Impact factor 5·9

Eurosurveillance

Europe's journal on infectious disease epidemiology, prevention and control

Vol. 22 | Weekly issue 2 | 12 January 2017

ecdc EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

www.eurosurveillance.org

Note from the editors: The year 2016 gone, 2017 just started

Eurosurveillance editorial team [1]
1. European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden

Correspondence: Eurosurveillance editorial team (eurosurveillance@ecdc.europa.eu)

Citation style for this article:
Eurosurveillance editorial team. Note from the editors: The year 2016 gone, 2017 just started. Euro Surveill. 2017;22(2):pii=30442. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30442

Article published on 12 January 2017

At the start of a new year, we provide our readers with a look back over the past 12 months and an outlook into the future. In 2016, *Eurosurveillance*'s 20th anniversary was an opportunity to review the evolution and achievements of the journal so far, and we summarised our personal views in an editorial accompanying a special anniversary print edition of Eurosurveillance [1].

For the journal, as well as for infectious diseases specialists and public health experts, Zika virus (ZIKV) remained one of the main topics in 2016. The virus continued to spread, while increasing evidence led to scientific consensus about a causal link between ZIKV infection and occurrence of Guillain–Barré syndrome (GBS) as well as microcephaly and other congenital brain abnormalities [2]. Further, knowledge emerged that ZIKV can be transmitted by routes other than the vector-borne one. In February 2016, when an article in *Eurosurveillance* reported on an autochthonous case of ZIKV disease caused by possible sexual transmission, evidence supporting the possibility of this transmission pathway was still limited [3]. This changed rapidly, and sexual transmission of the virus, even by asymptomatically infected individuals, became the focus of scientific studies and the centre of attention of many public health experts. International and national public health organisations took new findings into account and, whenever necessary, updated advice and guidance on how to prevent sexual transmission [4-6].

Colistin serves as last resort antibiotic for treatment of patients infected with highly resistant, e.g. carbapenem-resistant, Gram-negative bacteria. For public health microbiologists and those concerned with antimicrobial resistance, an important finding in 2016 was the extent of the diffusion of plasmid-mediated colistin resistance, conferred by the *mcr-1* gene. The first findings in China of this new threat to healthcare and patient safety were published in November 2015 [7]. By July 2016, we had published seven articles on detection of the *mcr-1* gene in Belgium, France, the Netherlands, Portugal and Spain, as well as Brazil and Tunisia [8-14]. Already in March 2016, an editorial in Eurosurveillance took stock of the reported evidence of the *mcr-1* gene in food animals, food and humans [15].

In addition, we dedicated special issues to methods and approaches for surveillance, diagnosis and strain typing of *Clostridium difficile* and the impact of anthropogenic changes to water on human pathogens. Influenza vaccine effectiveness, in particular the effectiveness of live attenuated influenza vaccines in children, the emergence and surveillance of enteroviruses (EV) such as of EV D-68 causing severe respiratory illness particularly in children, vaccine preventable diseases, HIV/AIDS and sexually transmitted infections, as well as food- and waterborne outbreaks, were among the variety of other subjects that we covered in 2016.

We published 195 articles (73 rapid communications, 122 regular articles) and a total of 39 editorials, letters and meeting reports. On average there were 72 submissions to *Eurosurveillance* per month. Of the 864 articles we received in 2016, we accepted a little less than 20% for publication.

Articles were selected for publication with the help of our wide network of reviewers. Nearly 500 experts worldwide dedicated their time in 2016 to guide us in the decision-making process by sharing their views and comments on articles. We are grateful to them and published a list with their names in our first 2017 issue [16]. In the choice and commissioning of articles we also rely on our associate editors and editorial advisors. They are our link to academia and public health practice and help us find the right strategy and mix of topics for our audience. We are glad to have them on board and value their contributions. We are also supported by experts, many of them our colleagues at the European Centre for Disease Prevention and Control

(ECDC), whose names do not necessarily appear in the reviewer list but who are ready to listen to us and provide us with their feedback and to whom we would like to express our gratitude. We acknowledge the continued funding, logistic support and encouragement from our publisher, ECDC. Together with the editorial independence which ECDC and its Director grant the editor-in-chief, the sustained funding has been a key element in the positive evolution and recognition of the journal over the past decade.

The joint forces of *Eurosurveillance* contributors over two decades have borne fruit. Also in 2016, commonly used indices of 'impact' such as the impact factor, SCImago journal rank and Google metrics positioned the journal well among others in the same field, and in a similar fashion to previous years.

For 2017, some changes are envisaged for the journal; the most important one will be the launch of a new website that should go live in the second part of the year. It will provide our audience with more modern features and user-friendly navigation. A minor change for readers of our national bulletin section will be that we will change the frequency of its publication from monthly to quarterly. The first 2017 'In the national epidemiological bulletins – a selection from current issues' will be published in the 30 March issue. In the last quarter of 2016, we had a restricted call for a special issue on Immunisation Information Systems and we envisage publication of the special issue at the end of April. A formal call to provide evidence for screening of infectious diseases in migrants will soon be published.

At the beginning of 2017, we do not know what surprises the year may bring, however, *Eurosurveillance* will remain a platform for the infectious disease and public health community to share findings that help protect and improve public health, and we look forward to receiving such contributions.

References

1. Steffens I. 20 years of communicating facts and figures. Euro Surveill. 2016;21(48):30415. DOI: 10.2807/1560-7917.ES.2016.21.48.30415 PMID: 27934580
2. World Health Organization (WHO). Zika situation report. Zika virus, Microcephaly and Guillain-Barré syndrome—31 March 2016. Geneva: WHO; 2016. Available from: http://www.who.int/emergencies/zika-virus/situation-report/31-march-2016/en/
3. Venturi G, Zammarchi L, Fortuna C, Remoli ME, Benedetti E, Fiorentini C, et al. An autochthonous case of Zika due to possible sexual transmission, Florence, Italy, 2014. Euro Surveill. 2016;21(8):30148. DOI: 10.2807/1560-7917.ES.2016.21.8.30148 PMID: 26939607
4. World Health Organization (WHO). Prevention of sexual transmission of Zika virus. Interim guidance update, 6 September 2016. Geneva: WHO; 2016. Available from: http://apps.who.int/iris/bitstream/10665/204421/1/WHO_ZIKV_MOC_16.1_eng.pdf?ua=1
5. European Centre for Disease Prevention and Control (ECDC). Rapid risk assessment: Zika virus disease epidemic. Ninth update, 20 October 2016. Stockholm, ECDC; 2016. Available from: http://ecdc.europa.eu/en/publications/Publications/rapid-risk-assessment-zika-october-2016.pdf
6. Petersen EE, Meaney-Delman D, Neblett-Fanfair R, Havers F, Oduyebo T, Hills SL, et al. Update: Interim Guidance for Preconception Counseling and Prevention of Sexual Transmission of Zika Virus for Persons with Possible Zika Virus Exposure - United States, September 2016. MMWR Morb Mortal Wkly Rep. 2016;65(39):1077-81. DOI: 10.15585/mmwr.mm6539e1 PMID: 27711033
7. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016;16(2):161-8. DOI: 10.1016/S1473-3099(15)00424-7 PMID: 26603172
8. Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, et al. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. Euro Surveill. 2016;21(27):30280. DOI: 10.2807/1560-7917.ES.2016.21.27.30280 PMID: 27416987
9. Perrin-Guyomard A, Bruneau M, Houée P, Deleurme K, Legrandois P, Poirier C, et al. Prevalence of mcr-1 in commensal Escherichia coli from French livestock, 2007 to 2014. Euro Surveill. 2016;21(6):30135. DOI: 10.2807/1560-7917.ES.2016.21.6.30135 PMID: 26898350
10. Kluytmans-van den Bergh MF, Huizinga P, Bonten MJ, Bos M, De Bruyne K, Friedrich AW, et al. Presence of mcr-1-positive Enterobacteriaceae in retail chicken meat but not in humans in the Netherlands since 2009. Euro Surveill. 2016;21(9):30149. DOI: 10.2807/1560-7917.ES.2016.21.9.30149 PMID: 26967540
11. Campos J, Cristino L, Peixe L, Antunes P. MCR-1 in multidrug-resistant and copper-tolerant clinically relevant Salmonella 1,4,[5],12:i:- and S. Rissen clones in Portugal, 2011 to 2015. Euro Surveill. 2016;21(26):30270. DOI: 10.2807/1560-7917.ES.2016.21.26.30270 PMID: 27387036
12. Prim N, Rivera A, Rodríguez-Navarro J, Español M, Turbau M, Coll P, et al. Detection of mcr-1 colistin resistance gene in polyclonal Escherichia coli isolates in Barcelona, Spain, 2012 to 2015. Euro Surveill. 2016;21(13):30183. DOI: 10.2807/1560-7917.ES.2016.21.13.30183 PMID: 27055477
13. Fernandes MR, Moura Q, Sartori L, Silva KC, Cunha MP, Esposito F, et al. Silent dissemination of colistin-resistant Escherichia coli in South America could contribute to the global spread of the mcr-1 gene. Euro Surveill. 2016;21(17):30214. DOI: 10.2807/1560-7917.ES.2016.21.17.30214 PMID: 27168587
14. Grami R, Mansour W, Mehri W, Bouallègue O, Boujaâfar N, Madec JY, et al. Impact of food animal trade on the spread of mcr-1-mediated colistin resistance, Tunisia, July 2015. Euro Surveill. 2016;21(8):30144. DOI: 10.2807/1560-7917.ES.2016.21.8.30144 PMID: 26940999
15. Skov RL, Monnet DL. Plasmid-mediated colistin resistance (mcr-1 gene): three months later, the story unfolds. Euro Surveill. 2016;21(9):30155. DOI: 10.2807/1560-7917.ES.2016.21.9.30155 PMID: 26967914
16. Eurosurveillance editorial team. Eurosurveillance reviewers in 2016. Euro Surveill. 2017;22(1):30432. DOI: 10.2807/1560-7917.ES.2017.22.1.30432

License and copyright

Experimental transmission of Zika virus by mosquitoes from central Europe

A Heitmann [1,2], S Jansen [1,2,3], R Lühken [1], M Leggewie [1,3], M Badusche [1], B Pluskota [4], N Becker [4,5], O Vapalahti [6], J Schmidt-Chanasit [1,3], E Tannich [1,3]

1. Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany
2. These authors contributed equally to this work
3. German Centre for Infection Research (DZIF), partner site Hamburg-Luebeck-Borstel, Hamburg, Germany
4. Institute for Dipterology (IfD), Speyer, Germany
5. University of Heidelberg, Heidelberg, Germany
6. University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Correspondence: Renke Lühken (renkeluhken@gmail.com)

Citation style for this article:
Heitmann A, Jansen S, Lühken R, Leggewie M, Badusche M, Pluskota B, Becker N, Vapalahti O, Schmidt-Chanasit J, Tannich E. Experimental transmission of Zika virus by mosquitoes from central Europe . Euro Surveill. 2017;22(2):pii=30437. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30437

Article submitted on 15 December 2016 / accepted on 10 January 2017 / published on 12 January 2017

Mosquitoes collected in Germany in 2016, including *Culex pipiens pipiens* biotype *pipiens*, *Culex torrentium* and *Aedes albopictus*, as well as *Culex pipiens pipiens* biotype *molestus* (in colony since 2011) were experimentally infected with Zika virus (ZIKV) at 18 °C or 27 °C. None of the *Culex* taxa showed vector competence for ZIKV. In contrast, *Aedes albopictus* were susceptible for ZIKV but only at 27 °C, with transmission rates similar to an *Aedes aegypti* laboratory colony tested in parallel.

In 2015, Zika virus (ZIKV) emerged in Columbia and Brazil and spread rapidly across the American continent and the Caribbean, causing an epidemic with notable numbers of associated clinical cases of microcephaly and Guillain–Barré syndrome [1]. Mosquitoes of the species *Aedes aegypti* and *Ae. albopictus* are considered the primary and secondary vectors of ZIKV [2]. However, with transmission rates below 50%, their vector competence for ZIKV in the laboratory is low [3]. The question therefore remains whether other common mosquito species such as *Culex* spp. play a role in the transmission cycle of ZIKV. The few studies performed so far have provided inconclusive results and suggested that at least *Culex quinquefasciatus* might be able to transmit ZIKV [4-9]. In addition, for an assessment of the risk of possible spread to regions with temperate climate such as central Europe, information is lacking on ZIKV vector competence of mosquitoes under reduced temperature conditions (< 20°C).

This study aimed to evaluate the vector competence of central European mosquito species for ZIKV. Therefore, German populations of *Culex pipiens pipiens* biotype *pipiens* (*Cx. p. pipiens*), *Culex pipiens pipiens* biotype *molestus* (*Cx. p. molestus*), *Culex torrentium* and *Ae. albopictus (Ae. albopictus,* GER) were experimentally infected with ZIKV, using *Ae. aegypti* and an Italian *Ae. albopictus (Ae. albopictus*, ITA) as positive controls.

Experimental infection of mosquitoes

Two long-established laboratory strains (*Ae. aegypti* (Bayer company) and *Cx. p. molestus* (in colony since 2011, collected in Heidelberg, Germany)) and four species collected in summer 2016 (*Cx. p. pipiens* Fo (collected in Hamburg, Germany), *Culex torrentium* Fo (collected in Hamburg, Germany), *Ae. albopictus* F7 (collected in Freiburg, Germany) and *Ae. albopictus* F7 (collected in Calabria, Italy)) were analysed and maintained as previously described [10,11]. All colonies tested negative in pan-flavivirus PCRs [12].

Between 150 and 200 female mosquitoes 4–14 days-old were starved for 24 h before application of infectious blood meals containing ZIKV (strain ZIKV_FB-GWUH-2016, GenBank KU870645, fifth passage) [13] at a final concentration of 107 plaque-forming units (PFU)/mL. Artificial feeding was performed using a Hemotek Feeder (*Aedes* spp.) or by cotton sticks (*Culex* spp.). Engorged females were incubated at 80% humidity at either 18 °C or 27 °C. Analyses for ZIKV were done 14 and 21 days post infection (dpi) for approximately 35 randomly selected females and twice the number for *Ae. aegypti* at 27°C. For salivation, mosquitoes were anaesthetised and the proboscises were inserted into cropped 10 µL filter tips containing 10 µL phosphate-buffered saline (PBS). After 30 min, tips were removed and saliva-containing PBS was analysed for the presence of infectious virus particles by measuring its cytopathic effect (CPE) on Vero cells within the following 8 days. ZIKV in the supernatant of cytopathic cells was confirmed by qRT-PCR using Real Star Zika Virus RT-PCR Kit (Altona diagnostics, Hamburg, Germany). In addition, bodies of all challenged mosquitoes,

Table

Susceptibility and transmission rates of mosquitoes experimentally infected with Zika virus (n = 856)

		14 days post infection			21 days post infection		
Mosquito taxa	T in °C	IR[a] (%)	Mean (SD) log10 RNA copies/specimen[b]	TR[c] (%)	IR[a] (%)	Mean (SD) log10 RNA copies/specimen	TR (%)
Aedes aegypti	18	17/31 (55)	4.70 (0.86)	0/17	18/33 (55)	4.33 (0.63)	0/18
	27	31/63 (49)	8.69 (1.60)	14/31 (45)	36/50 (72)	6.82 (1.75)	11/36 (31)
Aedes albopictus, ITA	18	19/30 (63)	4.05 (0.59)	0/19	14/39 (36)	5.52 (0.87)	0/14
	27	22/31 (71)	6.34 (2.14)	4/22 (18)	15/29 (52)	7.41 (2.22)	2/15 (13)
Aedes albopictus, GER	18	4/32 (13)	6.22 (1.25)	0/4	11/32 (34)	6.36 (1.39)	0/11
	27	20/31 (65)	6.78 (2.41)	4/20 (20)	18/34 (53)	8.61 (1.82)	6/18 (33)
Culex p. molestus	18	12/41 (29)	3.40 (0.38)	0/12	2/32 (6)	2.48 (0.29)	0/2
	27	7/29 (24)	3.73 (0.38)	0/7	12/38 (32)	4.02 (0.44)	0/12
Culex p. pipiens	18	16/34 (47)	3.38 (0.40)	0/16	3/32 (9)	3.88 (0.43)	0/3
	27	3/37 (8)	3.13 (0.45)	0/3	0/35 (0)	NA[d]	NA[d]
Culex torrentium	18	11/35 (31)	3.15 (0.47)	0/11	1/38 (3)	3.31 (NA)	0/1
	27	4/36 (11)	3.80 (1.79)	0/4	0/34 (0)	NA[d]	NA[d]

GER: from Germany; IR: infection rate; ITA: from Italy; NA: not available; SD: standard deviation; T: temperature; TR: transmission rate.

[a] Infection rate: number of ZIKV-positive mosquito bodies per number of fed females.

[b] RNA copies were averaged over all ZIKV-positive mosquito bodies excluding the zeros of ZIKV-negative mosquito bodies.

[c] Transmission rate: number of mosquitoes with ZIKV-positive saliva per number of ZIKV-positive mosquito bodies.

[d] Not available: Mean viral RNA copies and transmission rate could not be calculated for the species–temperature combinations with no ZIKV-positive bodies.

excluding legs and wings, were analysed for ZIKV RNA by qRT-PCR.

Results

At 14 or 21 dpi, ZIKV RNA was detected in the bodies of all challenged mosquito taxa, with infection rates ranging between 3 and 72% in the species–temperature combinations with ZIKV-positive bodies. Infection rates and virus titres were substantially higher in *Aedes* species, with viral RNA copies ranging from 10^2 to 10^4 in *Culex* spp. and from 10^4 to 10^9 in *Aedes* spp. (Table).

Virus load was generally higher at elevated incubation temperature (27 °C vs 18 °C). However, transmission of infectious virus particles as measured by CPE of Vero cells incubated with mosquito saliva was not detected in any of the *Culex* taxa. In contrast, saliva was positive for infectious virus particles in all *Aedes* species, but only at 27 °C incubation temperature. Interestingly, transmission rates at 21 dpi were similar in *Ae. aegypti* and *Ae. albopictus* from Germany but were substantially lower in *Ae. albopictus* from southern Italy (30% vs 13%).

Discussion

Culex species from central Europe are known as established vectors, able to transmit numerous viruses including West Nile, Sindbis and Usutu virus [14,15]. The results presented here indicate that the three most common *Culex* taxa in central Europe *(Cx. p. pipiens, Cx. p. molestus* and *Cx. torrentium*) do not have vector competence for ZIKV. This is in agreement with results from other parts of the world including Italy [4-7,9], which all showed a low degree of compentence of the *Cx. pipiens* complex for ZIKV transmission.

The invasive mosquito *Ae. albopictus* is established in large parts around the Mediterranean Sea and is considered to be the main vector in Europe for autochthonous human infections with chikungunya and dengue virus [16]. *Aedes albopictus* are regularly introduced into Germany as accidental cargo via road traffic from southern Europe [17]. In the winter 2015/16, successful overwintering of the species was observed for the first time in southern Germany [18]. The results presented here indicate that specimens of this overwintering population have considerable susceptibility to ZIKV, although only at elevated temperature of 27 °C.

Moreover, the transmission rate in this overwintering population was substantially higher than in *Ae. albopictus* from the Calabrian region in southern Italy. Whether the difference in virus susceptibility between German and Italian *Ae. albopictus* populations is due to an ongoing process of adaptation to a new environment or to experimental conditions remains to be determined. Nevertheless, the susceptibility of European *Ae. albopictus* to ZIKV demonstrates the risk of arbovirus transmission associated with the establishment and ongoing spread of this invasive mosquito species in Europe. Of note, none of the tested *Aedes* populations were susceptible to ZIKV at 18 °C, which may limit the spread of ZIKV in central Europe to short summer periods with high temperatures. However, for a comprehensive risk assessment of ZIKV transmission in central Europe, further infection studies are needed at intermediate temperatures (e.g. 21 °C and 24 °C) as well as with other common *Aedes* species such as *Ae. vexans* or the newly established *Ae. japonicus* [19].

Acknowledgements

We thank Ella Weinert and Michelle Helms for excellent technical assistance and Jessica Börstler for analyses of Culex mosquitoes. ML was supported by the Leibniz Association, grant number SAW-2014-SGN-3. This work was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE) with the grant number 28-1-91.048-15.

Conflict of interest

None declared.

Authors' contributions

Conceived and designed the study: AH, SJ, RL, JSC, ET. Performed the data collection: AH, SJ, ML. Analysed the data: AH, SJ, RL, JSC, ET. Provided the ZIKA virus strain: OV. Provided mosquito specimens: MB, BP, NB. Wrote the paper: AH, SJ, RL, ET. Contributed to the manuscript drafting: ML. All authors read and approved the final version of the manuscript.

References

1. Musso D, Gubler DJ. Zika Virus.Clin Microbiol Rev. 2016;29(3):487-524.DOI: 10.1128/CMR.00072-15 PMID: 27029595
2. World Health Organization Regional Office for Europpe (WHO/Europe). Zika virus technical report. Interim risk assessment WHO European Region. Copenhagen: WHO/Europe; 2016. Available from: http://www.euro.who.int/en/health-topics/emergencies/zika-virus/technical-reports-and-guidelines-on-zika-virus/zika-virus-technical-report.-interim-risk-assessment-for-who-european-region
3. Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, et al. Differential susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika virus. PLoS Negl Trop Dis. 2016;10(3):e0004543.
4. Aliota MT, Peinado SA, Osorio JE, Bartholomay LC. Culex pipiens and Aedes triseriatus Mosquito susceptibility to Zika virus.Emerg Infect Dis. 2016;22(10):1857-9.DOI: 10.3201/eid2210.161082 PMID: 27434194
5. Amraoui F, Atyame-Nten C, Vega-Rúa A, Lourenço-de-Oliveira R, Vazeille M, Failloux AB. Culex mosquitoes are experimentally unable to transmit Zika virus.Euro Surveill. 2016;21(35):30333. DOI: 10.2807/1560-7917.ES.2016.21.35.30333 PMID: 27605159
6. Boccolini D, Toma L, Di Luca M, Severini F, Romi R, Remoli ME, et al. Experimental investigation of the susceptibility of Italian Culex pipiens mosquitoes to Zika virus infection. Euro Surveill. 2016;21(35):30328.DOI: 10.2807/1560-7917.ES.2016.21.35.30328 PMID: 27605056
7. Fernandes RS, Campos SS, Ferreira-de-Brito A, de Miranda RM, Barbosa da Silva KA, de Castro MG, et al. Culex quinquefasciatus from Rio de Janeiro Is Not Competent to Transmit the Local Zika Virus. PLoS Negl Trop Dis. 2016;10(9):e0004993.
8. Guo XX, Li CX, Deng YQ, Xing D, Liu QM, Wu Q, et al. Culex pipiens quinquefasciatus: a potential vector to transmit Zika virus. Emerg Microbes Infect. 2016;5(9):e102.DOI: 10.1038/emi.2016.102 PMID: 27599470
9. Huang Y-JS, Ayers VB, Lyons AC, Unlu I, Alto BW, Cohnstaedt LW, et al. Culex species mosquitoes and Zika virus. Vector Borne Zoonotic Dis. 2016;16(10):673-6.DOI: 10.1089/vbz.2016.2058 PMID: 27556838
10. Vogels CBF, van de Peppel LJJ, van Vliet AJH, Westenberg M, Ibañez-Justicia A, Stroo A, et al. Winter activity and aboveground hybridization between the two biotypes of the West Nile virus vector Culex pipiens. Vector Borne Zoonotic Dis. 2015;15(10):619-26.DOI: 10.1089/vbz.2015.1820 PMID: 26394124
11. Leggewie M, Badusche M, Rudolf M, Jansen S, Börstler J, Krumkamp R, et al. Culex pipiens and Culex torrentium populations from Central Europe are susceptible to West Nile virus infection. One Health. 2016;2:88-94.DOI: 10.1016/j.onehlt.2016.04.001
12. Chao D-Y, Davis BS, Chang G-JJ. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes.J Clin Microbiol. 2007;45(2):584-9.DOI: 10.1128/JCM.00842-06 PMID: 17108075
13. Driggers RW, Ho C-Y, Korhonen EM, Kuivanen S, Jääskeläinen AJ, Smura T, et al. Zika virus infection with prolonged maternal viremia and fetal brain abnormalities. N Engl J Med. 2016;374(22):2142-51.DOI: 10.1056/NEJMoa1601824 PMID: 27028667
14. Jöst H, Bürck-Kammerer S, Hütter G, Lattwein E, Lederer S, Litzba N, et al. Medical importance of Sindbis virus in south-west Germany. J Clin Virol. 2011;52(3):278-9.DOI: 10.1016/j.jcv.2011.08.002 PMID: 21893428
15. Nikolay B. A review of West Nile and Usutu virus co-circulation in Europe: how much do transmission cycles overlap?Trans R Soc Trop Med Hyg. 2015;109(10):609-18.DOI: 10.1093/trstmh/trv066 PMID: 26286946
16. Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, Zeller H, et al. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector Borne Zoonotic Dis. 2012;12(6):435-47.DOI: 10.1089/vbz.2011.0814 PMID: 22448724
17. Becker N, Geier M, Balczun C, Bradersen U, Huber K, Kiel E, et al. Repeated introduction of Aedes albopictus into Germany, July to October 2012. Parasitol Res. 2013;112(4):1787-90.DOI: 10.1007/s00436-012-3230-1 PMID: 23242268
18. Pluskota B, Jöst A, Augsten X, Stelzner L, Ferstl I, Becker N. Successful overwintering of Aedes albopictus in Germany. Parasitol Res. 2016;115(8):3245-7.DOI: 10.1007/s00436-016-5078-2 PMID: 27112761
19. Huber K, Schuldt K, Rudolf M, Marklewitz M, Fonseca DM, Kaufmann C, et al. Distribution and genetic structure of Aedes japonicus japonicus populations (Diptera: Culicidae) in Germany. Parasitol Res. 2014;113(9):3201-10. 25056941DOI: 10.1007/s00436-014-4000-z

National molecular surveillance of recently acquired HIV infections in Germany, 2013 to 2014

A Hauser [1], A Hofmann [2,3], K Hanke [1], V Bremer [2], B Bartmeyer [2], C Kuecherer [1], N Bannert [1]
1. Division of HIV and Other Retroviruses, Robert Koch Institute, Berlin, Germany
2. Division of HIV/AIDS, STI and Blood-borne Infections, Robert Koch Institute, Berlin, Germany
3. Charité – Universitätsmedizin, Berlin, Germany

Correspondence: Norbert Bannert (BannertN@rki.de)

Citation style for this article:
Hauser A, Hofmann A, Hanke K, Bremer V, Bartmeyer B, Kuecherer C, Bannert N. National molecular surveillance of recently acquired HIV infections in Germany, 2013 to 2014. Euro Surveill. 2017;22(2):pii=30436. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30436

Article submitted on 01 February 2016 / accepted on 23 August 2016 / published on 12 January 2017

To enable an up-to-date molecular analysis of human immunodeficiency virus (HIV) genotypes circulating in Germany we have established a surveillance system based on recently acquired HIV infections. New HIV infections are reported to the Robert Koch Institute as a statutory duty for anonymous notification. In 2013 and 2014, a dried serum spot (DSS) sample was received from 6,371 newly diagnosed HIV-cases; their analysis suggested that 1,797 samples originated from a recent infection. Of these, 809 were successfully genotyped in the *pol* region to identify transmitted drug resistance (TDR) mutations and to determine the HIV-1 subtype. Total TDR was 10.8%, comprising 4.3% with mono-resistance to nucleoside reverse transcriptase inhibitors (NRTIs), 2.6% to non-NRTIs, 3.0% to protease inhibitors and 0.6% and 0.2%, respectively, with dual- and triple-class resistances. HIV-1 subtype B was most prevalent with 77.0%. Non-B infections were identified more often in men and women with heterosexual transmission compared with intravenous drug users or men who have sex with men (79% and 76%, 33%, 12%; all $p < 0.05$). Non-B subtypes were also more frequently found in patients originating from countries other than Germany (46% vs 14%; $p < 0.05$) and in patients infected outside of Germany (63% vs 14%; $p < 0.05$).

Introduction

Continuous molecular HIV surveillance provides valuable public health information concerning the transmission of drug-resistant viruses and the dynamics of currently circulating variants. Transmitted drug resistance (TDR) has significant clinical consequences as it is associated with an increase in the failure rate of antiretroviral therapy (ART) [1]. With prevalence of TDR (at least one resistance mutation) ranging between 10% and 15% in several European countries and in North America [2-6], the problem is of substantial concern. Genotypic resistance testing is therefore recommended before commencing first-line treatment [7]. The prevalence of TDR in a country is determined by the history of clinical therapy regimens and the failures resulting from resistance. TDR can vary depending on transmission route, country of origin of the patient and country of infection [6]. From a virological perspective, TDR is largely determined by the persistence of the associated mutation(s). Persistence of those mutations depends mainly on the fitness costs to the virus and on the viral genetic background (e.g. chance of compensatory mutations) [8].

Knowledge of currently circulating HIV subtypes in a country and the dynamics of their spread is of epidemiological and clinical relevance. This information affects the safety and accuracy of HIV diagnostics and is valuable for HIV prevention and vaccine development.

The objective of this study was to determine TDR and HIV-1 subtypes in a substantial subset of new HIV-1 infections diagnosed in 2013 and 2014 in Germany. We intended to examine transmissions from a restricted period of time and therefore, only recently acquired infections among the newly diagnosed cases were included. We aimed to analyse TDR and transmitted HIV-1 subtypes in the main transmission groups: men who have sex with men (MSM), persons with heterosexual contact (HET) and people who inject drugs (PWID), considering sex and origin of the infected individual and place of infection.

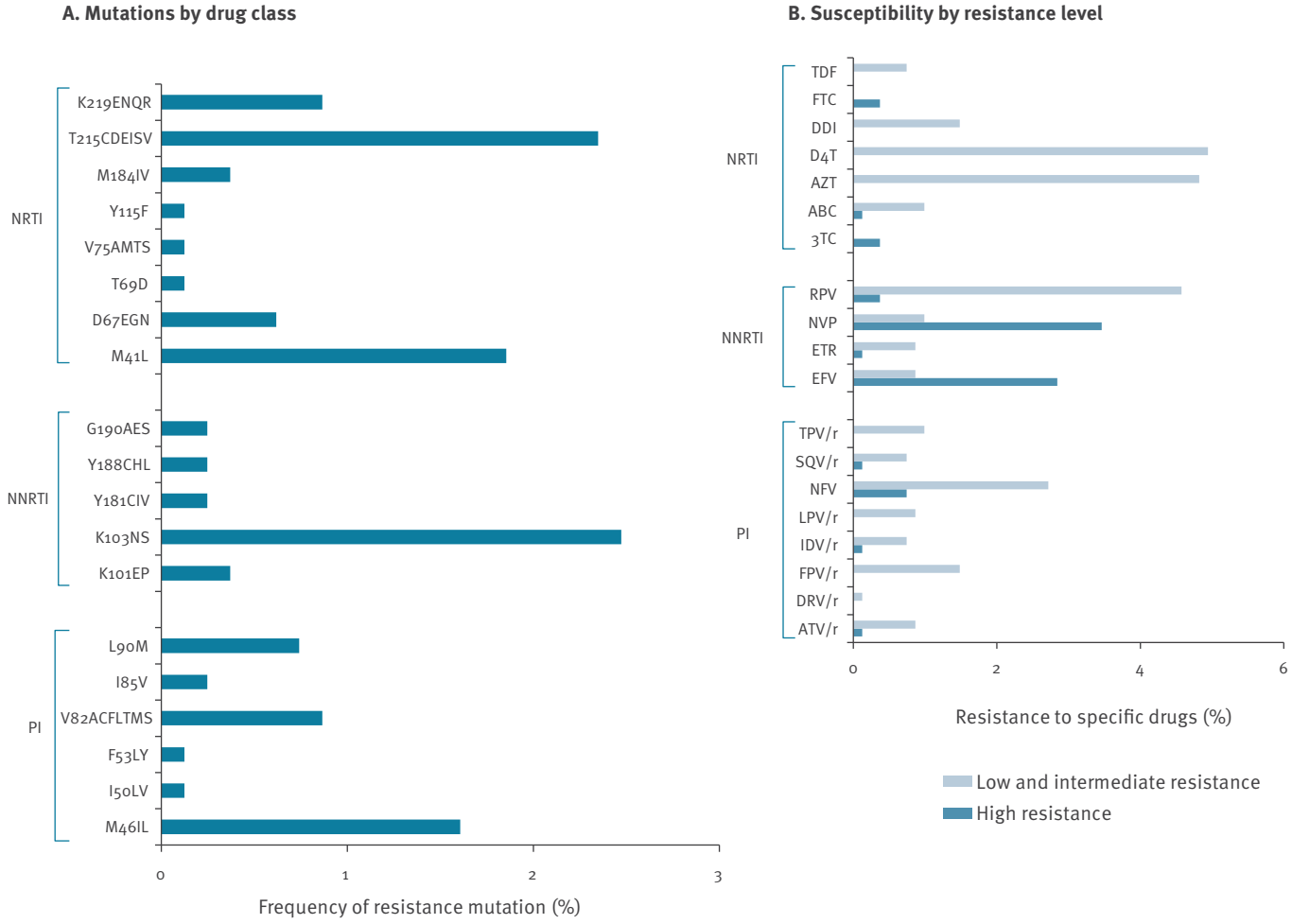
Methods

Clinical samples

The data protection officer of the RKI and the German Federal Commissioner for Data Protection and Freedom of Information approved the study protocol (III-401/008#0016).

Figure 1

Prevalence of transmitted drug resistance mutations by drug class (A) and predicted susceptibility to antiretroviral drugs by level of resistance (B) in the study population, Germany, 2013–14 (n = 809)



NRTI: nucleoside reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse transcriptase inhibitors; PI: protease inhibitor.

Panel B: Prediction according to Stanford SIR algorithm: intermediate levels of resistance (light colours) and high levels of resistance (dark colours); low and potentially low levels are not indicated.

RNA isolation and HIV sequencing

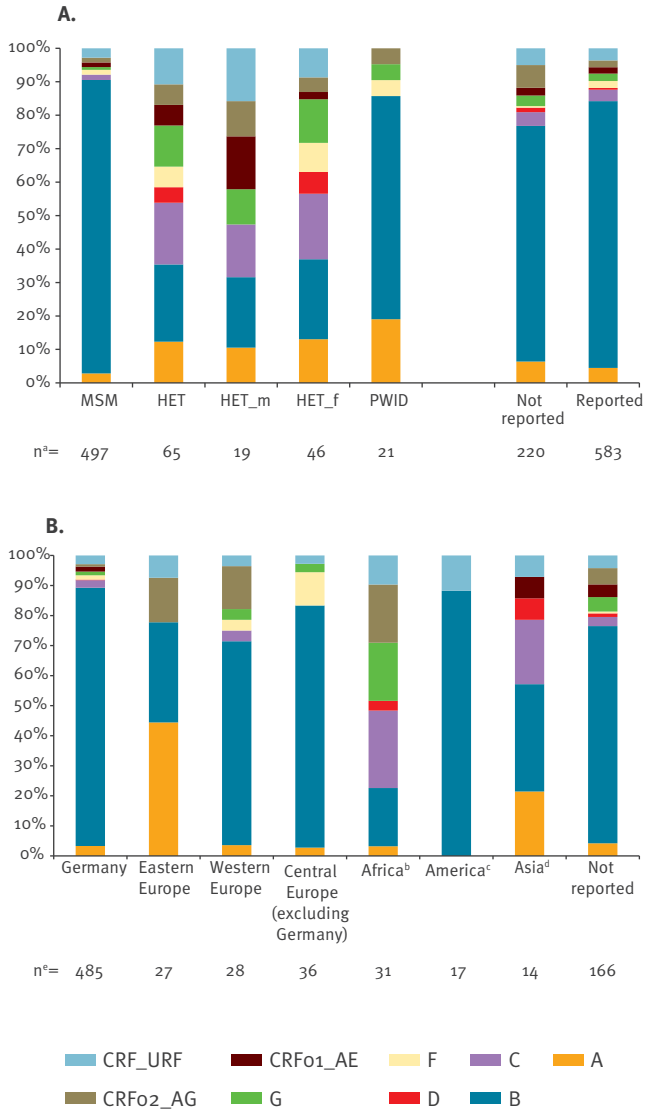
HIV RNA from 4 x 100 µL DSS was extracted according to the manufacturer's instructions with the manual NucliSense Magnetic Extraction method for samples collected in 2013 or the automated Biomerieux EasyMag platform for samples collected in 2014 (both bioMerieux, Capronne, France). Viral RNA was quantified using an in house LTR RT-TaqMan PCR according to a modified protocol [9]. As concentration controls, serial dilutions of the IIIB strain of HIV-1 in human HIV-negative plasma was dropped onto filter cards (DPS standard). Samples were analysed using a newly designed HIV-1 group M generic RT-PCR system (in house *pol* RT-PCR) covering the genomic region of HIV-1 protease (AS9–99) and reverse transcriptase (AS1–252) including all resistance-associated positions. The in house *pol* RT-PCR assay consists of two *pol* RT-PCRs yielding two overlapping amplicons: a fragment 1 of 576 bp (primer_A: CCCTCARATCACTCTTTggCARCgA, position 2,252–2,276; Primer_B: CCTAATTgAACYTCCCARAARTCYTgAgT, position 2,799–2,827) and a second fragment 2 of 718 bp (Primer_C: AAACAATggCCATTRACAgARgA, position 2,613–2,636; primer_D: CTAAYTTYTgTATRTCATTgACAgTCCA, position 3,303–3,330). All nucleotide positions refer to sequence of the HXB2 genome (accession number K03455). The detection limit of the in house *pol* RT-PCR was shown to be equivalent to 1,000 copies/mL DPS standard (highest dilution resulting in 100% positive PCR outcome). Population-based sequencing was performed and a consensus sequence from both fragments was obtained.

HIV-1 subtyping

The HIV-1 subtype was assigned by applying the REGA HIV-1 subtyping tool (REGA HIV Subtyping Tool Version

Figure 2

Subtype distribution (A) in the main transmission groups and (B) by country of origin, Germany, 2013–14 (n = 809)



HET: persons with heterosexual contact; HET_f: female persons with heterosexual contact; HET_m: male persons with heterosexual contact; MSM: men who have sex with men; PWID: people who inject drugs.

[a] Transmission group `other´(n=6, see Table 1) was excluded from the data set.

[b] Patients from the Middle East, North and sub-Saharan Africa.

[c] Patients from North and Latin America.

[d] Patients from South and South-east Asia.

[e] Two patients from the Caribbean, one patient from Oceania and two patients with unknown non-German origin were excluded.

3.0) and COMET HIV-1 (Version 1.0) [10] to the *pol* sequence. Only subtype classifications based on bootstrap values of›70% in the tree topology were taken into account. In cases where a subtype or circulating recombinant form (CRF) was not assigned by either subtyping tool, the strain was classified as unique recombinant form (URF). In addition, a distance-based neighbour-joining method and bootstrap (PHYLIP version 3.6) was calculated using an extended panel of subtype reference sequences from the Los Alamos HIV sequence database.

HIV drug resistance interpretation and calculation of the prevalence of transmitted drug resistance

The World Health Organization (WHO)'s surveillance drug resistance mutations (SDRM) list was used to interpret TDR [11]. Levels of expected resistance to each of the three drug classes nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) as mono, dual or triple class resistance were predicted using the Stanford algorithm (version 7.0). Three levels of resistance were scored: high (R), intermediate (I, intermediate and low), sensitive (S, potentially resistant and sensitive).

Statistical analysis

Descriptive statistics for continuous variables were calculated as medians and interquartile ranges (IQR). Differences in proportions, odds ratios (OR) and 95% confidence intervals (CI) were assessed by two-sided Fisher's exact test using EPICALC 2000 software (version 1.02; Gilman and Myatt 1997). A two-sided p value≤0.05 was considered significant.

Results

Characterisation of the study population

In 2013 and 2014, a total of 6,371 DSS prepared from residual serum of newly HIV-diagnosed cases were submitted to the RKI along with the anonymous report. Of these, 2,034 (32%) were serologically classified as recent HIV infections using the BED-CEIA. The viral load was reported for 700 and a CD4+ T-cell count for 543 of the 2,034 specimens. Specimens with CD4+ T-cell counts‹200 cells/µL (n = 108) or viral loads‹400 copies/mL (n = 50) or both (n=8) were reclassified as 'long-standing infections' and, together with repeatedly reported cases (n = 71), excluded, resulting in 1,797 DSS. Sufficient material (four serum spots à 100 µL) was available for 1,387 of those specimens. The *pol* RT-PCR amplification and sequencing was successful in 809 samples; these represented the final study panel of recent HIV infections. A viral load was reported for 298 of 809 cases, with a median of 184,481 copies/mL (IQR: 45,405–983,158). Among these 298 cases, CD4+ T-cell counts were available for 214, with a median of 357 cells/µL (IQR: 278–487). The proportional distribution of recently infected cases analysed by sex, transmission routes, country of origin and country of

Figure 3

Prevalence of HIV-1 subtypes stratified by (A) main transmission groups and (B) origin of patient, Germany 2013–14 (n = 809)

HET: persons with heterosexual contact; MSM: men who have sex with men; PWID: people who inject drugs.

[a] Transmission group `other´(n=6), see Table 1.

[b] Patients from South and South-east Asia.

[c] Patients from North and Latin America.

[d] Patients from Middle East, North and sub-Sahara Africa.

[e] Two patients from the Caribbean, one patient from Oceania and two patients with unknown non-German origin were excluded.

infection extracted from sociodemographic data submitted with the notification form are shown in Table 1.

Prevalence of transmitted drug resistance

The overall prevalence of TDR in patients with recently acquired HIV infection diagnosed in 2013 and 2014 was 10.8% (n = 87/809; 95% CI: 8.8–13.1). This comprised mono-resistance to NRTIs in 4.3% (35/809; 95% CI: 3.1–6.0) of the patients, to NNRTIs in 2.6% (21/809; 95% CI: 1.7–4.0) and to PIs in 3.0% (24/809; 95% CI: 2.0–4.5) of cases. Dual and triple class resistance was identified in 0.6% (n = 4 NRTI/NNRTI; n = 1 NNRTI/PI) and 0.2% (n = 2 NRTI/NNRTI/PI) of the patients, respectively (Table 2).

Of all 52 NRTI-associated mutations identified, 46 were thymidine analogue mutations (TAM), namely: the revertants of T215Y (T215CDEISV; 2.3%; 19/809), M41L (2.2%; 15/809), K219ENQR (0.9%; 7/809) and D67EGN (0.6%; 5/809). TAM were selected by the thymidine analogues and conferred intermediate resistance to azidothymidine (AZT) and stavudine (D4T). The most prevalent NNRTI resistance mutations were K103N and K103S (2.5%; 20/809), selected by first generation NNRTIs. The PI-selected resistance mutations M46I and M46L were present at a level of 1.6% (13/809) associated with resistance to a broad spectrum of PIs: atazanavir/ritonavir (ATV/r), fosamprenavir (FPV/r), indinavir (IDV/r), lopinavir (LPV/r), nelfinapir (NFV) and tripranavir (TPV/r). The PI-selected resistance mutations L90M (resistance to LPV/r, ATV/r, saquinavir (SQV)/r, IDV/r, NFV) and V82ACFLTMS (resistance to FPV/r, TPV/r, IDV/r, LPV/r, ATV/r) were present at levels of 0.7% (6/809) and 0.9% (7/809), respectively (Figure 1A and B).

Drug resistance mutations observed in transmitted HIV strains mainly induced intermediate levels of resistance to NRTI and PI (mainly resistance-associated singleton mutations). High levels of resistance were frequently observed to the NNRTIs efavirenz (EFV) and nevirapine (NVP) and were caused primarily by the prevalent resistance mutation K103N (Figure 1B).

We found no correlation between TDR and sex, transmission group, origin of patient or country of infection in general or for any resistance class (all p values › 0.05) (Table 2).

Prevalence of HIV-1 subtypes

The most prevalent HIV-1 subtype in the total study population (n = 809) was subtype B with 77.0% (623/809; 95% CI: 73.9–79.8), while 23.0% of HIV-1 infections (186/809; 95% CI: 20.2–26.1) were caused by non-B subtypes including A (5.1%, 41/809), C (3.6%, 29/809), D (0.7%, 6/809), F (1.6%, 13/809), G (2.7%, 22/809), CRF01_AE (2.0%, 16/809) and CRF02_AG (3.3%, 27/809) as well as 4.0% (32/809) other rare CRFs (06_cpx, 07_BC, 12_BF, 18_cpx, 19_cpx, 34_01B, 35_AD, 44_BF, 49_cpx) and URFs. These rare CRFs

Table 1

Characteristics of patients with recent infection included in molecular HIV surveillance, Germany, 2013–14 (n = 809)

Study population	n	%
Sex		
Male	718	88.8
Female	86	10.6
Not reported	5	0.6
Transmission group		
Men who have sex with men	497	61.4
Persons with heterosexual contacts	65	8.0
Persons with intravenous drug use	21	2.6
Other	6	0.7
Not reported	220	27.2
Country of origin		
Germany	485	60.0
Other	158	19.5
Not reported	166	20.5
Country of infection		
Germany	523	64.6
Other	91	11.2
Not reported	195	24.1

were grouped together with the URFs into the rare CRF/URF subgroup.

The subtype distribution in the subset of recent infections with available CD4+ T-cell counts and viral loads (n = 214) was very similar to the overall study population, with 77.1% (165/214) subtype B and 22.9% (49/214) non-B infections including A (4.2%, 9/214), C (5.1%, 11/214), D (0.9%, 2/214), F (0.9%, 3/214), G (2.8%, 6/214), CRF01_AE (1.9%, 4/214) and CRF02_AG (1.9%, 4/214) as well as 4.7% (10/214) rare CRF/URFs (all p values of pairwise comparisons > 0.2).

HIV-1 subtype B was associated with a significantly higher proportion of TDR (12.0%, 75/623) than the non-B subtypes (6.4%; 12/186; OR: 0.50; 95% CI: 0.27–0.95; p = 0.04), particularly with regard to the NRTI mutations (5.3% in B, 1.1% in non-B, p = 0.01) (Table 2).

With regard to the main transmission groups, MSM were predominantly infected by subtype B, with 87.7% (436/497) which is significantly higher than in PWID (14/21) or in HET (15/65; male HET: 4/19 and female HET 11/46; all p values MSM-PWID-HET < 0.05). In HET, subtype diversity was high and particularly non-B subtypes A (8/65), C (12/65) and G (8/65) were frequent. In PWID, subtype A (4/21) was the second most prevalent subtype (after B with 14/21), while subtypes C, D, CRF01_AE and the group of rare CRF/URF were not identified in PWID. Subtype distribution in the group that did not report transmission type mirrored the group that did report transmission type (MSM + HET + PWID), with a slightly lower proportion of subtype B (70.5%, 155/220 vs 79.8%, 465/583) (Figure 2A). The proportion of subtype non-B infections in Germans (14.0%, 68/485) was significantly lower than in individuals from other countries (45.6%, 72/158; p < 0.0001). Subtype B was predominant in Germans and in migrants originating from America and western and central Europe (417/485, 15/17, 19/28 and 29/36, respectively). Subtype A was the most frequently subtype identified in eastern Europeans (12/27), while migrants from Africa and Asia were infected with a greater variety of subtypes (Figure 2B).

For the subtypes A, B, F, CRF01_AE and the rare CRF/URF, the main transmission route was MSM (14/41, 436/623, 7/13, 7/16 and 14/32, respectively) while the subtypes C, D and G were mainly transmitted by heterosexual contacts (12/29, 3/6 and 8/22, respectively) (Figure 3A).

HIV subtype A was mostly identified in Germans (16/41) and eastern European patients (12/41), subtype C and G in Germans (12/29 and 6/22) and Africans (8/29 and 6/22), while for Subtype D and CRF02_AG, no main origin of patients could be identified. The rare CRF/URFs were mainly identified in Germans (14/31) but also in patients originating from other continents (Europe (4/31), Africa (3/31), America (2/31) and Asia (1/31)) (Figure 3B).

A stratification according to the geographical region of acquisition revealed that in Germany (86.2%; 451/523), America (3/5) and in western (14/21) and central Europe (7/12), subtype B infections were mainly acquired. Non-B subtypes were acquired more frequently abroad (62.6%; 57/91) than in Germany (13.8%; 72/523; p < 0.05).

Discussion

We have established a molecular HIV-1 surveillance strategy based on samples collected in association with the mandatory notification system of new HIV diagnoses in Germany. Analysis of samples was restricted to recent HIV-transmissions with a defined duration of less than 302 days since HIV infection. This approach permitted us to focus on the viruses circulating during any given period of time and to estimate current trends in the HIV epidemic, because late presenters who acquired an infection before that period were excluded.

The overall TDR prevalence among the 2013 and 2014 study samples was 10.8%. This is largely comparable to previously published cohort studies from Germany and several other western European countries with a long history of combination ART (cART) [3-5] and indicates in many respects that the rate of TDR is essentially stable: Patients included in the German HIV-1 seroconverter cohort between 1996 and 2010 revealed TDR in 11.9% [5]. Two recent comprehensive epidemiological studies involving 26 European countries carried

Table 2

Prevalence of transmitted drug resistance to the major antiretroviral drug classes in the study population stratified by relevant subgroups, Germany, 2013–14 (n = 809)

	TDR		NRTI		NNRTI		PI		Dual		Multi	
	n	%	n	%	n	%	n	%	n	%	n	%
Total	87	10.8	35	4.3	21	2.6	24	3.0	5	0.6	2	0.2
Sex												
Male (n = 718)	78	10.9	32	4.5	18	2.5	21	2.9	5	0.7	2	0.3
Female (n = 68)	9	10.5	3	3.5	3	3.5	3	3.5	0		0	
Not reported (n = 5)	0		0		0		0		0		0	
Transmission group												
MSM (n = 497)	59	11.9	22	4.4	14	2.8	17	3.4	4	0.8	2	0.4
HET (n = 65)	4	6.2	3	4.6	1	1.5	0		0		0	
PWID (n = 21)	2	9.5	1	4.8	0		1	4.8	0		0	
Other (n = 6)	0		0		0		0		0		0	
Not reported (n = 220)	22	10.0	9	4.1	6	2.7	6	2.7	1	0.5	0	
Country of origin												
Germany (n = 485)	56	11.5	24	4.9	12	2.5	16	3.3	3	0.6	1	0.2
Other (n = 158)	12	7.6	2	1.3	5	3.2	3	1.9	1	0.6	1	0.6
Not reported (n = 166)	19	11.4	9	5.4	4	2.4	5	3.0	1	0.6	0	
Country of infection												
Germany (n = 523)	63	12.0	23	4.4	15	2.9	18	3.4	5	1.0	2	0.4
Other (n = 91)	5	5.5	3	3.3	1	1.1	1	1.1	0		0	
Not reported (n = 195)	19	9.7	9	4.6	5	2.6	5	2.6	0		0	
HIV-1 Subtypes												
B (n = 623)	75	12.0	33	5.3	16	2.6	19	3.0	5	0.8	2	0.3
Non-B (n = 186)	12	6.5	2	1.1	5	2.7	5	2.7	0		0	

HET: persons with heterosexual contact; HIV: human immunodeficiency virus; MSM: men who have sex with men; NRTI: nucleotide reverse transcriptase inhibitors; NNRTI: non-nucleotide reverse transcriptase inhibitors; PI: protease inhibitor; PWID: people who inject drugs; TDR: transmitted drug resistance.

`Not reported´ data were excluded from the pairwise comparisons. All p values of pairwise comparison were › 0.05.

out on behalf of the SPREAD-programme reported levels of 8.9% (95% CI: 8.1–9.8) and 8.3% (95% CI: 7.7–9.5) for the period from 2002 to 2007 [6] and from 2008 to 2010, respectively [12]. Proportions of patients with mono-NRTI, mono-NNRTI, mono-PI, dual- and multi-class resistance (5.0%, 2.9%, 2.5%, 0.8% and 0.4%, respectively) [6] were very similar to our findings (4.3%, 2.6%, 3.0%, 0.6% and 0.2%). In both the SPREAD studies and in ours, the detected NRTI resistance mutations belonged to the TAM (mainly revertants of T215Y, followed by M41L) selected by AZT and D4T. The most prevalent NNRTI SDRMs were K103NS, and resistance to PIs was mainly due to the mutations L90M and M46IL [6,12]. TAM are very frequent and long-term persisting resistance mutations [8]. De novo selection by current therapies is unlikely because the use of AZT and D4T is declining [13]. We therefore suggest that these have become circulating strains by onward transmission from untreated, and probably undiagnosed, individuals. While TAM may not have any particular impact on the success of current first-line treatments, K103N and K103S may still be associated with their failure [7].

Sex was not associated with TDR in our study. However, the prevalence of TDR was slightly but not significantly higher among MSM, in Germans or in individuals infected in Germany. In studies carried out in some other European countries, the associations between TDR and MSM or subtype B (exhibiting higher TDR levels) were significant [6,12,14].

HIV-1 epidemiology is characterised by compartmentalised subepidemics of subtypes with a dynamic nature. The most prevalent subtype in western European countries is subtype B, mainly as a consequence of multiple introductions via migration, tourism and trade from different geographical areas [15]. The lowest proportion of subtype B was reported for Portugal with 48.3% for patients diagnosed between 2006 and 2012 [16] while the highest proportion was observed in Poland with 96.2% for patients diagnosed between 2002 and 2005 [17].

Determinants of subtype distribution are the transmission group, country of origin and country of infection. Subtype B is highly prevalent in MSM, most probably as a result of a founder effect rather than an increased

transmission potential of subtype B [18]. Non-B subtypes were significantly more frequently diagnosed in sub-populations originating from and often acquired in countries other than Germany. Most migrants with recent HIV infections carried subtypes dominating in their country of origin [19], suggesting that they may have acquired the infection during a visit to their home countries or within their community in Germany.

Interestingly, a considerable proportion of rare CRF/URFs are present in patients originating from all continents. However, the high proportion of Germans in the non-B clade group, in particular those with subtype A, F and rare CRF/URFs, indicates that non-B strains have become endemic in the German population. Although the number of PWID analysed was low, HIV-1 subtype A was strikingly more common in PWID than in other transmission groups.

One limitation of our study was that the restriction to recently acquired infections over-estimates patient groups that are tested more frequently due to their awareness of transmission risks. This might explain the slightly higher prevalence of MSM among recent infections in our study (61.4%) compared with the notification data for all (recent and prevalent) newly diagnosed HIV infections in 2013 and 2014 (57.7%) [20]. A second limitation was the overall genotyping success rate of 58% in samples from recent infections. This was mainly due to a lack of serum spots for RNA extraction or to RNA degradation after inappropriate handling or shipment. A technical limitation was the expected misclassification of long-standing as recent infections due to a false recent rate (FRR) of ca 4.7% for subtype B or even higher for the non-B subtypes, in particular A and D (FRR 18.9 and 18.2%, respectively), using the BED CEIA assay [21]. This may have led to an over-representation of non-B subtypes in the study. Taking into account CD4+ T-cell counts and viral load data as parameters for recency estimation is expected to diminish this bias [22]. Consideration of these clinical parameters that were available for about one third of the samples only marginally reduced the prevalence for the recent non-B infections from 23.0% to 22.9% but, more significantly, from 5.1% to 4.2% for subtype A, which is generally the most strongly affected by BED misclassification. An ancillary analysis of specimens that were reclassified as long-standing infections based on CD4+ T-cell counts < 200 cells/µL and viral loads < 400 copies/mL (n = 72) revealed 33.3% non-B and 11.1% subtype A (data not shown), confirming the anticipated over-representation introduced by the BED ELISA. Based on these data, the lack of CD4+ T-cell counts and viral loads for two thirds of the sample set is estimated to result in an over-representation of non-B subtypes of less than 2.5%. Being one of two commercially available recency tests, the BED CEIA is used worldwide for epidemiological surveillance studies [23-28], although efforts to evaluate and improve serological recency tests are ongoing [21].

Conclusion

The TDR prevalence in recent HIV infections among notified newly diagnosed HIV patients in Germany was still high (> 10%) in 2013 and 2014 and was within the range of other European countries, including the proportions of resistance classes. Although the selection for resistant HIV was dramatically reduced by the introduction of cART and new drugs [29], levels of TDR remain stable in all European countries and are still dominated by resistance to NRTI. This is most likely caused by a continued onward transmission of persisting NRTI-resistant strains that emerged as a result of failed treatment regimens during the pre-cART era (1987–96) and not by transmission of resistant viruses from patients failing cART. Therefore, genotypic resistance testing of HIV before first-line treatment needs to be continued. Since therapy-naïve and probably also undiagnosed patients are the predominant source of TDR, early detection of HIV infection followed by early treatment, as recommended in the current guidelines [7], could reduce the transmission of resistant virus [13,30]. Our data also demonstrate that subtype B remains the most frequently transmitted subtype in Germany because of its high prevalence in MSM.

Acknowledgements

The authors thank the laboratories participating in the InzSurv and MASTER HIV/HEP projects. We also thank the sequencing facility of the RKI. Many thanks to Steve Norley for proofreading of the manuscript. The study was funded by the German Federal Ministry of Health.

Conflict of interest

None declared.

Authors' contributions

Andrea Hauser: designed and performed the experiments, collected data, analysed the data, wrote the manuscript. Alexandra Hofmann: collected and analysed data; contributed to the writing of the manuscript. Kirsten Hanke: analysed the data. Viviane Bremer: analysed the data, contributed to the writing of the manuscript. Barbara Bartmeyer: collected and analysed data, contributed to the writing of the manuscript. Claudia Kuecherer: contributed significantly to the design of the study, contributed reagents/materials/analysis tools; analysed the data, contributed to the writing of the manuscript. Norbert Bannert: was mainly responsible for the design and supervision of the study, analysed the data, contributed to the writing of the manuscript.

References

1. Wittkop L, Günthard HF, de Wolf F, Dunn D, Cozzi-Lepri A, de Luca A, et al. Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. Lancet Infect Dis. 2011;11(5):363-71. DOI: 10.1016/S1473-3099(11)70032-9 PMID: 21354861
2. Wheeler WH, Ziebell RA, Zabina H, Pieniazek D, Prejean J, Bodnar UR, et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. AIDS. 2010;24(8):1203-12. DOI: 10.1097/QAD.0b013e3283388742 PMID: 20395786

3. Colafigli M, Torti C, Trecarichi EM, Albini L, Rosi A, Micheli V, et al. Evolution of transmitted HIV-1 drug resistance in HIV-1-infected patients in Italy from 2000 to 2010. Clin Microbiol Infect. 2012;18(8):E299-304. DOI: 10.1111/j.1469-0691.2012.03847.x PMID: 22536753
4. Baxter JD, Dunn D, White E, Sharma S, Geretti AM, Kozal MJ, et al. Global HIV-1 transmitted drug resistance in the INSIGHT Strategic Timing of AntiRetroviral Treatment (START) trial. HIV Med. 2015;16(Suppl 1):77-87. DOI: 10.1111/hiv.12236 PMID: 25711326
5. Zu Knyphausen F, Scheufele R, Kücherer C, Jansen K, Somogyi S, Dupke S, et al. First line treatment response in patients with transmitted HIV drug resistance and well defined time point of HIV infection: updated results from the German HIV-1 seroconverter study. PLoS One. 2014;9(5):e95956. DOI: 10.1371/journal.pone.0095956 PMID: 24788613
6. Frentz D, Van de Vijver DA, Abecasis AB, Albert J, Hamouda O, Jørgensen LB, et al. Increase in transmitted resistance to non-nucleoside reverse transcriptase inhibitors among newly diagnosed HIV-1 infections in Europe. BMC Infect Dis. 2014;14(1):407. DOI: 10.1186/1471-2334-14-407 PMID: 25047543
7. European AIDS Clinical Society (EACS). Guidelines. Version 8.0. European Guidelines for treatment of HIV-infected adults in Europe2015. Brussels: EACS; October 2015. Available from: http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html
8. Yang WL, Kouyos RD, Böni J, Yerly S, Klimkait T, Aubert V, et al. , Swiss HIV Cohort Study (SHCS). Persistence of transmitted HIV-1 drug resistance mutations associated with fitness costs and viral genetic backgrounds.PLoS Pathog. 2015;11(3):e1004722. DOI: 10.1371/journal.ppat.1004722 PMID: 25798934
9. Cleland A, Davis C, Adams N, Lycett C, Jarvis LM, Holmes H, et al. Development of multiplexed nucleic acid testing for human immunodeficiency virus type 1 and hepatitis C virus. Vox Sang. 2001;81(2):93-101. DOI: 10.1046/j.1423-0410.2001.00093.x PMID: 11555468
10. Struck D, Lawyer G, Ternes AM, Schmit JC, Bercoff DP. COMET: adaptive context-based modeling for ultrafast HIV-1 subtype identification.Nucleic Acids Res. 2014;42(18):e144. DOI: 10.1093/nar/gku739 PMID: 25120265
11. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, Kiuchi M, et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLoS One. 2009;4(3):e4724. DOI: 10.1371/journal.pone.0004724 PMID: 19266092
12. Hofstra LM, Sauvageot N, Albert J, Alexiev I, Garcia F, Struck D, et al. Transmission of HIV drug resistance and the predicted effect on current first-line regimens in Europe. Clin Infect Dis. 2016;62(5):655-63. DOI: 10.1093/cid/civ963 PMID: 26620652
13. Mbisa JL, Fearnhill E, Dunn DT, Pillay D, Asboe D, Cane PA, UK HIV Drug Resistance Database. Evidence of self-sustaining drug resistant HIV-1 lineages among untreated patients in the United Kingdom.Clin Infect Dis. 2015;61(5):829-36. DOI: 10.1093/cid/civ393 PMID: 25991470
14. Karlsson A, Björkman P, Bratt G, Ekvall H, Gisslén M, Sönnerborg A, et al. Low prevalence of transmitted drug resistance in patients newly diagnosed with HIV-1 infection in Sweden 2003-2010. PLoS One. 2012;7(3):e33484. DOI: 10.1371/journal.pone.0033484 PMID: 22448246
15. Magiorkinis G, Angelis K, Mamais I, Katzourakis A, Hatzakis A, Albert J, et al. The global spread of HIV-1 subtype B epidemic. Infect Genet Evol. 2016;46:169-79.
16. Pereira NR, Duro R, Piñero C, Malheiro L, Soares J, Serrão R, et al. Characterization of HIV-1 subtypes in a Portuguese cohort. J Int AIDS Soc. 2014;17(4) Suppl 3;19683.PMID: 25397433
17. Abecasis AB, Wensing AM, Paraskevis D, Vercauteren J, Theys K, Van de Vijver DA, et al. HIV-1 subtype distribution and its demographic determinants in newly diagnosed patients in Europe suggest highly compartmentalized epidemics. Retrovirology. 2013;10(1):7. DOI: 10.1186/1742-4690-10-7 PMID: 23317093
18. Foley B, Pan H, Buchbinder S, Delwart EL. Apparent founder effect during the early years of the San Francisco HIV type 1 epidemic (1978-1979).AIDS Res Hum Retroviruses. 2000;16(15):1463-9. DOI: 10.1089/088922200750005985 PMID: 11054259
19. Hemelaar J, Gouws E, Ghys PD, Osmanov S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004.AIDS. 2006;20(16):W13-23. DOI: 10.1097/01.aids.0000247564.73009.bc PMID: 17053344
20. Robert Koch Institute (RKI). Regional distribution of new HIV diagnoses within Germany. [Regionale Verteilung der HIV-Neudiagnosen innerhalb Deutschlands]. Epidemiologisches Bulletin. 2015;27/2015. German. Available from: http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2015/Ausgaben/27_15.pdf?__blob=publicationFile.
21. Kassanjee R, Pilcher CD, Keating SM, Facente SN, McKinney E, Price MA, et al. Independent assessment of candidate HIV incidence assays on specimens in the CEPHIA repository. AIDS. 2014;28(16):2439-49. DOI: 10.1097/QAD.0000000000000429 PMID: 25144218
22. European Centre for Disease Prevention and Control (ECDC). Monitoring recently acquired HIV infections in the European context. Stockholm: ECDC; 2013. Available from: http://www.ecdc.europa.eu/en/publications/Publications/monitoring-recently-acquired-HIV-infections-european-context.pdf
23. Kim AA, McDougal JS, Hargrove J, Rehle T, Pillay-Van Wyk V, Puren A, et al. Evaluating the BED capture enzyme immunoassay to estimate HIV incidence among adults in three countries in sub-Saharan Africa. AIDS Res Hum Retroviruses. 2010;26(10):1051-61. DOI: 10.1089/aid.2009.0218 PMID: 20849299
24. Scheer S, Nakelsky S, Bingham T, Damesyn M, Sun D, Chin CS, et al. Estimated HIV Incidence in California, 2006-2009. PLoS One. 2013;8(2):e55002. DOI: 10.1371/journal.pone.0055002 PMID: 23405106
25. Grinberg G, Giron LB, Knoll RK, Galinskas J, Camargo M, Arif MS, et al. High prevalence and incidence of HIV-1 in a counseling and testing center in the city of Itajaí, Brazil. Braz J Infect Dis. 2015;19(6):631-5. DOI: 10.1016/j.bjid.2015.08.001 PMID: 26361837
26. Zhang J, Guo Z, Yang J, Pan X, Jiang J, Ding X, et al. Genetic diversity of HIV-1 and transmitted drug resistance among newly diagnosed individuals with HIV infection in Hangzhou, China. J Med Virol. 2015;87(10):1668-76. DOI: 10.1002/jmv.24223 PMID: 25899877
27. Lunar MM, Matković I, Tomažič J, Vovko TD, Pečavar B, Poljak M. Longitudinal trends of recent HIV-1 infections in Slovenia (1986-2012) determined using an incidence algorithm.J Med Virol. 2015;87(9):1510-6. DOI: 10.1002/jmv.24209 PMID: 25970253
28. Santos-Hövener C, Zimmermann R, Kücherer C, Bätzing-Feigenbaum J, Wildner S, Hamouda O, et al. Conversation about Serostatus decreases risk of acquiring HIV: results from a case control study comparing MSM with recent HIV infection and HIV negative controls. BMC Public Health. 2014;14(1):453. DOI: 10.1186/1471-2458-14-453 PMID: 24885694
29. Gill VS, Lima VD, Zhang W, Wynhoven B, Yip B, Hogg RS, et al. Improved virological outcomes in British Columbia concomitant with decreasing incidence of HIV type 1 drug resistance detection. Clin Infect Dis. 2010;50(1):98-105. DOI: 10.1086/648729 PMID: 19951169
30. Drescher SM, von Wyl V, Yang WL, Böni J, Yerly S, Shah C, et al. Treatment-naive individuals are the major source of transmitted HIV-1 drug resistance in men who have sex with men in the Swiss HIV Cohort Study. Clin Infect Dis. 2014;58(2):285-94. DOI: 10.1093/cid/cit694 PMID: 24145874

License and copyright

A joint cross-border investigation of a cluster of multidrug-resistant tuberculosis in Austria, Romania and Germany in 2014 using classic, genotyping and whole genome sequencing methods: lessons learnt

L Fiebig [1,2], TA Kohl [2,3], O Popovici [4], M Mühlenfeld [5], A Indra [6], D Homorodean [7], D Chiotan [8], E Richter [9], S Rüsch-Gerdes [10], B Schmidgruber [11], P Beckert [3,12], B Hauer [1], S Niemann [3,10,12], F Allerberger [6], W Haas [1]

1. Respiratory Infections Unit, Department for Infectious Disease Epidemiology, Robert Koch Institute, Berlin, Germany
2. These authors contributed equally to this work
3. Molecular and Experimental Mycobacteriology, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany
4. National Institute of Public Health – National Center for Communicable Diseases Surveillance and Control, Bucharest, Romania
5. Local Public Health Office Ingolstadt, Ingolstadt, Germany
6. Austrian Reference Laboratory for Mycobacteria, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria
7. Clinical Hospital of Pneumology, Tuberculosis National Reference Laboratory, Cluj-Napoca, Romania
8. Pneumology Institute "Marius Nasta", Bucharest, Romania
9. Labor Dr. Limbach, TB Laboratory, Heidelberg, Germany
10. National Reference Center (NRC) for Mycobacteria, Research Center Borstel, Borstel, Germany
11. Tuberculosis Patient Service, Health Service of the City of Vienna, Vienna, Austria
12. German Center for Infection Research, Partner Site Hamburg-Borstel-Lübeck, Borstel, Germany

Correspondence: Lena Fiebig (FiebigL@rki.de)

Citation style for this article:
Fiebig L, Kohl TA, Popovici O, Mühlenfeld M, Indra A, Homorodean D, Chiotan D, Richter E, Rüsch-Gerdes S, Schmidgruber B, Beckert P, Hauer B, Niemann S, Allerberger F, Haas W. A joint cross-border investigation of a cluster of multidrug-resistant tuberculosis in Austria, Romania and Germany in 2014 using classic, genotyping and whole genome sequencing methods: lessons learnt. Euro Surveill. 2017;22(2):pii=30439. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30439

Article submitted on 30 May 2016 / accepted on 28 September 2016 / published on 12 January 2017

Molecular surveillance of multidrug-resistant tuberculosis (MDR-TB) using 24-loci MIRU-VNTR in the European Union suggests the occurrence of international transmission. In early 2014, Austria detected a molecular MDR-TB cluster of five isolates. Links to Romania and Germany prompted the three countries to investigate possible cross-border MDR-TB transmission jointly. We searched genotyping databases, genotyped additional isolates from Romania, used whole genome sequencing (WGS) to infer putative transmission links, and investigated pairwise epidemiological links and patient mobility. Ten isolates from 10 patients shared the same 24-loci MIRU-VNTR pattern. Within this cluster, WGS defined two subgroups of four patients each. The first comprised an MDR-TB patient from Romania who had sought medical care in Austria and two patients from Austria. The second comprised patients, two of them epidemiologically linked, who lived in three different countries but had the same city of provenance in Romania. Our findings strongly suggested that the two cases in Austrian citizens resulted from a newly introduced MDR-TB strain, followed by domestic transmission. For the other cases, transmission probably occurred in the same city of provenance. To prevent further MDR-TB transmission, we need to ensure universal access to early and adequate therapy and collaborate closely in tuberculosis care beyond administrative borders.

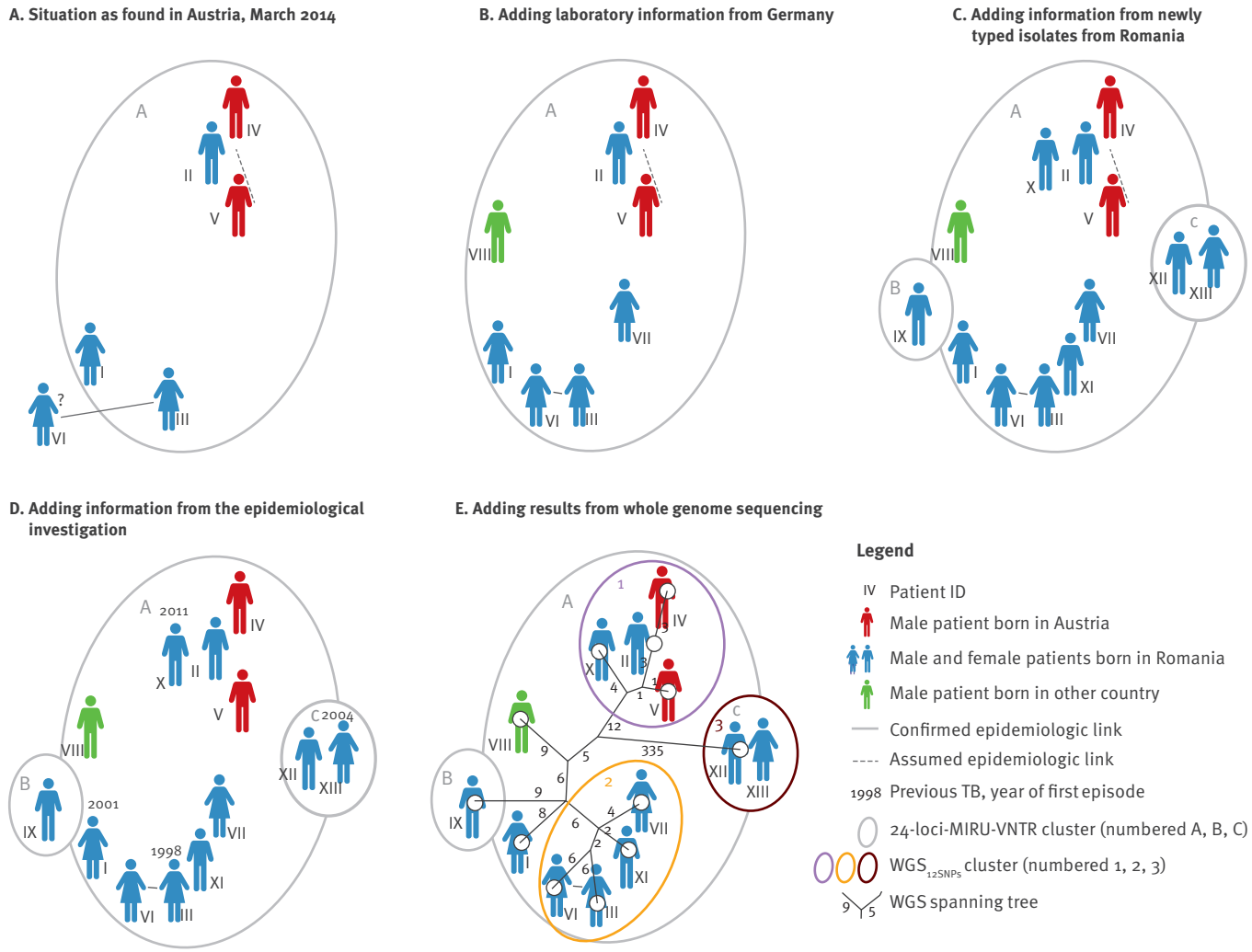
Background

Tuberculosis (TB) and its multi- and extensively drug-resistant forms (M/XDR-TB) are a major global public health concern. The World Health Organization (WHO) estimates that 9.6 million people worldwide fell ill with TB in 2014, of those ca 480,000 cases with MDR-TB [1]. Where second-line drug susceptibility testing (DST) is available, (pre)XDR-TB is frequently detected [2,3]. These patients have a high risk of death [3].

To control this infectious disease, it is key to understand and interrupt the spread of TB and M/XDR-TB. TB transmission can be traced by classic and by molecular epidemiological methods. Classic methods include contact and source case investigations based on patient interviews. Molecular methods examine the genetic relationship between the isolates of the *Mycobacterium tuberculosis* complex. Common genotyping methods include spacer oligonucleotide typing (spoligotyping) and 24-loci mycobacterial interspersed repetitive units variable number of tandem repeats (24-loci MIRU-VNTR) analysis, both targeting specific small parts of the genome. Whole genome sequencing (WGS) queries the entire mycobacterial genomic

Figure 1

Cluster of multidrug-resistant tuberculosis in Austria, Romania and Germany, 2010 to 2014 (n = 13)



ID: unique patient identifier; TB: tuberculosis; WGS: whole genome sequencing.

The panels present the findings of our cluster investigation in chronological order.

material. It has higher discriminatory power and may indicate the directionality and sequence of transmission events [4-7]. Moreover, WGS permits identification of genes and mutations that mediate drug resistance [8-11]. WGS has been employed to analyse and review TB outbreaks in different settings [5,12,13]. Recently, it has become increasingly affordable and routinely applicable [8,14,15].

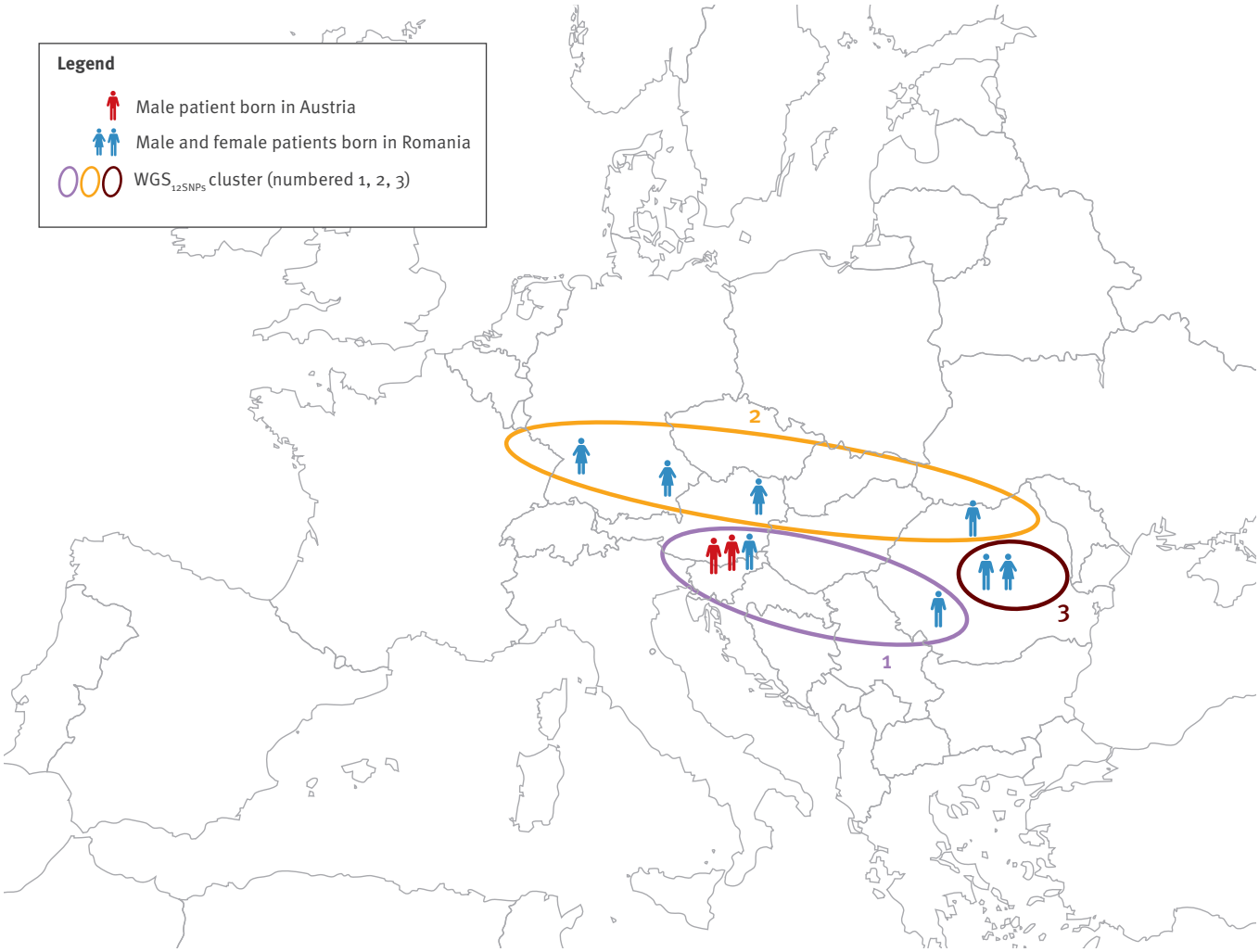
Austria, Romania and Germany are European Union (EU) Member States with, respectively, TB notification rates of 6.8, 79.7 and 5.6 cases per 100,000 population, rather similar proportions of MDR-TB among new laboratory-confirmed TB cases with DST results of 4.8%, 6.4% and 3.1%, yet very different absolute numbers of detected MDR-TB cases with 20, 517 and 87 cases in 2014 [2].

None of the three countries has an area-wide integrated molecular surveillance for TB as established in the Netherlands [16], the United Kingdom (UK) [17] or the United States (US) [18]. However, the National Reference Laboratories (NRLs) for Mycobacteria in Austria and Germany systematically type M/XDR-TB isolates. Germany submits the results to the genotyping database of the European Centre for Disease Prevention and Control (ECDC) [19].

In March 2014, the Austrian NRL at the Austrian Agency for Health and Food Safety (AGES) detected a molecular cluster of five MDR-TB cases. The question arose

Figure 2

Geographical dimension of the three WGS_{12SNPs} cluster of multidrug-resistant tuberculosis, Austria, Romania and Germany, 2010 to 2014 (n = 10)



WGS: whole genome sequencing.

The persons are depicted in their country of residence at the beginning of the investigation, the colour refers to their country of birth (blue – Romania, red – Austria). The map background is used from RegioGraph (version 2015, GfK GeoMarketing GmbH, Bruchsal, Germany).

whether MDR-TB transmission had occurred within Austria, which had never been observed before. Links to Romania and Germany prompted the three countries to investigate the MDR-TB cluster jointly within given legal contexts and with unchanged in-country responsibilities, with the aim of tracing the MDR-TB transmission.

Methods

Collaboration

The investigation team consisted of the national TB contact points for WHO and ECDC or representatives acting on their behalf, the NRLs and the responsible local public health authorities in Austria, Romania and Germany. Collaboration was maintained by monthly telephone conferences from April to October 2014.

Case inclusion

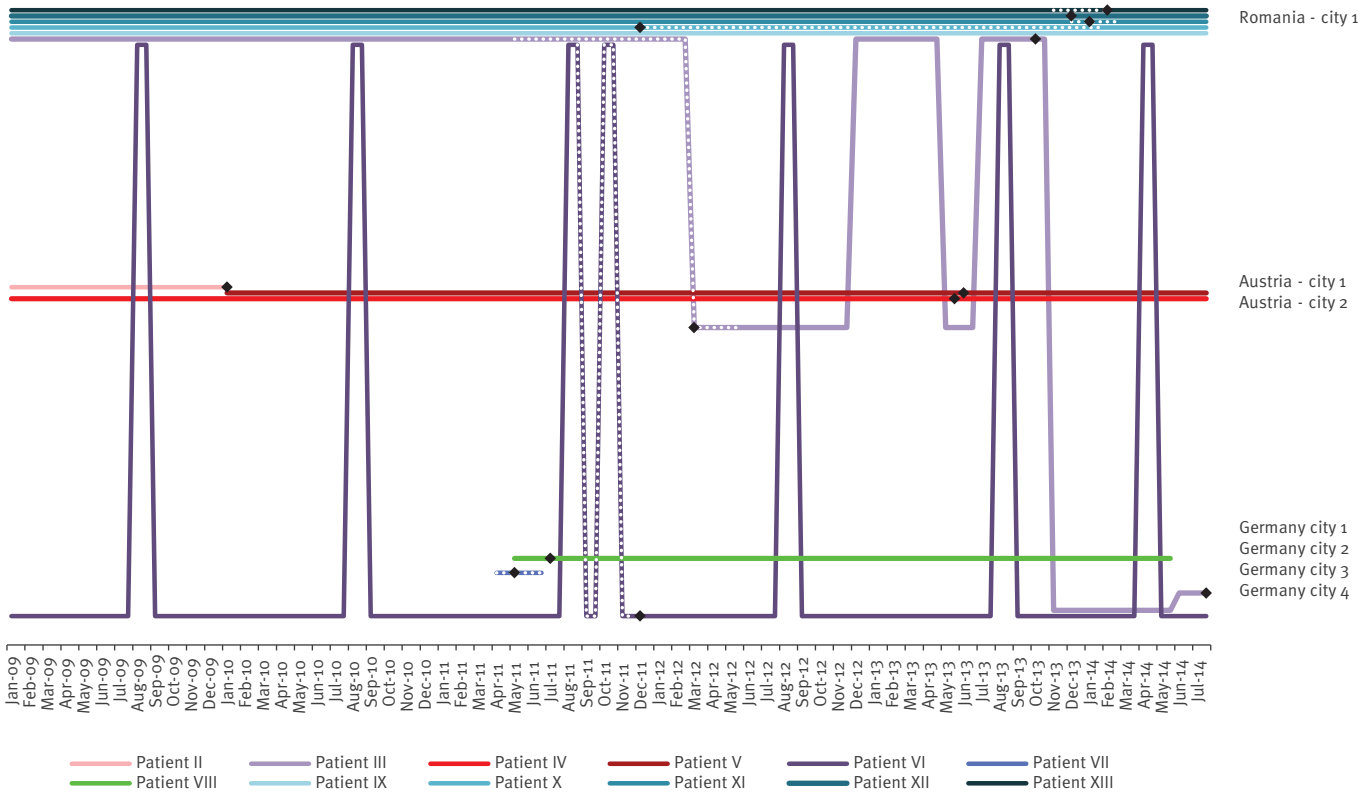
Cases were included without restriction in time when the isolate, collected in any of the three countries and recorded in any typing databases by the NRLs, shared the same spoligotype and 24-loci MIRU-VNTR pattern as in the initial cluster detected in Austria in March 2014. Five MDR-TB cases from one administrative district in Romania were included based on epidemiological information in the absence of molecular typing data. No epidemiological links pointing to other districts in Romania were identified.

Drug susceptibility testing

Isolates were gained by culturing specimens in liquid (BACTEC MGIT 960, Becton Dickinson Diagnostic Systems, Sparks, US) and on solid Löwenstein-Jensen (LJ) media.

Figure 3

Patient mobility per city/country and month, cluster of multidrug-resistant tuberculosis, Austria, Romania and Germany, 2010 to 2014 (n = 10)



Dotted lines: assumed period of infectiousness; black diamonds: dates of notification for patients II–XIII.

In Austria and Germany, DST was done using the Mycobacteria Growth Indicator Tube (MGIT) system with BACTEC MGIT 960 growth supplement for DST in the MGIT 960 instrument (Becton Dickinson Diagnostic Systems, Sparks, MD). For cycloserine, the proportion method employed was modified according to Canetti [20]. In Romania, specimens were cultured on LJ medium. The proportion method was used to test isoniazid, rifampicin, ethambutol, streptomycin, kanamycin, amikacin, capreomycin, ofloxacin and ethionamide.

Genotyping

On extracted genomic DNA from the mycobacterial strains, spoligotyping and 24-loci MIRU-VNTR was done following standard protocols [21,22].

Whole genome sequencing and sequence data analysis

Libraries for sequencing were prepared from extracted genomic DNA with the Nextera XT library preparation kit and sequenced on the Illumina MiSeq next generation sequencing (NGS) platform in a 2 × 301 bp paired-end run (Illumina, San Diego, US).

WGS data of sequenced isolates were submitted to the EMBL-EBI ENA sequence read archive (accession number: ERP013444). Resulting reads were mapped to the *M. tuberculosis* H37Rv genome (GenBank accession number: NC_000962.3) with the SARUMAN exact alignment tool [23]. The mean genomic coverage was at least 45-fold, with more than 99% of the reference genome covered for all isolates. Variants were called from mapped reads by in-house Perl scripts, asking for a minimum coverage of 10 reads and a minimum allele frequency of 75% as detection thresholds. Combining detected single nucleotide polymorphisms (SNPs) of all isolates, positions that matched the threshold levels in at least 95% of all isolates were considered as valid and used for a concatenated sequence alignment excluding variants in resistance-associated or repetitive regions of the genome.

We employed the BioNumerics software (Applied Maths NV, Belgium) to build a neighbour-joining tree from the 708 concatenated SNP positions. Putative transmission groups were predicted with a cut-off of 12 distinct SNP positions (referred to as WGS12SNPs clusters) [24].

All variants located on genes that were previously associated with mutations conferring drug resistance were extracted from the full set of detected variants, and the derived subset of variants was manually annotated with published data [8,25-30].

Table 1

Cluster of multidrug-resistant tuberculosis in Austria, Romania and Germany, demographic and clinical characteristics of the investigated patients, 2010 to 2014 (n = 13)

Patient ID	Country of residence at the beginning of the investigation	Sex	Age group (years)	Country of birth	Month and year of diagnosis of current episode	Previous TB (year of diagnosis)	Site of disease
I	Austria	Female	30–39	Romania	03/2010	Yes (2001)	Pulmonary
II	Austria	Male	50–59	Romania	01/2011	No	Pulmonary
III	Austria	Female	30–39	Romania	03/2012	Yes (1998, 2003)	Pulmonary
IV	Austria	Male	40–49	Austria	06/2013	No	Pulmonary
V	Austria	Male	50–59	Austria	06/2013	No	Pulmonary
VI	Germany	Female	30–39	Romania	12/2011	No	Pulmonary
VII	Germany	Female	30–39	Romania	05/2011	No	Pulmonary
VIII	Germany	Male	30–39	Nigeria	07/2011	No	Extrapulmonary
IX	Romania	Male	40–49	Romania	01/2004	No	Pulmonary
X	Romania	Male	50–59	Romania	12/2011	Yes (2011)	Pulmonary
XI	Romania	Male	30–39	Romania	01/2014	No	Pulmonary
XII	Romania	Male	20–29	Romania	12/2013	No	Pulmonary
XIII	Romania	Female	60–69	Romania	01/2014	Yes (2004)	Pulmonary

ID: unique patient identifier; TB: tuberculosis.

WGS was performed at the NRL at the Research Center Borstel in Germany.

Epidemiological investigation

We used a self-designed form in all three countries to systematically compile patient information, direct epidemiological links (exposure of at least 8 hours or at least 40 hours to, respectively, a sputum smear- or culture-positive but sputum smear-negative source case) [31,32], and spatio-temporal information in terms of the patients' city and country of stay per month from January 2009 to July 2014. The data sources were records of the responsible authorities and re-interviews of the patients III, IV, V, VI. The others could not be contacted, had reportedly moved away or did not follow the invitation by the authorities.

We compiled these data into a line list using Microsoft Excel and analysed them descriptively.

Legal framework and data protection

Patient data had been collected as part of routine case notification and contact investigation according to the Tuberculosis Law (Tuberkulosegesetz) in Austria, Law Number 95/2006 on Health Reform in Romania, and the Protection against Infection Act (Infektionsschutzgesetz; IfSG) in Germany.

The collection of direct person-to-person links required international sharing of all patients' names. The Decision Number 1082/2013/EU of the European Parliament and of the Council [33] stipulates that proper authorities may communicate personal data for contact tracing purposes through selective exchanges in the European Early Warning and Response System (EWRS). In Germany, authorisation to collect personal data under the terms of section 16(1) IfSG lies with local public health authorities while the national authority's administrative involvement in handling personal data (section 25(1), IfSG) is restricted to international travellers (section 12(7) International Health Regulation Implementation Act).

Accordingly, in Germany, one of the responsible local authorities compiled the patients' names, assigned random unique identifiers (IDs) and redistributed the key to authorities in charge of the patients in the three countries. The form was completed using the ID, the key destroyed and anonymous data shared with the German national TB contact point at the Robert Koch Institute (RKI) for analysis.

The investigation protocol had been positively evaluated by data protection and legal departments of the RKI.

Results

Austria

In March 2014, *M. tuberculosis* (non-Beijing genotype) isolates from five MDR-TB patients in Austria were found to share the same spoligotype and 24-loci MIRU-VNTR pattern 'A'. Three patients (I–III) diagnosed from 2010 to 2012, originated from the same city in Romania

Table 2

Cluster of multidrug-resistant tuberculosis in Austria, Romania and Germany, bacteriological confirmation and spoligotype and 24-loci MIRU-VNTR pattern of the isolates from the investigated patients, 2010 to 2014 (n = 13)

ID	Bacterial confirmation	Archive run accession	Spoligotype	24-loci MIRU-VNTR																							
				154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
I	ND	ERR1163047	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
II	Culture-pos, NAAT-pos, ssm-pos	ERR1163048	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
III	Culture-pos, NAAT-pos, ssm-pos	ERR1163049	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
IV	Culture-pos, NAAT-pos	ERR1163050	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
V	Culture-pos, NAAT-pos	ERR1163051	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
VI	Culture-pos, NAAT-pos, ssm-pos	ERR1163052	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
VII	Culture-pos, NAAT-pos, ssm-pos	ERR1163053	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
VIII	Microscopy of EP specimen-pos	ERR1163054	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
IX	Culture-pos	ERR1163055	1111111100111111111111111111111100001111111	2	2	3	2	3	3	3	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
X	Culture-pos, ssm-pos	ERR1163056	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
XI	Culture-pos, ssm-pos	ERR1163057	1111111100111111111111111111111100001111111	2	2	3	2	2	3	4	2	2	4	2	4	2	2	5	1	5	3	3	2	3	5	2	2
XII	Culture-pos, ssm-pos	ERR1163058	1111111111111111111111111111111100001111111	2	3	4	2	4	3	1	2	2	3	2	3	2	3	6	1	5	3	3	2	2	5	2	2
XIII	Culture-pos, ssm-pos	ERR1163059	1111111111111111111111111111111100001111111	2	3	4	2	4	3	1	2	2	3	2	3	2	3	6	1	5	3	3	2	2	5	2	2

ID: unique patient identifier; MIRU-VNTR ND: 24-loci mycobacterial interspersed repetitive units variable number of tandem repeats; no data; ssm: sputum smear microscopy; shaded cells: distinct molecular typing patterns.

Table 3

Phenotypic drug susceptibility testing results, cluster investigation of multidrug-resistant tuberculosis, Austria, Romania and Germany, 2010 to 2014 (n = 13)

ID	H	R	Z	E	Eth	Pt	PAS	Rb	Cs	S	Amk	Kan	Cap	Ofl	Mox	Lev
I	Res	Res	Res	Sus	ND	Res	Sus	Res	Sus	Sus	Sus	ND	Sus	Res	Res	ND
II	Res	Res	Res	Res	ND	Res	Sus	Res	Sus	Res	Sus	ND	Sus	Sus	ND	ND
III	Res	Res	Res	Res	Res	Res	Sus	Res	Res	Res	Sus	ND	Sus	Res	Res	Res
IV	Res	Res	Res	Sus	ND	Res	Sus	Res	Sus	Res	Sus	ND	Sus	Sus	Sus	ND
V	Res	Res	Res	Sus	Res	Res	Sus	Res	Sus	Res	Sus	ND	Sus	Sus	Sus	ND
VI	Res	Res	Res	Res	Res	Res	Sus	Res	Sus	Res	Res	ND	Res	Sus	ND	ND
VII	Res	Res	Res	Res	Res	Res	Sus	Res	Sus	Res	Sus	ND	Sus	Sus	ND	ND
VIII	Res	Sus	Res	Sus	Res	Res	ND	ND	ND	Res	ND	ND	Sus	Sus	ND	ND
IX	Res	Res	ND	ND	ND	ND	ND	ND	ND	Res	ND	ND	ND	ND	ND	ND
X	Res	Res	ND	Res	Sus	ND	ND	ND	ND	Res	Res	Res	Res	Sus	ND	ND
XI	Res	Res	ND	Res	Sus	ND	ND	ND	ND	Res	Sus	Sus	Sus	Sus	ND	ND
XII	Res	Res	ND	Sus	Sus	ND	ND	ND	ND	Res	Sus	Sus	Sus	Sus	ND	ND
XIII	Res	Res	ND	Sus	Sus	ND	ND	ND	ND	Res	Sus	Sus	Sus	Sus	ND	ND

Amk: amikacin; Cap: capreomycin; E: ethambutol; Eth: ethionamide; ID: unique patient identifier; Kan: kanamycin; Lev: levofloxacin; Mox: moxifloxacin; ND: no data; H: isoniazid; Ofl: ofloxacin; PAS: para-aminosalicylic acid; Pt: protionamide; R: rifampicin; Rb: rifabutin; Res: resistant; Cs: cycloserine; S: streptomycin; Sus: susceptible; Z: pyrazinamide.

(Figure 1A, Table 1). They had moved to two different cities in Austria, seeking medical care for their complicated MDR-TB. Two patients (IV and V) had been diagnosed with new MDR-TB in June 2013. They were residents of the same Austrian city to which patients I and II had moved and had no history of migration or international travel.

Contact tracing did not confirm any epidemiological link between patients I to IV. However, a link between patients IV and V was assumed; they had both frequented the vicinity of the railway station and had problematic alcohol use.

Patient III reported having a sister diagnosed with MDR-TB living in Germany. This prompted the AGES to share the spoligotype and MIRU-VNTR pattern (Table 2) with Germany.

Germany

In early April, the NRL in Germany identified three isolates with MIRU-VNTR pattern 'A'. One isolate referred to the sister of patient III (patient VI), the second to another woman born in Romania (patient VII), and the third to a man born in West Africa with extrapulmonary non-MDR-TB (patient VIII; Figure 1B).
As five patients (I–III, VI and VII) reportedly originated from the same city in Romania, the Romanian national TB contact point was informed. In mid-April 2014, all three countries held their first telephone conference and agreed upon a joint investigation.

Romania

In Romania, in the absence of systematic MIRU-VNTR typing of MDR-TB strains, isolates from all five MDR-TB patients (IX–XIII) ever reported in the corresponding district were typed at the Austrian NRL. The isolate from patient IX had a unique MIRU-VNTR pattern 'B', the isolates from patients X and XI shared pattern 'A', and the ones from patients XII and XIII shared a distinct pattern 'C' and a different spoligotype (Figure 1C, Table 2).

Epidemiological investigation

Investigation forms were completed for patients II–XIII by seven public health authorities by September 2014. For patient I, only a laboratory report was available.

All patients were adults, five women and eight men; six had experienced migration (I–III and VI–VIII). Nine had new TB, four (I, III, X and XIII) had had previous TB, the first TB diagnosis dating back to year 1998 (III). All but patient VIII had pulmonary TB (Table 1).

The two sisters (III and VI) were confirmed to have a direct epidemiological link between them. Direct links were ruled out for persons II, III, IV, V, VII, IX and XII, and unknown for VI, VIII, X, XI and XIII. The assumed link between cases IV and V was negated when re-interviewing the persons (Figure 1D).

The two sisters (III and VI) had crossed borders presumably while being infectious (Figures 2 and 3). Other patients with migration background had moved before 2009 (II) or at an unknown date (I, VII, VIII). The mobility pattern did not preclude TB transmission events from patient II to patients IV and V in Austria, nor from patient III to patients X–XIII in Romania. The sisters III and VI had a space–time correlation in Romania in August 2011, however, only about one month before the beginning of the assumed infectious period of patient VI.

Table 4

Genotypic drug susceptibility testing results, cluster of multidrug-resistant tuberculosis, Austria, Romania and Germany, 2010 to 2014 (n = 13)

ID	H	R	R	Z	E	E	E	Eth/Pt	Eth/Pt	Ami	S	SM	PAS	FQ
	Rv1908c	Rv0667	Rv0667	Rv2043c	Rv3795	Rv3795	Rv3795	Rv3854c	Rv3854c	MTB000019	Rv3919c	Rv0682	Rv2764c	Rv0007
	katG [26]	rpoB [8,25]	rpoB [8,25]	pncA [27]	embB [8,28]	embB [8,28]	embB [8,28]	ethA [29]	ethA [29]	Rrs [8]	gidB [25]	rpsL [25]	thyA [30]	gyrA [8]
I	S315T [a]	WT	S450L [a]	A146V [a]	WT	WT	D1024N [b]	WT	Ins 802 ag [b]	WT	Q125_ [b]	WT	R222C [b]	A288D [b]
II	S315T [a]	WT	S450L [a]	A146V [a]	M306I [a]	WT	WT	Ins 1391 a [b]	WT	WT	Q125_ [b]	WT	WT	WT
III	S315T [a]	WT	S450L [a]	A146V [a]	WT	G406S [a]	D1024N [b]	WT	WT	WT	Q125_ [b]	WT	WT	WT
IV	S315T [a]	WT	S450L [a]	A146V [a]	M306I [a]	WT	WT	Ins 1391 a [b]	WT	WT	Q125_ [b]	WT	WT	WT
V	S315T [a]	WT	S450L [a]	A146V [a]	M306I [a]	WT	WT	Ins 1391 a [b]	WT	WT	Q125_ [b]	WT	WT	WT
VI	S315T [a]	WT	S450L [a]	A146V [a]	WT	G406S [a]	D1024N [b]	WT	WT	1401 A->G [a]	Q125_ [b]	WT	WT	WT
VII	S315T [a]	WT	S450L [a]	A146V [a]	WT	G406S [a]	D1024N [b]	WT	WT	WT	Q125_ [b]	WT	WT	WT
VIII	S315T [a]	WT	WT	A146V [a]	WT	WT	WT	WT	WT	WT	Q125_ [b]	WT	WT	WT
IX	S315T [a]	WT	S450L [a]	A146V [a]	M306I [a]	WT	D1024N [b]	WT	WT	WT	Q125_ [b]	WT	WT	WT
X	S315T [a]	WT	S450L [a]	A146V [a]	M306I [a]	WT	WT	Ins 1391 a [b]	WT	1401 A->G [a]	Q125_ [b]	WT	WT	WT
XI	S315T [a]	WT	S450L [a]	A146V [a]	WT	G406S [a]	D1024N [b]	WT	WT	WT	Q125_ [b]	WT	WT	WT
XII	S315T [a]	T400A [a]	S450L [a]	WT	WT	WT	WT	WT	WT	WT	WT	K43R [1]	WT	WT
XIII	S315T [a]	T400A [a]	S450L [a]	WT	WT	WT	WT	WT	WT	WT	WT	K43R [1]	WT	WT

Ami: aminoglycoside; E: ethambutol; Eth: ethionamide; FQ: fluroquinolones; ID: unique patient identifier; H: isoniazid; PAS: para-aminosalicylic acid; Pt: protionamide; R: rifampicin; S: streptomycin; Z: pyrazinamide; WT: wild type.

[a] resistance mediating mutation.

[b] resistance associated variant.

Whole genome sequencing

WGS was completed by August 2014. WGS12SNPs divided cluster 'A' into two subgroups (one comprising patients II, IV, V and X, the other patients III, VI, VII and XI), and two separate cases (I and VIII). The third WGS12SNPs cluster was congruent with genotyping pattern 'C' (Figure 1E). The isolates from patients II and IV, as well as II and V were distinct by 3 and 4 SNPs, respectively. Isolates from patients XII and XIII were genetically identical (0 SNPs). The isolates from the epidemiologically linked sisters were distinct by 12 SNPs.

The first two WGS12SNPs clusters spanned across borders, while the third was domestic (Figure 2).

The detected mutations mediating resistance to first-line drugs correlated with phenotypic DST results. The isoniazid resistance-conferring mutation S315T in *katG* fully matched phenotypic isoniazid resistance; the same was observed for S450L or T400A in *rpoB* and rifampicin/rifabutin resistance and A146V in *pncA* and pyrazinamid resistance (information missing for patients IX–XIII). Two phenotypical ethambutol-susceptible isolates harboured the known resistance-mediating mutation M306I in *embB*; the resistant isolates showed either the mutation M306I or a combination of two mutations G406S and D1024N.

In addition, we detected resistance-mediating mutations for streptomycin (*rpsL* K43R) and kanamycin/amikacin (*rrs* 1401 A->G). One of two quinolone-resistant isolates shows a mutation in *gyrA* (A288D), a quinolone resistance-associated gene. Among the five isolates phenotypically resistant to ethionamide, one harboured a frameshift insertion in *ethA*. Four out of eight phenotypical protionamide-resistant isolates, showed frameshift insertions in *ethA* (Tables 3 and 4). Patients in one WGS cluster shared a cluster-specific set of resistance-mediating mutations, patient X in cluster 1 and patient VI in cluster 2 had acquired an additional aminoglycoside resistance (*rrs* 1401 A->G).

Discussion

We investigated a molecular cluster of MDR-TB in Austria, Romania and Germany. WGS combined with epidemiological information showed that isolates from patient II, seeking medical care in Austria, differed from the subsequently diagnosed Austrian patients IV and V by only 3 and 4 SNPs, respectively. This suggested that two MDR-TB transmission events had occurred in Austria. Isolates from patients III, VI, VII and XI, who

lived in three different countries but had the same city of provenance, differed by 6–12 SNPs from each other. Here, transmission is likely to have occurred before the patients moved abroad.

Close genetic similarity of isolates from different patients is highly unlikely to occur by chance. From well-described TB outbreaks we know that isolates gained within three years from patients with a direct epidemiological link usually differ by 5 or fewer SNPs [34,35]. In an outbreak of nine drug-susceptible TB cases in San Francisco, US, the isolates differed by 0–2 SNPs per any transmission event that had resulted in a secondary case [6]. In a similar investigation in Germany, differences of 0–3 SNPs were found (n = 31) [7]. From a retrospective study of TB outbreaks, Walker and colleagues derived that epidemiological linkage is expected to be consistent with sequenced isolates differing in up to 5 SNPs; the absence of an epidemiological link is consistent with more than 12 SNPs, while pairs of 6–12 SNPs were considered to be indeterminate [24].

In our investigation, isolates from the two epidemiologically linked sisters differed by 12 SNPs. This strongly suggests one or more missing links in the transmission chain, namely a common source case for both sisters with possibly additional intermediate cases. Missing links may be the result of undetected TB cases, the restriction of our investigation to only one district in Romania, unavailable genotyping results, or from selection based on identical MIRU-VNTR patterns when a mutation affected a VNTR locus even though isolates differed only by few SNPs [6].

We investigated a single scenario and may not draw conclusions about the extent of cross-border transmission of MDR-TB in the EU. The ECDC MDR-TB molecular surveillance project investigated 2,092 MIRU-VNTR patterns of isolates from 24 contributing EU Member States from 2003 to 2011 [19]. In total, 941 cases in 79 European multiple-country clusters were detected and 1,086 cases were allocated to national clusters. That study was solely based on genotyping data. In the UK, 24-loci MIRU-VNTR typing and epidemiological surveillance data were linked and jointly interpreted, and 8.5% of the MDR-TB cases were attributed to recent domestic transmission [36]. Similar nationwide evaluations are missing for our countries.

A high proportion of imported MDR-TB in low-incidence countries does not necessarily entail ongoing MDR-TB transmission when early case detection, infection control and adequate treatment succeed [19]. A systematic review for the EU/European Economic Area indicates that TB in the foreign-born population has no significant influence on TB in the native population [37].

Beyond higher resolution in TB outbreak investigation, WGS provided us in addition with information on drug resistance of the bacteria. We could identify mutations mediating pyrazinamide resistance in previously not tested isolates and mutations mediating ethambutol resistance in two samples with susceptible phenotypic DST results. However, our data on mutations mediating drug resistance to ethionamide, protionamide and the quinolones showed discrepancies between phenotypical and genotypical DST. A comprehensive database of characterised mutations is needed to extend the usability of WGS in predicting drug resistance, e.g. in order to provide rapid and effective treatment in outbreaks of drug-resistant TB. The concordance of resistance-mediating mutations in each WGS cluster confirmed transmission of MDR strains rather than treatment failure and new acquisition of MDR in each patient [10].

Our investigation was subject to limitations. The collection of direct epidemiological links yielded little information. It was difficult to differentiate whether a specific contact was absent (e.g. due to missing links), unknown (exposures in public space, recall bias) or non-reported (reluctance to name persons). Spatio-temporal data did not cover all patients' presumed infectious periods and travel history. Their low resolution (per city/country and month) allowed us to judge whether a contact was possible at all, but not to explore new exposure settings or events. More detailed investigations are difficult given long infectious periods and serial intervals in TB transmission chains.

The clinical characteristics 'cavitary disease' and 'HIV status' were not assessed as they are not notifiable everywhere, although relevant to assessing infectiousness and transmission risks. For patient I, it remained unclear which local public health authority was in charge. This highlights the challenge in transferring patient reports when patients are highly mobile.

We learned that the choice of methods and the order in which we use them can play a significant role. If WGS had been used initially and had led to the detection of the close genetic relationship between isolates from patients II, IV and V in Austria, a cross-border investigation might not have been initiated.

The cross-border investigation of a single genotyping cluster of TB can become complex and labour-intensive with uncertain public health benefits. In our case, there were no implications for contact tracing, which had already been completed. However, such investigation as ours may detect previously undetected individuals with TB. While investigations might get more efficient with increasing routine, each cluster brings together a new group of competent authorities that need to establish collaboration. Systematic and timely integration of genotyping and sequencing data into TB surveillance improves the understanding of transmission in a given country and internationally [38].

Topical issues remain: Should WGS replace 24-loci MIRU-VNTR as a standard? By when? How should we collect, analyse and interpret sequencing data within

routine TB surveillance [39] and evaluate utility? How should we prioritise cluster investigations? Are there reliable predictors of cluster growth [40-43]? Will epidemiological links remain an essential component in TB outbreak definitions, i.e. may we use the term 'outbreak' solely based on WGS results when epidemiological links cannot confirmed? How can we collaborate most efficiently across borders when contact networks are complex and personal data are to be shared by everyone with everyone else? Could a secure interactive online platform complement communication channels such as EWRS?

Conclusion

Our joint cross-border investigation clarified a transboundary MDR-TB transmission scenario. The applied methods complemented each other: genotyping results prompted our investigation, classic epidemiological data anchored the cluster in time and space, and WGS allowed a high resolution of transmission and new information on drug-resistance.

To prevent further MDR-TB transmission within and between countries, we need to ensure universal access to early and adequate therapy in order to reduce incentives to seek medical care abroad and to ensure infection control and seamless collaboration in TB care beyond administrative borders [44].

Acknowledgements

We would like to thank the following persons from the responsible authorities and TB patient services in Romania, Austria and Germany for their support of our investigation: Dr Georgeta Marga, Dr Lavinia Danciu, Dr Ioan Campean, Ursula Tuch, Dr Ulrike Meixner-Gruber, Dr Marianne Wassermann-Neuhold, Dr Johannes Donhauser, Andrea Hingerl, Ludger Staufenbiel, Dr Wolfgang Hautmann, Dr Johannes Dreesman, and Dr Dr Günther Pfaff. We further thank Dr Alyssa Finlay and the CDC Division of TB Elimination for helpful comments on the analysis.

We acknowledge the European Union PathoNgenTrace (FP7-278864-2) project and the German Center for Infection Research (DZIF) for their financial support for WGS.

Declaration of interests

The authors declare that they have no competing interests.

Role of the funding source: The WGS has been financially supported by the European Union PathoNgenTrace (FP7–278864–2) project and the German Center for Infection Research (DZIF). For other parts of the investigation there was no external funding source. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

Designed and led the investigation via phone conferences: LF, FA, OP, SN, ER, SRG, AI, MM, BS, DC, DH, BH, WH; collected epidemiological data: MM, OP, BS, AI, LF; analysed epidemiological data: LF; interpreted epidemiological findings: LF, WH, BH, FA, OP; performed laboratory analyses and genotyping: AI, DH, ER, SRG, SN; performed WGS and sequence data analysis: TK, PB, SN; wrote the manuscript: LF; helped to draft the manuscript: TK, PB. All authors critically revised the manuscript and approved the final version.

References

1. World Health Organization (WHO). Global tuberculosis report 2015. Geneva: WHO; 2015. WHO/HTM/TB/2015.22. Available from: http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf
2. European Centre for Disease Prevention and Control (ECDC)/WHO Regional Office for Europe. Tuberculosis surveillance and monitoring in Europe 2016. Stockholm: ECDC; 2016. Available from: http://ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?List=4f55ad51-4aed-4d32-b960-af70113dbb90&ID=1452
3. Balabanova Y, Ignatyeva O, Fiebig L, Riekstina V, Danilovits M, Jaama K, et al. Survival of patients with multidrug-resistant tuberculosis in Eastern Europe: what makes a difference? Thorax. 2016;71(9):854-61.
4. Niemann S, Köser CU, Gagneux S, Plinke C, Homolka S, Bignell H, et al. Genomic diversity among drug sensitive and multidrug resistant isolates of Mycobacterium tuberculosis with identical DNA fingerprints. PLoS One. 2009;4(10):e7407. DOI: 10.1371/journal.pone.0007407
5. Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, et al. Whole genome sequencing versus traditional genotyping for investigation of a Mycobacterium tuberculosis outbreak: a longitudinal molecular epidemiological study. PLoS Med. 2013;10(2):e1001387. DOI: 10.1371/journal.pmed.1001387
6. Kato-Maeda M, Ho C, Passarelli B, Banaei N, Grinsdale J, Flores L, et al. Use of whole genome sequencing to determine the microevolution of Mycobacterium tuberculosis during an outbreak. PLoS One. 2013;8(3):e58235. DOI: 10.1371/journal.pone.0058235
7. Schürch AC, Kremer K, Daviena O, Kiers A, Boeree MJ, Siezen RJ, et al. High-resolution typing by integration of genome sequencing data in a large tuberculosis cluster. J Clin Microbiol. 2010;48(9):3403-6. DOI: 10.1128/JCM.00370-10
8. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2011;55(5):2032-41. DOI: 10.1128/AAC.01550-10
9. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, et al. Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. Nat Genet. 2011;44(1):106-10. DOI: 10.1038/ng.1038
10. Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. , Modernizing Medical Microbiology (MMM) Informatics Group. Whole-genome sequencing for prediction of Mycobacterium tuberculosis drug susceptibility and resistance: a retrospective cohort study.Lancet Infect Dis. 2015;15(10):1193-202. DOI: 10.1016/S1473-3099(15)00062-6
11. Outhred AC, Jelfs P, Suliman B, Hill-Cawthorne GA, Crawford AB, Marais BJ, et al. Added value of whole-genome sequencing for management of highly drug-resistant TB. J Antimicrob Chemother. 2015;70(4):1198-202.
12. Gardy JL, Johnston JC, Ho Sui SJ, Cook VJ, Shah L, Brodkin E, et al. Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. N Engl J Med. 2011;364(8):730-9. DOI: 10.1056/NEJMoa1003176
13. Stucki D, Ballif M, Bodmer T, Coscolla M, Maurer AM, Droz S, et al. Tracking a tuberculosis outbreak over 21 years: strain-specific single-nucleotide polymorphism typing combined with targeted whole-genome sequencing. J Infect Dis. 2015;211(8):1306-16.
14. Köser CU, Ellington MJ, Cartwright EJP, Gillespie SH, Brown NM, Farrington M, et al. Routine use of microbial whole genome sequencing in diagnostic and public health microbiology. PLoS Pathog. 2012;8(8):e1002824. DOI: 10.1371/journal.ppat.1002824
15. Walker TM, Monk P, Smith EG, Peto TE. Contact investigations for outbreaks of Mycobacterium tuberculosis: advances through whole genome sequencing.Clin Microbiol Infect. 2013;19(9):796-802. DOI: 10.1111/1469-0691.12183
16. Lambregts-van Weezenbeek CS, Sebek MM, van Gerven PJ, de Vries G, Verver S, Kalisvaart NA, et al. Tuberculosis

contact investigation and DNA fingerprint surveillance in The Netherlands: 6 years' experience with nation-wide cluster feedback and cluster monitoring. Int J Tuberc Lung Dis. 2003;7(12) Suppl 3;S463-70.

17. Mears J, Abubakar I, Crisp D, Maguire H, Innes JA, Lilley M, et al. Prospective evaluation of a complex public health intervention: lessons from an initial and follow-up cross-sectional survey of the tuberculosis strain typing service in England. BMC Public Health. 2014;14(1):1023. DOI: 10.1186/1471-2458-14-1023
18. Centers for Disease Prevention and Control (CDC). TB genotyping information management system (TB GIMS). Atlanta: CDC. [Accessed: 31 March 2016]. Available from: http://www.cdc.gov/tb/programs/genotyping/tbgims/default.htm
19. De Beer JL, Kodmon C, van der Werf MJ, van Ingen J, van Soolingen D. ECDC MDR-TB molecular surveillance project participants. Molecular surveillance of multi- and extensively drug-resistant tuberculosis transmission in the European Union from 2003 to 2011.Euro Surveill. 2014;19(11):20742. DOI: 10.2807/1560-7917.ES2014.19.11.20742
20. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bull World Health Organ. 1969;41(1):21-43.
21. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993;31(2):406-9.
22. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol. 1997;35(4):907-14.
23. Blom J, Jakobi T, Doppmeier D, Jaenicke S, Kalinowski J, Stoye J, et al. Exact and complete short-read alignment to microbial genomes using Graphics Processing Unit programming. Bioinformatics. 2011;27(10):1351-8. DOI: 10.1093/bioinformatics/btr151
24. Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ, et al. Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: a retrospective observational study. Lancet Infect Dis. 2013;13(2):137-46. DOI: 10.1016/S1473-3099(12)70277-3
25. Feuerriegel S, Oberhauser B, George AG, Dafae F, Richter E, Rüsch-Gerdes S, et al. Sequence analysis for detection of first-line drug resistance in Mycobacterium tuberculosis strains from a high-incidence setting. BMC Microbiol. 2012;12(1):90. DOI: 10.1186/1471-2180-12-90
26. Marttila HJ, Soini H, Huovinen P, Viljanen MK. katG mutations in isoniazid-resistant Mycobacterium tuberculosis isolates recovered from Finnish patients.Antimicrob Agents Chemother. 1996;40(9):2187-9.
27. Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniewski F, Rodionova Y, et al. Mycobacterium tuberculosis pyrazinamide resistance determinants: a multicenter study. MBio. 2014;5(5):e01819-14. DOI: 10.1128/mBio.01819-14
28. Plinke C, Cox HS, Zarkua N, Karimovich HA, Braker K, Diel R, et al. embCAB sequence variation among ethambutol-resistant Mycobacterium tuberculosis isolates without embB306 mutation. J Antimicrob Chemother. 2010;65(7):1359-67. DOI: 10.1093/jac/dkq120
29. DeBarber AE, Mdluli K, Bosman M, Bekker LG, Barry CE. Ethionamide activation and sensitivity in multidrug-resistant Mycobacterium tuberculosis.Proc Natl Acad Sci USA. 2000;97(17):9677-82. DOI: 10.1073/pnas.97.17.9677
30. Rengarajan J, Sassetti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria.Mol Microbiol. 2004;53(1):275-82. DOI: 10.1111/j.1365-2958.2004.04120.x
31. Diel R, Loytved G, Nienhaus A, Castell S, Detjen A, Geerdes-Fenge H, et al. Neue Empfehlungen für die Umgebungsuntersuchungen bei Tuberkulose. [New recommendations for contact tracing in tuberculosis]. Gesundheitswesen. 2011;73(6):369-88. German. http://dx.doi.org/DOI: 10.1055/s-0030-1256574 PMID:21695661
32. Bogyi M, Hagel E, Hirtl T, Klein JP, Rumetshofer R, Wolf K, et al. Österreichische Leitlinie zur Tuberkulose – Umgebungsuntersuchung. [Austrian guidelines for tuberculosis contact tracing]. Vienna: Bundesministerium für Gesundheit (BMG). [Accessed: 31 March 2016]. German. Available from: http://www.bmgf.gv.at/home/Leitlinie_Tuberkulose_Umgebungsuntersuchung
33. Decision No. 1082/2013/EU of the European Parliament and the council of the European Union of 22 October 2013 on serious cross-border threats to health and repealing Decision No 2119/98/EC. Offical Journal of the European Union. Luxembourg: Publications Office of the European Union. 5.11.2013:L 293. Available from: http://ec.europa.eu/health/preparedness_response/docs/decision_serious_crossborder_threats_22102013_en.pdf
34. Perez-Lago L, Comas I, Navarro Y, Gonzalez-Candelas F, Herranz M, Bouza E et al. Whole genome sequencing analysis of intrapatient microevolution in Mycobacterium tuberculosis: potential impact on the inference of tuberculosis transmission. J Infect Dis. 2014;209:98e108.
35. Schürch AC, Kremer K, Kiers A, Daviena O, Boeree MJ, Siezen RJ, et al. The tempo and mode of molecular evolution of Mycobacterium tuberculosis at patient-to-patient scale. Infect Genet Evol. 2010;10(1):108-14. DOI: 10.1016/j.meegid.2009.10.002
36. Anderson LF, Tamne S, Brown T, Watson JP, Mullarkey C, Zenner D, et al. Transmission of multidrug-resistant tuberculosis in the UK: a cross-sectional molecular and epidemiological study of clustering and contact tracing. Lancet Infect Dis. 2014;14(5):406-15. DOI: 10.1016/S1473-3099(14)70022-2
37. Sandgren A, Schepisi MS, Sotgiu G, Huitric E, Migliori GB, Manissero D, et al. Tuberculosis transmission between foreign- and native-born populations in the EU/EEA: a systematic review. Eur Respir J. 2014;43(4):1159-71. DOI: 10.1183/09031936.00117213
38. Coscolla M, Barry PM, Oeltmann JE, Koshinsky H, Shaw T, Cilnis M, et al. Genomic epidemiology of multidrug-resistant Mycobacterium tuberculosis during transcontinental spread. J Infect Dis. 2015;212(2):302-10. DOI: 10.1093/infdis/jiv025
39. Kohl TA, Diel R, Harmsen D, Rothgänger J, Walter KM, Merker M, et al. Whole-genome-based Mycobacterium tuberculosis surveillance: a standardized, portable, and expandable approach. J Clin Microbiol. 2014;52(7):2479-86. DOI: 10.1128/JCM.00567-14
40. Driver CR, Macaraig M, McElroy PD, Clark C, Munsiff SS, Kreiswirth B, et al. Which patients' factors predict the rate of growth of Mycobacterium tuberculosis clusters in an urban community? Am J Epidemiol. 2006;164(1):21-31. DOI: 10.1093/aje/kwj153
41. Kik SV, Verver S, van Soolingen D, de Haas PE, Cobelens FG, Kremer K, et al. Tuberculosis outbreaks predicted by characteristics of first patients in a DNA fingerprint cluster. Am J Respir Crit Care Med. 2008;178(1):96-104. DOI: 10.1164/rccm.200708-1256OC
42. Althomsons SP, Kammerer JS, Shang N, Navin TR. Using routinely reported tuberculosis genotyping and surveillance data to predict tuberculosis outbreaks.PLoS One. 2012;7(11):e48754. DOI: 10.1371/journal.pone.0048754
43. Oeltmann JE, Click ES, Moonan PK. Using tuberculosis patient characteristics to predict future cases with matching genotype results.Public Health Action. 2014;4(1):47-52. DOI: 10.5588/pha.13.0098
44. Dara M, de Colombani P, Petrova-Benedict R, Centis R, Zellweger JP, Sandgren A, et al. Minimum package for cross-border TB control and care in the WHO European region: a Wolfheze consensus statement. Eur Respir J. 2012;40(5):1081-90. DOI: 10.1183/09031936.00053012

License and copyright

Research article

Anti-hepatitis C virus seroprevalence in the working age population in Poland, 2004 to 2014

B Walewska-Zielecka [1,2], U Religioni [1], G Juszczyk [1], ZM Wawrzyniak [3,4], A Czerw [1], P Soszyński [2], A Fronczak [1]
1. Department of Public Health, Medical University of Warsaw, Poland
2. Medicover Sp. z o.o., Poland
3. Department of Prevention of Environmental Hazards and Allergology, Medical University of Warsaw, Poland
4. Faculty of Electronics and Information Technology, Warsaw University of Technology, Poland

Correspondence: Bożena Walewska-Zielecka (bozena.walewska-zielecka@medicover.pl)

Citation style for this article:
Walewska-Zielecka B, Religioni U, Juszczyk G, Wawrzyniak ZM, Czerw A, Soszyński P, Fronczak A. Anti-hepatitis C virus seroprevalence in the working age population in Poland, 2004 to 2014. Euro Surveill. 2017;22(2):pii=30441. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30441

Article submitted on 10 November 2015 / accepted on 10 October 2016 / published on 12 January 2017

Hepatitis C virus (HCV) infection is considered by the World Health Organization (WHO) to be a serious public health concern and one of the major public health priorities. In 2005, it was estimated that there are 185 million anti-HCV positive people in the world, which constitutes 2.8% of the global population. Our study estimates the anti-HCV seroprevalence in the working age population (15–64 years-old), mostly urban and suburban residents, in Poland from 2004 to 2014. The studied group consisted of 61,805 working-age population representatives whose data were obtained from electronic medical records of an outpatient clinic network operating on a countrywide level. Positive anti-HCV test results were obtained in 957 patients, representing 1.5% of the whole population studied throughout the analysed period. The average age of all anti-HCV positive patients was 36.8 years. Analysis of the data suggests that the proportion of anti-HCV positive patients decreased over the study period (mean positive anti-HCV = -0.0017 × year + 3.3715; $R^2 = 0.7558$). In 2004, positive results were noted among 3.2% of patients undergoing HCV antibody tests, but in 2014, the percentage of patients with a positive result stood at 1.1%. The apparent decrease affected men and women similarly. Our study also provides evidence that screening people born before 1965 could be beneficial.

Introduction

Liver cirrhosis, liver failure and hepatocellular carcinoma are possible long-term consequences of untreated hepatitis C virus (HCV) infection [1-5], which the World Health Organization (WHO) considers as a serious public health concern and one of the major public health priorities [6]. HCV is transmitted mostly by percutaneous exposure to blood [7,8], including intravenous drug injection, which is becoming an important route, especially in developed countries [9,10]. Mother-to-child transmission occurs as well; however, it is relatively uncommon, affecting an estimated 4% of children of HCV-infected mothers [11,12].

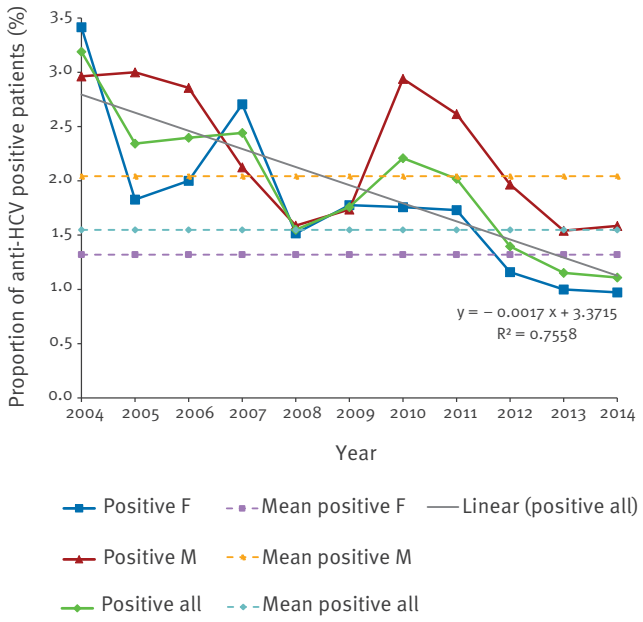
In 2005, ca 185 million people in the world, corresponding to approximately 2.8% of the global population, were estimated to be anti-HCV positive [13]. The prevalence of HCV infection ranges from 1.2% to 3.8% in different parts of the world and is highest in central Asia (3.8%), east Asia (3.7%) and North Africa/Middle East (3.6%) [14,15]. In the United States (US), HCV infection prevalence is at 1.6% (2.1% in men and 1.2 % in women) and higher (75% of all cases) in people born between 1945 and 1965 [16]. For this reason, both the Centers for Disease Control and Prevention (CDC) as well as the American Gastroenterology Association (AGA) recommend screening for all individuals born in this period [17,18].

A study from 2014, based on comprehensive literature search anti-HCV prevalence, found the prevalence in Europe to vary from 0.9% in western Europe, through 1.3% in central Europe to 3.3% in eastern Europe [19]. A report from the European Centre for Disease Control and Prevention estimates that in European Union (EU)/ European Free Trade Association (EFTA) countries over half of persons with HCV infection in 2006 are in the 25–44 year age group and overall men (64.4%) are more affected than women (35.6%) [20]. From the 1990s up to 2007, new infections appeared to decline in western Europe, while they increased in eastern Europe, possibly due to rising numbers of people who inject drugs (PWIDs) in the east and effective needle sharing programmes in the west [15,21,22].

In Poland, newly diagnosed HCV infections are registered and monitored by the National Institute of Public Health since 1997 [23,24]. The data are based on formal notifications from local Sanitary Inspectorates of newly diagnosed HCV infections according to the national case definition [25]. A regulation of the

Figure 1

Proportions of hepatitis C virus antibody positive tests among the study population, stratified by year and sex, Poland, 2004–2014 (n = 61,805 patients)



F: female; M: male; HCV: hepatitis C virus.

Minister of Health of 20 September 2012 made anti-HCV tests mandatory in all pregnant women from that year onwards [26].

The latest estimates for HCV infection incidence in the country are 7.99 newly diagnosed cases per 100,000 inhabitants in 2014 [27] and, preliminarily, 11.14 newly diagnosed cases per 100,000 inhabitants in 2015 [24]. HCV infection incidence is much higher in the cities (10.7/100,000 inhabitants) than in rural areas (4.82/100,000 inhabitants) and in men (8.58/100,000) than in women (7.44/100,000) [27]. As acute HCV infection is usually asymptomatic, 86% of infected people in Poland are estimated to be unaware of their infection [14,28]. Therefore increasing the diagnosis rate of infected persons is important [2], not only to more timely treat hepatitis C, but also to stop further spread of HCV.

Research on HCV prevalence in Poland has so far mainly focused on specific groups (healthcare workers, patients, volunteers, students, blood donors, pregnant women) or on selected areas of Poland [28-36]. There are no epidemiological data for the prevalence of HCV in the general working age population over the whole country, especially based on a large population sample. The purpose of this study is therefore to estimate the anti-HCV seroprevalence in the working age population of Poland, using real-life data obtained from medical records of countrywide outpatient clinics, and accordingly formulate recommendations on age-related HCV infection screening.

Methods

Data source

Data were obtained in February 2015 from electronic medical records of a large countrywide outpatient clinic network operating mainly in big cities (with more than 300,000 inhabitants) representing the capitals of 11 of the 16 regions in Poland (Bialystok, Bydgoszcz, Gdańsk, Katowice, Krakow, Lublin, Łódź, Poznań, Szczecin, Warszawa, Wrocław). The clinics provide medical services predominantly to urban and suburban inhabitants with a negligible share of patients from rural areas. It is estimated that study clinics are accessible to a total of 6 million city dwellers (15% of the Polish population).

Testing for hepatitis C virus antibodies

In order to estimate the seroprevalence in the study population, only the results of anti-HCV were analysed. Anti-HCV in serum was detected by electrochemiluminescence (Roche, ECLIA) and the detection method did not change throughout the study period. All patients with positive results had been referred to special infectious disease clinical departments in order to undergo confirmatory HCV RNA tests if necessary; therefore, those results were not available in the anonymous dataset. Anti-HCV true positive results were not confirmed by immunoblotting. Such a limited approach without final confirmation of anti-HCV positivity was applied on the basis of the European Association for the Study of the Liver (EASL) recommendations, stating that immunoblotting is not recommended to distinguish false positive and true positive anti-HCV result. In order to confirm current viraemia, an HCV RNA test ought to be performed, however this was not the aim of this study [37].

The clinical sensitivity of the test used to detect anti-HCV is estimated at 100% (95% confidence interval (CI): 99.61–100%), the specificity at 99.62% (95%CI: 99.71%–99.92%) [38].

Study population

The total population aimed to be investigated in the study consisted of patients who had been tested for anti-HCV at least once in the period from 2004 to 2014. The study group was extracted from the pool of all medical records of 1.5 million individuals who had been consulted by any doctor in this period. Available records included information on: unique patient number, sex, date of test, age at the date of testing, diagnosis related to the test referral and test result. Only the latest result of testing was included into the study pool, which finally comprised 61,805 single test results of unique patients. The study group was limited to working age population representatives, aged 15–64 years. The working age population was defined according to the definition of the Organisation for Economic

Table 1

Aggregated ICD-10 diagnoses accompanying referrals for testing hepatitis C virus antibodies, Poland, 2004–2014 (n = 36,356 patients)

Diagnosis	ICD-10- codes	Group
Pregnancy and pregnancy-related conditions	O20, O24, O26, Z32, Z34, Z35	1
Preventive consultations of generally healthy persons	Z00, Z01, Z02, Z10, Z24, Z29, Z31, Z71, Z76	2
Various symptoms and signs	R10, R53, R68, R69, R72, R79, Z03, Z04	3
Fatty liver disease	K76, E78	4
Hypertransaminasaemia	R74	5
Others	Other than the above	6

Co-operation and Development (OECD) [39]. In Poland, the working age population consists of 25 million people including 11.768 million women and 12.971 million men.

Data analysis

Analysis of the population tested for hepatitis C virus antibodies

The total population tested for anti-HCV was divided into 10-year age groups stratified by sex and the year of testing for time analysis. In each subgroup, the total number of patients tested was used as a denominator. Analysis of the population testing positive for hepatitis C virus antibodies

The number of persons with a positive result for HCV antibody were available each year along with demographical data (sex and age). The rate of total positive tests was calculated and stratified by sex and age. The analyses by age group were conducted by comparing the number of patients, the number of tests and the number of positive/negative results for HCV antibody. Two classifications according to age were used. In the first classification the study population age range was split into 10 year-age groups. In the second classification, since the US data indicated a higher prevalence of HCV in people now aged 50 to 70 years [16], the percentage of positive anti-HCV test results was accordingly analysed in age groups 15 to 49 years and over 50 years.

The mean rates of positive patients were analysed over time by regression analysis and stratified by sex.

Analysis of testing and positive tests by referral group

A number of referrals for anti-HCV test (n = 36,356) had preliminary diagnosis information (according to ICD-10 coding) [40]. We compiled those diagnoses into specific groups for further analyses (Table 1). Both 3-digital and 5-digital ICD-10 codes were aggregated.

Statistical methods

Data were analysed using STATISTICA (data analysis software system), version 12, (www.statsoft.com) StatSoft, Inc. (2014) US, to calculate the incidence of newly diagnosed cases per year, and the prevalence in the entire examined population. The independent-sample t-test was used for normally distributed variables, and the nonparametric Mann–Whitney U test was used for not normally distributed parameters. Significance was set at $p<0.05$. Using linear regression analysis, the trend of the number of the incidence as a function of time (years) was calculated and the R-square value evaluated the goodness of fit of the regression.

Results

Characteristics of the study population

Overall characteristics

A total of 61,805 single patient records were considered in the study, spanning the period from 2004 to 2014 (Table 2). Men (n = 19,531) accounted for 31.6% of the total study group. The overall average age of patients was 34.4 years (standard deviation (SD): 8.6). The average age of men was 36.5 years (SD: 9.6). The average age of women was 33.4 years (SD: 7.9) (Table 3).

Analysis by age group

The most represented age group in terms of number of individuals was the one comprising 25 to 34 year-olds (n = 35,047 patients; 56.7%) and the least numerous group comprised persons over 55 years (n = 2,626 patients; 4.2%) (Table 4).

Time analysis of testing practices

The number of patients examined for anti-HCV increased steadily with time, from 815 patients in 2004, to 14,963 in 2014 (Table 2).

The percentage of all medical-facility-patients tested yearly increased from 0.9% (815/88,177) in 2004 to 4.0% (14,963/376,637) in 2014. Data showed a growing proportion of women being examined. In 2004, a similar number of men and women underwent anti-HCV tests (50.3% (410/815) of women and 49.7% (405/815) of men), whereas in 2014, women accounted for 79.1% (11,620/14,693). This increase may reflect

Table 2

Annual numbers of patients tested for hepatitis C virus (HCV) antibodies and proportions testing positive, stratified by sex, Poland, 2004–2014 (n = 61,805 patients)

Year	All patients			Women			Men			P value
	Number of anti-HCV tests	Number of positive results	Percentage of positive results	Number of anti-HCV tests	Number of positive results	Percentage of positive results	Number of anti-HCV tests	Number of positive results	Percentage of positive results	
2004	815	26	3.2%	410	14	3.4%	405	12	3.0%	0.7143
2005	1,366	32	2.3%	766	14	1.8%	600	18	3.0%	0.1553
2006	1,210	29	2.4%	650	13	2.0%	560	16	2.9%	0.3314
2007	1,761	43	2.4%	961	26	2.7%	800	17	2.1%	0.4322
2008	3,033	47	1.6%	1,648	25	1.5%	1,385	22	1.6%	0.8740
2009	4,263	75	1.8%	2,477	44	1.8%	1,786	31	1.7%	0.9208
2010	5,250	116	2.2%	3,243	57	1.8%	2,007	59	2.9%	0.0075
2011	7,378	149	2.0%	4,970	86	1.7%	2,408	63	2.6%	0.0180
2012	9,527	133	1.4%	6,730	78	1.2%	2,797	55	2.0%	0.0059
2013	12,239	141	1.2%	8,799	88	1.0%	3,440	53	1.5%	0.0216
2014	14,963	166	1.1%	11,620	113	1.0%	3,343	53	1.6%	0.0090
Total	61,805	957	1.5%	42,274	558	1.3%	19,531	399	2.0%	0.0001

Table 3

Characteristics of the study population and that testing positive for hepatitis C virus antibodies, Poland, 2004–2014 (n = 61,805 patients)

Participants	Sex	Mean age	Standard deviation	Number of persons	Column percentage
Study participants	F	33.4	7.9	42,274	68.4%
	M	36.5	9.6	19,531	31.6%
	Total	34.4	8.6	61,805	100.0%
Study participants testing positive	F	36.0	9.8	558	58.3%
	M	37.8	9.7	399	41.7%
	Total	36.8	9.8	957	100.0%

F: female; M: male.

legal requirements for prenatal care during pregnancy in Poland, with HCV testing becoming compulsory for pregnant women from 2012 onwards (Table 5) [26].

Characteristics of the population testing positive for hepatitis C virus antibodies

Overall characteristics

Throughout the analysed period, 1.5% patients (957/61,805) undergoing anti-HCV examination tested positive. Averaged positive results for women and men were 1.3% (558/42,274) and 2.0% (399/19,531) respectively (p = 0.0001). The average age of all anti-HCV positive patients was 36.8 years (SD: 9.8). The average age of anti-HCV positive women was 36.0 years (SD: 9.8), and the average age of men with positive test results was 37.8 years (SD: 9.7).

Analysis by age group

Most anti-HCV positive cases occurred in patients aged 45–54 years (2.9% of tested patients 147/5,107) and in patients older than 55 years (2.6% of tested patients 68/2,626). The lowest proportions of positive test results were noted in the youngest patients: 1.2% (436/35,047) among patients aged 25 to 34 years and 1.5% (52/3,411) among patients aged 15 to 24 years.

In the group of tested women, among those older than 25 years, the percentage of positive anti-HCV test results increased with age, being lowest among women aged 25–34 years (1.1%; 285/26,632) and 35–44 years (1.4%; 132/9,444), and highest for women aged over 55 years (3.2%; 43/1,394). For the youngest group comprising 15 to 24 year-olds, the value 1.5% (33/2,234) was similar to that of the group of 35 to 44 year-olds (1.4%; 132/9,444). In the group of tested men, the smallest proportion of infections was found in the 15 to 24 years age group (1.6%; 19/1,177) and increased

Table 4

Results of anti-hepatitis C virus tests stratified by patient age groups and sex in a study estimating hepatitis C seroprevalence, Poland, 2004–2014 (n = 61,805 patients)

Age group (years)	All patients			Women			Men			P value
	Number	Number testing positive for HCV antibodies	Percentage testing positive for HCV antibodies	Number	Number testing positive for HCV antibodies	Percentage testing positive for HCV antibodies	Number	Number testing positive for HCV antibodies	Percentage testing positive for HCV antibodies	
15–24	3,411	52	1.5%	2,234	33	1.5%	1,177	19	1.6%	0.7561
25–34	35,047	436	1.2%	26,632	285	1.1%	8,416	151	1.8%	0.0001
35–44	15,614	254	1.6%	9,444	132	1.4%	6,170	122	2.0%	0.0051
45–54	5,107	147	2.9%	2,591	65	2.6%	2,536	82	3.2%	0.1318
55–64	2,626	68	2.6%	1,394	43	3.2%	1,232	25	2.0%	0.0893
15–49	56,921	825	1.4%	39,676	480	1.2%	17,245	345	2.0%	0.0001
50–64	4,884	132	2.7%	2,598	78	3.0%	2,286	54	2.4%	0.1687
Total	**61,805**	**957**	**1.5%**	**42,274**	**558**	**1.3%**	**19,531**	**399**	**2.0%**	**0.0001**
Mean age in years (SD)	34.4 (8.6)	36.8 (9.8)	100.0%	33.4 (7.9)	36.0 (9.8)	58.3%	36.5 (9.6)	37.8 (9.7)	41.7%	0.0001

HCV: hepatitis C virus; SD: standard deviation.

Table 5

Proportions of patients undergoing anti-hepatitis C virus tests, Poland, 2004–2014 (n = 61,805 patients)

Year	All patients			Women			Men		
	Number	Number tested	Percentage tested	Number	Number tested	Percentage tested	Number	Number tested	Percentage tested
2004	88,177	815	0.9%	45,417	410	0.9%	42,760	405	0.9%
2005	106,464	1,366	1.3%	54,484	766	1.4%	51,980	600	1.2%
2006	127,195	1,210	1.0%	64,518	650	1.0%	62,677	560	0.9%
2007	157,238	1,761	1.1%	80,478	961	1.2%	76,760	800	1.0%
2008	200,031	3,033	1.5%	103,257	1,648	1.6%	96,774	1,385	1.4%
2009	219,905	4,263	1.9%	114,630	2,477	2.2%	105,275	1,786	1.7%
2010	240,307	5,250	2.2%	125,217	3,243	2.6%	115,090	2,007	1.7%
2011	269,140	7,378	2.7%	139,882	4,970	3.6%	129,258	2,408	1.9%
2012	303,813	9,527	3.1%	157,812	6,730	4.3%	146,001	2,797	1.9%
2013	335,526	12,239	3.6%	173,902	8,799	5.1%	161,624	3,440	2.1%
2014	376,637	14,963	4.0%	195,787	11,620	5.9%	180,850	3,343	1.8%
Total	2,424,433	61,805	2.5%	1,255,384	42,274	3.4%	1,169,049	19,531	1.7%

with age up to 3.2% (82/2,536) in the age group including 45 to 54 year-olds. For individuals over 55 years the value was similar (2.0%; 25/1,232) to that of the age group with 35 to 44 year-olds (2.0%; 122/6,170) (Table 3).

Because a higher prevalence of HCV was reported in 50 to 70 year-olds in the US [16], the percentage of positive anti-HCV test results was also analysed in age groups 15 to 49 years (representing 92.1% of those tested 56,921/61,805) and over 50 years (7.9%; 4,884/61,805). A higher percentage of anti-HCV positive patients was found in those aged over 50 years (2.7%; 132/4,884) compared with younger participants (1.4%; 825/56,921) ($p < 0.0001$). This percentage was higher for both women and men aged over 50 years, with, in women 3.0% (78/2,598) vs 1.2% (480/39,676) in those aged under 50 years ($p = 0.0001$) and, in men, 2.4% (54/2,286) vs 2.0% (345/17,245) in those less than 50 years-old ($p = 0.2507$).

Time analysis of patients testing positive for hepatitis C virus antibodies

An analysis of the data in the years 2004 to 2014 suggests a downward trend for the proportion of positive anti-HCV results (mean positive

Table 6

Primary diagnoses resulting in the referral for anti-hepatitis C virus tests, Poland, 2004–2014 (n = 36,356 patients)

LBID012_Diagnosis	Number of patients tested	Number of patients with positive results	Proportion of positive patients	Proportion of positive women	Proportion of positive men	Proportion positive in column	Proportion of diagnosed patients
Pregnancy and pregnancy related conditions	16,130	122	0.8%	0.8%	NA	25.4%	44.4%
Preventive consultations of generally healthy persons[a]	6,456	75	1.2%	0.7%	1.0%	15.6%	17.8%
Various symptoms and signs	5,151	108	2.1%	1.0%	2.1%	22.5%	14.2%
Hypertransaminasaemia[b]	1,093	25	3.7%	1.1%	1.7%	5.2%	3.0%
Fatty liver disease	1,115	23	3.4%	0.8%	1.7%	4.8%	3.1%
Others[c]	6,411	127	1.5%	0.9%	2.6%	26.5%	17.6%
Total	36,356	480	1.1%	0.8%	1.9%	100.00%	100.0%

NA: not applicable.

[a] Spontaneous or obligatory health check-ups.

[b] Excluding people with elevated transaminase level as a reason of additional anti-HCV test.

[c] Most often before surgical procedures.

anti-HCV = -0.0017 × year + 3.3715; R2 = 0.7558) (Figure).

In 2004, positive results were noted in 3.2% (26/815) of patients examined for anti-HCV, but in 2014 the percentage of patients with a positive result stood at 1.1% (166/14,693). Similar tendencies were observed in both women and men. In 2004, the percentage of anti-HCV positive results in women was 3.4% (14/410), and in men 3.0% (12/405) ($p > 0.05$), whereas in 2014, anti-HCV positive results were noted among 1.0% (113/11,620) of women and 1.6% (53/3,343) of men ($p = 0.0090$).

Referral group testing and test results

We analysed the diagnoses ascribed to each anti-HCV test referral. The predominant reason for anti-HCV testing was pregnancy and pregnancy-related conditions – 44.4% (16,130/36,356) –, followed by preventive testing of otherwise healthy people (occupational health or preventive screening) with 17.8% (6,456/36,356). Various symptoms and signs were the reason for testing in 14.2% of patients (5,151/36,356), fatty liver disease in 3.1% (1,115/36,356) and elevated alanine transaminase levels (ALT) as single diagnosis in 3.0% (1,093/36,356) (Table 6). Only 0.8% (122/16,130) of patients with a diagnosis of pregnancy and pregnancy-related conditions were anti-HCV positive. Preventive action and anti-HCV testing revealed positive results in 1.2% (75/6,456), of patients, while 2.1% (108/5,151) of patients with various symptoms and signs were anti-HCV positive, as well as 3.4% (23/1,115) of patients with a diagnosis of fatty liver disease and 3.7% (25/1,093) of people with elevated ALT (Table 6).

Discussion

Our study presents an evaluation of anti-HCV prevalence in a large country-wide sample of (sub)urban working-age Polish people between 2004 and 2014. Data from electronic medical records of ambulatory patients visiting doctors due to different conditions, including prophylactic and screening reasons, were analysed. The overall anti-HCV prevalence in our study was 1.5%. Similar to previous studies [29,30] and studies from other countries [41-43], anti-HCV positivity was significantly more frequent in men than women (2.0% vs 1.3% respectively; $p = 0.0001$).

We also found that in contrast to younger age groups (15 to 49 years), anti-HCV prevalence in people aged between 50 and 64 years was higher (2.7% vs 1.4%; $p < 0.0001$), and surprisingly more frequent in women (3.0%) than men (2.4%) although, this difference was not statistically significant. Higher HCV infection prevalence in people born before 1965 has also been observed in the US [16], therefore, the recommendation of CDC and AGA to screen people born before 1965 [17,18] might also be justified in Poland and could be implemented as part of primary healthcare. According to the authors' own research on 16,130 pregnant women, the prevalence of HCV in this group was only 0.8% which is close to the European average (1%) [44]. Low anti-HCV prevalence in pregnant women and high prevalence in people of post-reproductive age might be a subject of debate in terms of allocating effective financial resources for HCV screening in these two groups.

To our knowledge, our study constitutes one of the currently largest performed in the Polish population of working age. Indeed, although previous studies in the country have attempted to assess HCV prevalence, these have either been conducted either some time ago, or have been mainly based on small samples and/or on selected population groups – pregnant women, students, blood donors or deceased organ donors

– with, in some cases, only a single district or town in the country considered [28,29,31-35,45]. For example, the study by Bielawski et al. in 1999, which is still the point of reference for many epidemiological studies on HCV infection in Poland, was conducted on a group of 2,561 volunteers enrolled by a laboratory in Gdansk in response to press advertisements. It estimated the overall HCV infection prevalence at 1.9% [29], with 2.3% of men versus 1.7% of women seropositive for hepatitis C virus antibodies. Limitations of this study were however the restricted geographical area and the group chosen to be tested (volunteer bias) [29]. Since then, larger studies have been performed, an important one being that of Seyfried et al., where 4,233,119 blood donors were screened between 1994 and 2003. Anti-HCV prevalence in this group was found to be on average 0.5% [36]. The most recent study by Flisiak et al. in 2011, which was performed from 2009 to 2010 in 26,057 Polish adults (healthcare workers and hospital patients), presented anti-HCV prevalence in healthcare workers at 1.4%, whereas in ambulatory patients of different general practice and specialist outpatient clinics it was 1.9% [30]. Here we investigate 61,805 people of working-age over the whole country between 2004 and 2014 and find a prevalence of 1.5%, similar to what is currently estimated for central Europe (1.3%) [19].

Hepatitis C infection has been registered as a distinct disease entity in Poland since 1997. Until 2004, the annual number of newly diagnosed cases of HCV infection, logged by the National Institute of Hygiene, did not exceed 2,000. In the years 2005 and 2006 the number increased to 3,000 (2,997 and 2,949 in each year respectively), but this increase was most likely caused by the modification of disease reporting methods. In the following years, the number of newly diagnosed cases decreased steadily (2,753 in 2007, 2,353 in 2008). Since 2009 however, another upward trend can be observed. The number of reported cases of new HCV infections in Poland accumulated to 1,891 in 2009, 2,178 in 2010, 2,189 in 2011, 2,265 in 2012 and increased to 2,600 cases in 2013 [46].

Our analysis on the proportions of persons with HCV antibodies in working age people from 2004 to 2014 does not find such an increasing trend in the latest years of the study. Instead we find a higher proportion of patients with HCV antibodies in the first year of the study compared to the end of the analysed period (3.2% in 2004 vs 1.1% in 2014). This could be due to a general drop in HCV infections in working age people, but also to a change of indication for testing. Indeed, the most common indication for evaluating HCV serological status in the early years of the study was elevated serum ALT, which per se is regarded as a laboratory manifestation of liver injury. With the introduction of obligatory anti-HCV testing in all pregnant women from 2012 onwards, the proportion of anti-HCV positive persons dropped significantly, possibly coming closer to the actual prevalence of HCV infection in this relatively young and overall healthy group.

This study presents however a number of limitations. First, the prevalence of HCV infection is known to vary according to risk groups. The study by Flisiak et al. on 17,930 persons found that significant factors of HCV infection in Poland are more than three hospitalisations during a life time (odds ratio (OR) = 1.8), blood transfusion before 1992 (OR = 2.9) and intravenous drug use (OR = 6.2) [30]. Transmission of HCV via intravenous drug use has been increasingly observed and in 2007, 10 of 16 million of PWID worldwide were estimated to be HCV positive. The number of active PWIDs in the EU is estimated at ca 1 million [10]. In Poland, 70% of PWIDs are infected with HCV, predominantly men under 45 years of age [47]. In our study, we had no access to individual medical records; therefore, intravenous drug use could not be accounted for. Moreover, we did not have information on patients' profession either, so we could not evaluate any possible occupational risk.

Second, our study only analysed HCV antibody prevalence, and western blot tests were not performed. Thus results do not distinguish between current infections and probable infections in the past (resolved infection). A final diagnosis of current HCV infection requires the finding of HCV RNA in serum samples by RT-PCR. We had no access to HCV RNA results that were stored in the form of scans and required patients' consent for access. The results of this study can therefore not be compared with any studies using the EU HCV case definition [48]. A Polish study in 2011 showed that only 31% of those with HCV antibodies were also positive for HCV RNA by RT-PCR [30]. Moreover the sensitivity of the electrochemiluminescence (Roche, ECLIA) assay used in this study is very high, so specificity will be lower, which may result in false positive results. Taking these points into consideration, the prevalence of current HCV infection in the Polish urban working population is likely to be lower than 1.5%. Further studies on positive anti-HCV test results and HCV RNA detection may reveal if HCV infection is resolved more frequently than has been presumed up to now (ca 30% of cases being resolved [49]).

Finally, although our study was conducted on a large number of patients, another important limitation was the inclusion of only people living in big cities and their suburbs. One risk factor for HCV infection is the use of medical care, which is less frequent in inhabitants of rural areas. Accordingly, anti-HCV prevalence in rural areas has been shown to be lower than in urban areas [27,50]. Therefore our results cannot be extrapolated to the whole population and it can be assumed that the prevalence of HCV infection among people of working age in the country may be lower than 1.5%.

Conclusions

There is evidence that an improvement of diagnostics and treatment effectiveness may significantly reduce the burden of HCV infections in Poland [5,51]. A study using a modelling approach estimated that, until 2030, the HCV prevalence is projected to decrease by 5%. In

contrast, an increase in the number of treated patients to 15,000 yearly would reduce the number of total infections by 90% until 2030, which would also contribute to a decrease of HCV-mortality by 80% [49]. The results obtained in this study suggest that the proportion of people infected with HCV in Poland in the working population is decreasing, which may be a consequence of increasing social awareness, including preventative activities after or before exposure to blood-borne infections. Moreover, a higher prevalence of anti-HCV was found in the population of post-reproductive age. We therefore recommend screening HCV tests mainly in individuals over 45 years-old. Examining healthy and young people should not be carried out as part of screening, however testing may be recommended to individuals who are subjected to risk factors. The continuous monitoring of HCV prevalence and incidence in Poland is important to estimate the resources needed for screening and treatment as well as their costs. Knowing the age groups at higher risk for infection will help to establish recommendations for more effective detection of cases of HCV infection, which in turn is also crucial to reduce further transmission.

Acknowledgements

The Authors would like to acknowledge and expert IT help of Mrs Irena Zawalska.

Conflict of interest

Bożena Walewska-Zielecka and Piotr Soszyński are current employees of Medicover Sp. z o.o.

Authors' contributions

Conception or design of the work: BWZ, GJ; data acquisition: BWZ; data analysis: ZW, BWZ, GJ, UR, PS; interpretation of the data for the work: BWZ, UR, GJ, ZW, AC, PS, AF; drafting the work or revising it critically for important intellectual content: All; approval of manuscript submission: All.

References

1. Stanaway JD, Flaxman AD, Naghavi M, Fitzmaurice C, Vos T, Abubakar I, et al. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. Lancet. 2016;388(10049):1081-8. DOI: 10.1016/S0140-6736(16)30579-7 PMID: 27394647
2. Wedemeyer H, Dore GJ, Ward JW. Estimates on HCV disease burden worldwide - filling the gaps.J Viral Hepat. 2015;22(Suppl 1):1-5. DOI: 10.1111/jvh.12371 PMID: 25560838
3. Poynard T, Ratziu V, Benmanov Y, Di Martino V, Bedossa P, Opolon P. Fibrosis in patients with chronic hepatitis C: detection and significance.Semin Liver Dis. 2000;20(1):47-55. DOI: 10.1055/s-2000-9258 PMID: 10895431
4. Poynard T, Bedossa P, Opolon P, for the OBSVIRC, METAVIR, CLINIVIR and DOSVIRC groups. Natural history of liver fibrosis progression in patients with chronic hepatitis C.Lancet. 1997;349(9055):825-32. DOI: 10.1016/S0140-6736(96)07642-8 PMID: 9121257
5. Razavi H, Waked I, Sarrazin C, Myers RP, Idilman R, Calinas F, et al. The present and future disease burden of hepatitis C virus (HCV) infection with today's treatment paradigm. J Viral Hepat. 2014;21(Suppl 1):34-59. DOI: 10.1111/jvh.12248 PMID: 24713005
6. World Health Organization (WHO). Prevention and Control of Viral Hepatitis Infection: Framework for Global Action. Geneva: WHO; 2012. Available from: http://www.who.int/hiv/pub/hepatitis/Framework/en/
7. Suzuki K, Mizokami M, Lau JY, Mizoguchi N, Kato K, Mizuno Y, et al. Confirmation of hepatitis C virus transmission through needlestick accidents by molecular evolutionary analysis. J Infect Dis. 1994;170(6):1575-8. DOI: 10.1093/infdis/170.6.1575 PMID: 7527827
8. Foberg U, Ekermo B, Widell A, Mathiesen U, Fryden A. Hepatitis C virus transmission, 1988-1991, via blood components from donors subsequently found to be anti-HCV-positive.Scand J Infect Dis. 1996;28(1):21-6. DOI: 10.3109/00365549609027144 PMID: 9122627
9. Verbaan H, Andersson K, Eriksson S. Intravenous drug abuse--the major route of hepatitis C virus transmission among alcohol-dependent individuals?Scand J Gastroenterol. 1993;28(8):714-8. DOI: 10.3109/00365529309098279 PMID: 7692588
10. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). EMCDDA 2010 Selected Issue-Trends in Injecting Drug use in Europe European Union. Annual Report. Selected Issues/European Monitoring Centre for Drugs and Drug Addiction. European Monitoring Centre for Drugs and Drug Addiction. EMCDDA: Lisbon; 2010. pp.22.
11. Mazza C, Ravaggi A, Rodella A, Padula D, Duse M, Lomini M, et al. Prospective study of mother-to-infant transmission of hepatitis C virus (HCV) infection. J Med Virol. 1998;54(1):12-9. DOI: 10.1002/(SICI)1096-9071(199801)54:1<12::AID-JMV3>3.0.CO;2-S PMID: 9443104
12. Mast EE, Hwang LY, Seto DS, Nolte FS, Nainan OV, Wurtzel H, et al. Risk factors for perinatal transmission of hepatitis C virus (HCV) and the natural history of HCV infection acquired in infancy. J Infect Dis. 2005;192(11):1880-9. DOI: 10.1086/497701 PMID: 16267758
13. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence.Hepatology. 2013;57(4):1333-42. DOI: 10.1002/hep.26141 PMID: 23172780
14. Wei L, Lok AS. Impact of new hepatitis C treatments in different regions of the world. Gastroenterology. 2014;146(5):1145-1150.e 4. PMID: 24662488
15. World Health Organization (WHO). Guidelines for the screening, care and treatment of persons with hepatitis C infection. Geneva: WHO; April 2014.
16. Pawlotsky JM. New hepatitis C therapies: the toolbox, strategies, and challenges.Gastroenterology. 2014;146(5):1176-92. DOI: 10.1053/j.gastro.2014.03.003 PMID: 24631495
17. Smith BD, Morgan RL, Beckett GA, Falck-Ytter Y, Holtzman D, Teo CG, et al. . Recommendations for the identification of chronic hepatitis C virus infection among persons born during 1945-1965.MMWR Recomm Rep. 2012;61(RR-4):1-32.PMID: 22895429
18. Hepatitis C Task Force, Boldt MD, Brill JV, Davis GL, Gordon SC, Kim AY, et al. Hepatitis C screening: summary of recommendations from the clinical decision tool. Gastroenterology. 2013;145(5):1146-9. DOI: 10.1053/j.gastro.2013.09.007 PMID: 24139703
19. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection.J Hepatol. 2014;61(1) Suppl;S45-57. DOI: 10.1016/j.jhep.2014.07.027 PMID: 25086286
20. European Centre for Disease Prevention and Control (ECDC). Annual Epidemiological Report on Communicable Diseases in Europe 2008. Stockholm: ECDC; 2008. Available from: http://ecdc.europa.eu/en/publications/publications/0812_sur_annual_epidemiological_report_2008.pdf
21. Cornberg M, Razavi HA, Alberti A, Bernasconi E, Buti M, Cooper C, et al. A systematic review of hepatitis C virus epidemiology in Europe, Canada and Israel. Liver Int. 2011;31(Suppl 2):30-60. DOI: 10.1111/j.1478-3231.2011.02539.x PMID: 21651702
22. Esteban JI, Sauleda S, Quer J. The changing epidemiology of hepatitis C virus infection in Europe.J Hepatol. 2008;48(1):148-62. DOI: 10.1016/j.jhep.2007.07.033 PMID: 18022726
23. Meldunek roczny 1997 o zachorowaniach na choroby zakazne, zatruciach i zakazeniach szpitalnych zgłoszonych. [Annual report on the incidence of infectious diseases, poisonings and hospital infections reported in 1997]. Warsaw: National Institute of Hygiene, Scientific Research Institute of the Ministry of Health and Social Welfare Department of Epidemiology and The Ministry of Health and Social Welfare Department of Public Health; 1997. Polish. Available from: http://wwwold.pzh.gov.pl/oldpage/epimeld/1997/M_97_rok.pdf
24. Infectious diseases and poisonings in Poland in 2015. Warsaw: National Institute of Public Health – National Institute of Hygiene – Department of Epidemiology and Chief Sanitary

Inspectorate – Department for Communicable Disease and Infection Prevention and Control; 2016. Available from: http://wwwold.pzh.gov.pl/oldpage/epimeld/2015/Ch_2015.pdf

25. Narodowego Instytutu Zdrowia Publicznego - Państwowego Zakładu Higieny (NIPH-PZH). [Infectious diseases case definitions for the purpose of epidemiological surveillance]. Draft (3). Warszawa: Division of Epidemiology at NIPH-PZH; Jan 2014. Available from: http://wwwold.pzh.gov.pl/oldpage/epimeld/inne/Def_PL2_3.pdf
26. Regulation of the Minister of Health of 20 September 2012 on the standards of medical management and procedures during provision of perinatal health care to women during physiological pregnancy, childbirth and the puerperium, and neonatal care. Journal of Laws. 2012; item 1100.
27. Infectious diseases and poisonings in Poland in 2014. Warsaw: National Institute of Public Health – National Institute of Hygiene – Department of Epidemiology and Chief Sanitary Inspectorate – Department for Communicable Disease and Infection Prevention and Control; 2015. page 97. Available from: http://wwwold.pzh.gov.pl/oldpage/epimeld/2014/Ch_2014.pdf
28. Stepień M, Rosińska M. Badania rozpowszechnienia HCV w Polsce--gdzie jesteśmy? [Studies on HCV infection prevalence in Poland--where are we?].Przegl Epidemiol. 2011;65(1):15-20. PMID: 21735830
29. Bielawski K, Wlasiuk M, Truskolawska M, Falkiewicz B. HCV infection in Poland.Arch Med Res. 2000;31(5):532-5. DOI: 10.1016/S0188-4409(00)00100-4 PMID: 11179591
30. Flisiak R, Halota W, Horban A, Juszczyk J, Pawlowska M, Simon K. Prevalence and risk factors of HCV infection in Poland. Eur J Gastroenterol Hepatol. 2011;23(12):1213-7. DOI: 10.1097/MEG.0b013e32834d173c PMID: 22002000
31. Pszenny A, Hreńczuk M, Czerwiński J, Danielewicz R, Małkowski P. HBV and HCV infections among deceased organ donors in Poland.Probl Hig Epidemiol.2012;93(3):579-85.
32. Chlabicz S, Bonifatiuk I, Radziwon P. Prevalence of hepatitis C virus antibodies among blood donors in north-eastern Poland.Hepatol Res. 2005;33(3):206-10. DOI: 10.1016/j.hepres.2005.09.001 PMID: 16266823
33. Ganczak M, Korzeń M, Szych Z. Seroprevalence of hepatitis C virus infection among surgical nurses, their patients and blood donation candidates in Poland.J Hosp Infect. 2012;82(4):266-70. DOI: 10.1016/j.jhin.2012.08.021 PMID: 23110996
34. Zagożdżon P, Parszuto J, Raj A, Całus-Kania D, Korczak A, Ejsmont J. Czestość wystepowania i czynniki ryzyka zakazenia HCV wśród pracowników ochrony zdrowia w województwie pomorskim. [Prevalence and risk factors of chronic hepatitis C virus infection among health-care workers in Pomeranian voivodeship].Przegl Epidemiol. 2009;63(1):39-43.PMID: 19522224
35. Ślusarczyk J, Małkowski P, Bobilewicz D, Juszczyk G. Cross-sectional, anonymous screening for asymptomatic HCV infection, immunity to HBV, and occult HBV infection among health care workers in Warsaw, Poland.Przegl Epidemiol. 2012;66(3):445-51.PMID: 23230715
36. Seyfried H, Brojer E, Grabarczyk P, Rosińska M, Gronowska A, Letowska M, Viral Study Group of Regional Blood Transfusion Centers. Analizaczestości wykrywania markerów zakazenia wirusem zapalenia watroby typu C (HCV) u polskich dawców krwi w latach 1994-2003. [Prevalence of hepatitis C virus markers in Polish blood donors in 1994-2003].Przegl Epidemiol. 2005;59(4):807-14.PMID: 16729421
37. European Association for Study of Liver. EASL Clinical Practice Guidelines: Management of hepatitis C virus infection. J Hepatol. 2014;60(2):392-420.
38. Esteban JI, van Helden J, Alborino F, Bürgisser P, Cellerai C, Pantaleo G, et al. Multicenter evaluation of the Elecsys® anti-HCV II assay for the diagnosis of hepatitis C virus infection. J Med Virol. 2013;85(8):1362-8. DOI: 10.1002/jmv.23536 PMID: 23765774
39. Organisation for Economic Co-operation and Development (OECD). Working age population (indicator). Paris: OECD; 2016. doi: DOI: 10.1787/d339918b-en . [Accessed on 19 July 2016]. Available from: http://www.oecd-ilibrary.org/social-issues-migration-health/working-age-population/indicator/english_d339918b-en
40. World Health Organization (WHO). International Statistical Classification of Diseases and Related Health Problems 10th Revision. Geneva: WHO; 2016. [Accessed 11 Jan 2017]. Available from: http://apps.who.int/classifications/icd10/browse/2016/en
41. Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan.J Gastroenterol. 2009;44(19) Suppl 19;102-7. DOI: 10.1007/s00535-008-2251-0 PMID: 19148802
42. Meffre C, Le Strat Y, Delarocque-Astagneau E, Dubois F, Antona D, Lemasson JM, et al. Prevalence of hepatitis B and hepatitis C virus infections in France in 2004: social factors are important predictors after adjusting for known risk factors. J Med Virol. 2010;82(4):546-55. DOI: 10.1002/jmv.21734 PMID: 20166185
43. Bruggmann P, Berg T, Øvrehus AL, Moreno C, Brandão Mello CE, Roudot-Thoraval F, et al. Historical epidemiology of hepatitis C virus (HCV) in selected countries. J Viral Hepat. 2014;21(Suppl 1):5-33. DOI: 10.1111/jvh.12247 PMID: 24713004
44. Cornberg M, Razavi HA, Alberti A, Bernasconi E, Buti M, Cooper C, et al. A systematic review of hepatitis C virus epidemiology in Europe, Canada and Israel. Liver Int. 2011;31(Suppl 2):30-60. DOI: 10.1111/j.1478-3231.2011.02539.x PMID: 21651702
45. Aniszewska M, Kowalik-Mikołajewska B, Pokorska-Lis M, Kalinowska M, Cianciara J, Marczyńska M. Czestość wystepowania przeciwciał anty-HCV u kobiet ciezarnych. Analiza czynników ryzyka zakazenia HCV. [Seroprevalence of anti-HCV in pregnant women. Risk factors of HCV infection]. Przegl Epidemiol. 2009;63(2):293-8.PMID: 19799264
46. Chief Sanitary Inspectorate (GIS). State sanitary condition in 2013. Warsaw: GIS; 2014.
47. Panasiuk A, Flisiak R, Mozer-Lisewska I, Adamek A, Tyczyno M, Halota W, et al. Distribution of HCV genotypes in Poland. Przegl Epidemiol. 2013;67(1):11-6, 99-103.PMID: 23745369
48. European Commission. Commission Implementing Decision of 8 August 2012 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (notified under document C(2012) 5538). Official Journal of the European Union. Luxembourg: Publications Office of the European Union. 27.09.2012:L 262/1. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L:2012:262:FULL&from=EN
49. Flisiak R, Halota W, Tomasiewicz K, Kostrzewska K, Razavi HA, Gower EE. Forecasting the disease burden of chronic hepatitis C virus in Poland.Eur J Gastroenterol Hepatol. 2015;27(1):70-6. DOI: 10.1097/MEG.0000000000000237 PMID: 25426979
50. Hartleb M, Gutkowski K, Zejda JE, Chudek J, Więcek A. Serological prevalence of hepatitis B virus and hepatitis C virus infection in the elderly population: Polish nationwide survey--PolSenior.Eur J Gastroenterol Hepatol. 2012;24(11):1288-95. PMID: 22864260
51. Sibley A, Han KH, Abourached A, Lesmana LA, Makara M, Jafri W, et al. The present and future disease burden of hepatitis C virus infections with today's treatment paradigm - volume 3. J Viral Hepat. 2015;22(22) Suppl 4;21-41. DOI: 10.1111/jvh.12476 PMID: 26513446

License and copyright

Letter to the editor: Need for a European network for enterovirus D68 surveillance after detections of EV-D68 of the new B3 lineage in Sweden and Italy, 2016

E Pariani [1] , L Pellegrinelli [1] , ADC Merlone [2] , A Piralla [3], F Baldanti [3] , S Binda [1]
1. Department of Biomedical Sciences for Health, University of Milan, Milan, Italy
2. Unità Operativa Complessa di Pediatria, ASST Lariana Como, Italy
3. Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

Correspondence: Elena Pariani (elena.pariani@unimi.it)

Citation style for this article:
Pariani E, Pellegrinelli L, Merlone ADC, Piralla A, Baldanti F, Binda S. Letter to the editor: Need for a European network for enterovirus D68 surveillance after detections of EV-D68 of the new B3 lineage in Sweden and Italy, 2016 . Euro Surveill. 2017;22(2):pii=30440. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30440

Article submitted on 19 December 2016 / accepted on 12 January 2017 / published on 12 January 2017

To the editor: In response to the rapid communication entitled 'Outbreak of enterovirus D68 of the new B3 lineage in Stockholm, Sweden, August to September 2016' by Dyrdak et al. [1] we would like to share our recent experience.

As the regional reference laboratory for acute flaccid paralysis (AFP) surveillance (Lombardy, northern Italy), a case of acute flaccid myelitis (AFM) came to our attention at the end of July 2016 when a previously healthy 4-year-old child with febrile (body temperature › 38 °C) respiratory illness and headache was hospitalised at a hospital in our region. The child's condition suddenly worsened with the occurrence of severe neurological manifestations such as stiff neck, flaccid limb weakness associated with hyporeflexia, and bulbar muscle weakness, requiring intensive care unit care.

Clinical specimens (cerebral-spinal fluid, blood, nasal swab, nasopharyngeal aspirate, and stool) were collected at the onset of symptoms and laboratory tests ruled out poliomyelitis as differential diagnosis. Nasal swab, nasopharyngeal aspirate and stool samples all tested positive for enterovirus (EV) by real-time reverse transcription-polymerase chain reaction (RT-PCR) [2], and were then assessed for EV-D68 by a specific assay [3]. EV-D68 RNA was detected in all clinical samples examined and this result was further confirmed by sequence analysis of viral protein (VP)3/VP1/2A gene [4]. The phylogenetic analysis of the obtained sequence revealed the presence of an EV-D68 belonging to the recently described lineage B3 [5,6]. The nt sequence also shared high similarity (› 98.6% identity) with EV-D68 lineage B3 sequences.

This case evidences the occurrence of EV-D68 belonging to the new B3 lineage in Italy in the same period than the Swedish outbreak, that is during 2016 summer, and with similar neurological outcome as some cases reported by Dyrdak et al. [1]. Two years after the unexpected spread of EV-D68 worldwide in 2014, a number of epidemics occurred throughout Europe (Sweden, the Netherlands, and the United Kingdom) [7]. Since these epidemics were only reported by countries with an active EV surveillance the impact of EV/EV-D68 in Europe is likely to be more important. For other countries, such as Italy, this information is missing. The extent of circulation of EV-D68 is of course underestimated because of the scarcity of laboratories equipped to detect the virus and because of the poor awareness among healthcare workers of the problems the virus can cause.

It is now imperative to implement a surveillance system to monitor the spread and circulation of EV-D68 (in Italy and in Europe) due to its public health impact and consequences on childrens' health. Such a surveillance scheme could be included in the framework of the AFP surveillance system [8], thus taking advantage of an existing and efficient network that includes skilled healthcare specialists, and that is endowed with equipped laboratories, with a wide expertise on EV detection and sequencing. It is becoming more and more essential to monitor not merely the virus circulation but also its molecular characteristics in order to identify any association between a specific lineage and disease severity.

The implementation of EV-D68 surveillance in the framework of the existing AFP network will entail: (i) a reevaluation of the case definition [8] to comprise severe respiratory illness and neurological impairment; (ii) a reappraisal of the panel of specimens to be collected (respiratory samples to be included always); (iii) the inclusion of EV-D68 identification assay(s) and

sequencing, which should be within the laboratory capacity.

The realisation of a European network for EV-D68 surveillance will help to obtain the information needed to clarify viral epidemiology, to put in force proper control and preventive measures, so as to protect children and keep them safe and healthy.

Conflict of interest

None declared.

Authors' contributions

ADCM identified the case. LP and SB carried out virological analysis. AP and FB performed sequence analysis. EP and SB wrote the letter. All authors contributed to the discussion of the case.

References

1. Dyrdak R, Grabbe M, Hammas B, Ekwall J, Hansson KE, Luthander J, et al. Outbreak of enterovirus D68 of the new B3 lineage in Stockholm, Sweden, August to September 2016. Euro Surveill. 2016;21(46):30403. DOI: 10.2807/1560-7917.ES.2016.21.46.30403 PMID: 27918255
2. Bubba L, Pellegrinelli L, Pariani E, Primache V, Amendola A, Binda S. A novel multiplex one-step real-time RT-PCR assay for the simultaneous identification of enterovirus and parechovirus in clinical fecal samples.J Prev Med Hyg. 2015;56(2):E57-60.PMID: 26789989
3. Poelman R, Schuffenecker I, Van Leer-Buter C, Josset L, Niesters HG, Lina B, ESCV-ECDC EV-D68 study group. European surveillance for enterovirus D68 during the emerging North-American outbreak in 2014.J Clin Virol. 2015;71:1-9. DOI: 10.1016/j.jcv.2015.07.296 PMID: 26364237
4. Piralla A, Girello A, Grignani M, Gozalo-Margüello M, Marchi A, Marseglia G, et al. Phylogenetic characterization of enterovirus 68 strains in patients with respiratory syndromes in Italy. J Med Virol. 2014;86(9):1590-3. DOI: 10.1002/jmv.23821 PMID: 24155220
5. Gong YN, Yang SL, Shih SR, Huang YC, Chang PY, Huang CG, et al. Molecular evolution and the global reemergence of enterovirus D68 by genome-wide analysis. Medicine (Baltimore). 2016;95(31):e4416. DOI: 10.1097/MD.0000000000004416 PMID: 27495059
6. Knoester M, Schölvinck EH, Poelman R, Smit S, Vermont CL, Niesters HG, et al. Upsurge of Enterovirus D68, the Netherlands, 2016. Emerg Infect Dis. 2017;23(1):140-3. DOI: 10.3201/eid2301.161313 PMID: 27660916
7. European Centre for Disease Prevention and Control (ECDC). Rapid risk assessment. Enterovirus detections associated with severe neurological symptoms in children and adults in European countries. Stockholm: ECDC; 8 August 2016. Available from: http://ecdc.europa.eu/en/publications/Publications/01-08-2016-RRA-Enterovirus%2071-Spain,%20France,%20Netherlands.pdf
8. World Health Organization (WHO). WHO-recommended surveillance standard of poliomyelitis. Geneva: WHO. [Accessed 11 Jan 2017]. Available from: http://www.who.int/immunization/monitoring_surveillance/burden/vpd/surveillance_type/active/poliomyelitis_standards/en/

License and copyright