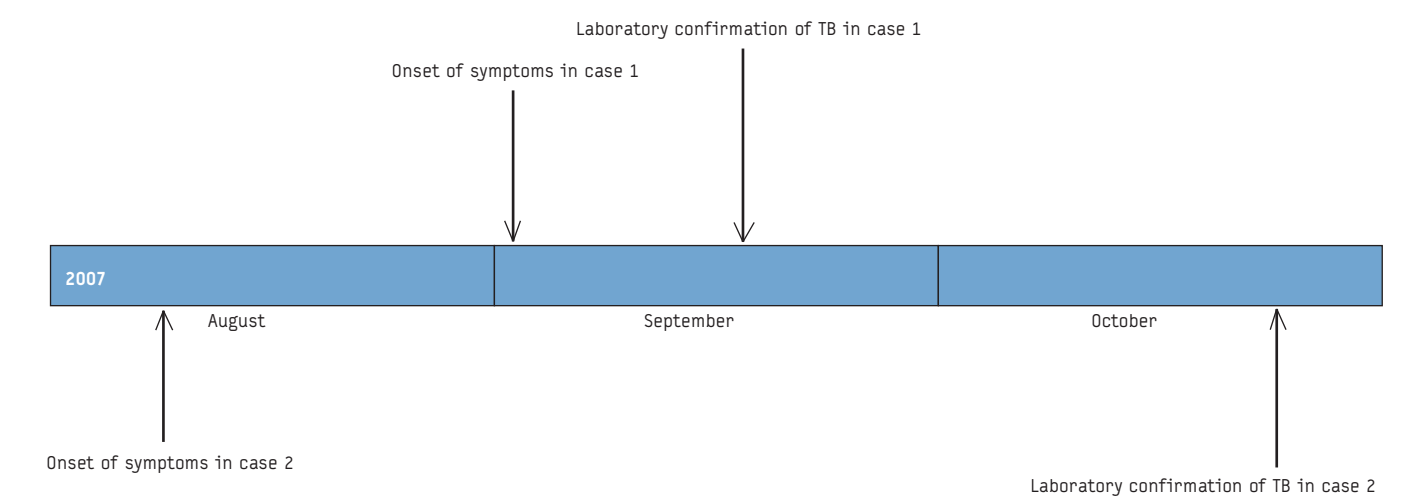
After the results from genotyping had revealed the connection between the two cases, and the coffee shop was identified as the place where the two cases had met, a new investigation was conducted directed at the coffee shop customers. Sixtyseven people were screened and LTBI was diagnosed in ten of them. Consequently, eight individuals were treated with isoniazid for 6 months. Two cases with LTBI had underlying conditions such as alcoholism and chronic hepatic disease which meant that they were at risk for developing hepatic toxicity from being treated with isoniazid. They were kept under clinical surveillance by the clinic as outpatients for 2 years.

Discussion and conclusion

The two main factors determining the risk of progression from latent to active TB are patient age and immune status. Immaturity of both the innate and adaptive immune systems of young children plays a critical role in increased susceptibility to active TB. Children below five years of age who are infected, have the highest risk of

FIGURE 1

Timeline of onset of symptoms and of diagnosis of tuberculosis in case 1 (child) and case 2 (adult source case), Portugal 2007



TABLE

Results of mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing (15 loci), tuberculosis cases Portugal, 2007

	Mtub04	ETRC	MIRU04	MIRU40	MIRU10	MIRU16	Mtub21	QUB11b	ETRA	Mtub30	MIRU26	MIRU31	Mtub39	QUB26	QUB4156
Case 1 (child)	1	4	2	1	N/A	1	3	2	2	1	4	3	2	N/A	2
Case 2 (adult source case)	1	4	2	1	N/A	1	3	2	2	1	4	3	2	N/A	2

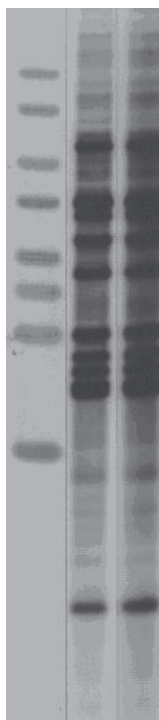
Legend: N/A= not available

progression to disease [6, 7]. The two strategies for managing TB in children are searching for secondary cases in close contacts and searching for the infectious adult source case. Whenever a TB case in a small child is diagnosed, family members are interviewed to identify contacts in the sphere of the patient's daily activities. The public health authority investigation team visits the child's home, school or other identified places where the child has social contacts in order to identify those at risk and to find the infected adult. Progression from primo-infection to illness in children under five years old is very fast, so one can be sure that there is an infectious adult nearby who transmitted the disease to the child [6, 7].

Due to the low probability of transmission between very young children, screening of other children is usually largely unproductive. There is however evidence that TB in young children is occasionally transmissible particularly in the presence of cavitation, consolidation or bronchial lesions [8, 9]. Although case 1 did not present cavities in the chest radiography, she was smear and culture positive so the risk of transmission could not be excluded. Therefore, all family and school contacts were screened. This activity did not prove to be fruitful as all children tested negative, only one adult was found to have LTBI (probably not connected with this situation) and it did not lead to the identification of the source case. At this point in time, we were reconsidering our strategy when another case appeared in the same geographic area. Further inquiries revealed a seemingly improbable contact between the adult and the child in an unusual place for a child to be – a coffee shop. Genotyping of the *M. tuberculosis* isolates from both cases however, confirmed the connection.

FIGURE 2

IS6110 restriction fragment-length polymorphism (IS6110-RFLP) typing fingerprints, tuberculosis cases Portugal, 2007



The delay of the diagnosis of TB in case 2, by two months and a prolonged infectious period were the causes of the high rate of TB transmission, resulting in three cases of pulmonary TB (two family members and case 1) and 18 LTBI among contacts which can most probably be attributed to the event.

Transmission of TB in bars has been described before and may pose a risk to public health [10,11]. This case report is intended as a reminder to health professionals that all TB transmission scenarios are possible and need to be considered in an investigation around a case, even the less likely. Moreover, a delay in the diagnosis of the infectious case results in transmission of the disease. Finally, the link between the child and the adult would not have been proved without the use of the *M. tuberculosis* fingerprinting techniques.

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