AN ACUTE GASTROENTERITIS OUTBREAK IN A KINDERGARTEN

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ABSTRACT

Objective. To detect the etiological cause of an acute gastroenteritis outbreak at St. Anna kindergarten in the village of Resilovo, region Kyustendil.

Materials and Methods. A total of 22 faecal specimens from children (n = 18) and staff (n = 4) were tested. Multiplex RT-PCR with specific primer pairs detecting the most common viral causes of gastroenteritis (noroviruses, rotaviruses, sapoviruses, intestinal adenoviruses and intestinal astroviruses) was applied to detect the viral causative agent. Noroviruses were detected and sequenced and subsequent phylogenetic analysis was carried out.

Results. Genogroup II noroviruses were detected in five samples from children and one sample from staff (6/22) or in 27.3% of the specimens. According to WHO criteria, this proves that noroviruses have caused the epidemic outbreak. Detected noroviruses were subjected to sequencing and subsequent phylogenetic analysis, with data identifying genotype 17 (GII.17) as the causative agent.

Conclusion. Norovirus genotype 17 (GII.17) was first detected in Bulgaria in 2015 as the causative agent of an outbreak in a secondary school in the town of Pravets. In 2016, the circulation of this genotype was again established in sporadic cases, and in 2022 it was found to be the cause of the epidemic gastroenteritis outbreak in a kindergarten in the village of Resilovo. To protect public health, continuous monitoring and targeted search for viral intestinal agents is essential, regardless of the transient and usually mild course of the disease.

Keywords: multiplex RT-PCR, sequencing, epidemic outbreak

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INTRODUCTION

Viral enteric infections (VEI) are a serious problem worldwide that is sometimes neglected, though diarrheal diseases are at the forefront as causes of mortality (1). Symptoms of acute gastroenteritis (AGE) include general malaise, abdominal pain and cramps, nausea, vomiting, and diarrhea, usually lasting 1 to 5 days, sometimes up to 14 days. The most common viral cause of AGE are rotaviruses (RVs), but with the introduction of vaccines against them, they are increasingly being replaced by noroviruses (NoVs). Other enteric viruses such as sapoviruses (SaVs), astroviruses (AstVs), as well as a few DNA viruses such as the intestinal adenoviruses (AdVs) may also be found in cases of gastroenteritis.

Globally, 1 in 5 cases of gastroenteritis leading to vomiting and diarrhea is caused by noroviruses (2).

Norovirus is a genus from the family *Caliciviridae* and its representatives contain a positive-sense, single-stranded RNA genome. They are naked viruses with icosahedral symmetry of the capsid. The genome contains 3 open reading frames (ORFs) - ORF 1 (which encodes six non-structural proteins, including RNA-dependent RNA polymerase [RdRp]), ORF2 (encodes for the major capsid protein VP1) and ORF3 (encodes for minor capsid protein VP2) (3,4).

The genus is currently subdivided into 10 genogroups and 49 genotypes based on the complete nucleotide sequence encoding the major capsid protein VP1 (5). Genogroups are denoted by the capital Latin letter G and the corresponding Roman numeral, and genotypes are denoted by Arabic numerals.

NoV genotype 17 (GII.17) was established as the dominant one in 2014 in Italy and several other countries around the world, and its circulation in

Bulgaria was detected in 2015 as the causative agent of an outbreak in a secondary school in the town of Pravets.

In May 2022, at St. Anna kindergarten in the village of Resilovo, region Kyustendil, some of the children fell ill.

✓ On May 10th, 2022, a child was brought to the kindergarten after having vomited the previous night. Due to the subsidence of the complaints, he was not left at home (May 9th is the supposed start of the outbreak);

✓ On that day a total of 17 children were in the kindergarten; around noon (11:30 a.m.) one of the children developed symptoms of illness, 30 minutes later another child was sick, and within the next 3-4 hours 7 more children fell ill; in the remaining 5 children symptoms appeared by 9 p.m.;

✓ The symptoms were repeated fountain vomiting and abdominal pain. Fever and diarrhea were not observed;

✓ Two of the children were hospitalized with an initial diagnosis of VEI;

Based on the clinical course that led to vomiting and rapid resolution of symptoms, the infectious disease specialist at the hospital suspected a norovirus infection. To detect the etiological cause of the AGE outbreak in St. Anna kindergarten in the village of Resilovo, region Kyustendil, clinical samples were sent for investigation to the National Reference Laboratory for Enteroviruses at the National Center for Infectious and Parasitic Diseases.

MATERIALS AND METHODS

Sample collection

A total of 22 fecal specimens from children and staff (18 children and 4 staff) were collected and sent for testing in the National Reference Laboratory "Enteroviruses".

Viral RNA extraction and reverse transcription

Viral genomes were extracted from 10% (w/v in phosphate buffer saline) supernatant of stool samples using NucleoSpin® Dx Virus kit based on the spin-column procedure and following the manufacturer's instructions (Macherey-Nagel GmbH & Co KG, Düren, Germany). The reverse transcription (RT) to a complementary DNA was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (hexa-deoxyribonucleotide mixture) (Invitrogen, Carlsbad, CA, USA). Before reverse transcription RNAs were denatured by heating at 95°C for 5 min and cooled on ice. Reverse transcription thermal cycling conditions were as follows: 37⁰C for 1 h and 95°C for 5 min. Copy-DNAs were stored at -20⁰C until required for further analysis.

Viral detection by PCR

Monoplex and multiplex PCR assays with three primer sets comprising eight specific pairs of primers that detected RVs groups A, B and C, NoVs genogroups I and II, sapoviruses, enteric AdVs and AsVs, were applied (6). Sequences of primer pairs are presented in Table 1 (7,8,9).

The DNA amplification was conducted with 2x HotBegan™ Red-Taq Master Mix (Canvax Biotech, S.L., Cordoba, Spain). The master mix in 20 µl total volume was prepared as shown in Table 2 and the PCR was performed at 94⁰C for 10 min, followed by 30 cycles of 94°C for 35 sec, 50°C for 35 sec, 72°C for 1 min, and a final extension at 72°C for 7 min, and then held at 4°C. Positive controls (known cDNA from patient samples for RVs and NoVII) and negative controls were included in each run.

Electrophoresis

Visualization of the PCR products was performed by 2% gel electrophoresis. The presence of the respective viruses was determined based on the size of the expected PCR product corresponding to each virus (Table 1).

Nucleotide Sequencing and Phylogenetic Analysis

A Sanger dideoxy sequencing of the detected NoVs was performed with an automatic sequencer model GenomeLab GeXP (Beckman Coulter, USA). The primers used for sequencing were the same as the ones used for detection. They target the portion of the gene encoding VP 1 (near the ORF1-ORF2 junction, a hot point for mutation). The resulting raw sequences were processed manually using BioEdit v.7.2 computer software (10). The Norovirus genotyping tool (11) was used to determine the genotype/variant of the NoV strain, and the programs BLAST (12) and BioEdit v.7.2 were implemented to compare

Table 1. Specific primers' sequences and size of the expected PCR products for the detection of enteric viruses by multiplex RT-PCR assay.

Set	Target virus	Primer sequences 5'-3'	PCR product size (bp)	Reference
A	RVs group A	F- GGCTTTAAAAGAGAGAATTTC R - ACTGATCCTGTTGGCCATCCTTT	395	[7]
B	RVs group B	F - GGCAATAAAATGGCTTCATTGC R - GGGTTTTTACAGCTTCGGCT	814	[7]
	RVs group C	F - ATTATGCTCAGACTATCGCCAC R - GTTTCTGTACTAGCTGGTGAAC	351	[7]
	AdVs	F - TTCCCCATGGCICAYAACAC R - CCCTGGTAKCCRATRTTGTA	482	[7]
	AsVs	F - GATTGGACTCGATTTGATGG R - CTGGCTTAACCCACATTCC	409	[8]
C	NoVs geno-group I	F - CTGCCCGAATTYGTAAATGA R - CCAACCCARCCATTRTACA	330	[9]
	NoVs geno-group II	F - CARGARBCNATGTTYAGRTGGATGAG R - CCRCCNGCATRHCCRTTRTACAT	387	[9]
	SaVs	F - CTCGCCACCTACRAWGCBTGGTT R - CGGRCYTCAAAVSTACCBCCCCA	434	[9]

Table 2. Ingredients for the performance of the PCR.

Buffer and reagents	Volume		
	Set A	Set B	Set C
2 x Master Mix	10.0 µl	10.0 µl	10.0 µl
F-primer (20 pmoles/µl)	0.5 µl	x 0.5 µl each	x 0.5 µl each
R-primer (20 pmoles/µl)	0.5 µl	x 0.5 µl each	x 0.5 µl each
Sterilized D.W.	6.5 µl	3.5 µl	4.5 µl
Template	2.5 µl	2.5 µl	2.5 µl

the obtained sequence with those published in the GeneBank genome bank (http://blast.ncbi.nlm.nih.gov). The phylogenetic analysis was done by the Neighbor-Joining Method, Kimura 2-parameter, 1,000 bootstrap replications of the MEGA11 program (13).

RESULTS

Genogroup II noroviruses were detected in five samples from children and in one sample from staff (6/22) or in 27.3% of the specimens. According to WHO criteria (14), this proves that NoVs have caused the epidemic outbreak. Detected NoVs were subjected to sequencing with the same pair of primers in Table 1 that detect a part of the VP1 gene encoding the major capsid protein (near the ORF1-ORF2 junction, a hot point for mutation). Four of the samples were successfully sequenced and the genotyping tool identified genotype 17 (GII.17). When comparing the Bulgarian strains with the Genome bank, the greatest similarity was found with those established in Japan, China and Italy, and for this reason these sequences were included in the phylogenetic analysis. Subsequent phylogenetic analysis revealed that the Bulgarian strains from Resilovo were genetically related to GII.17, variant

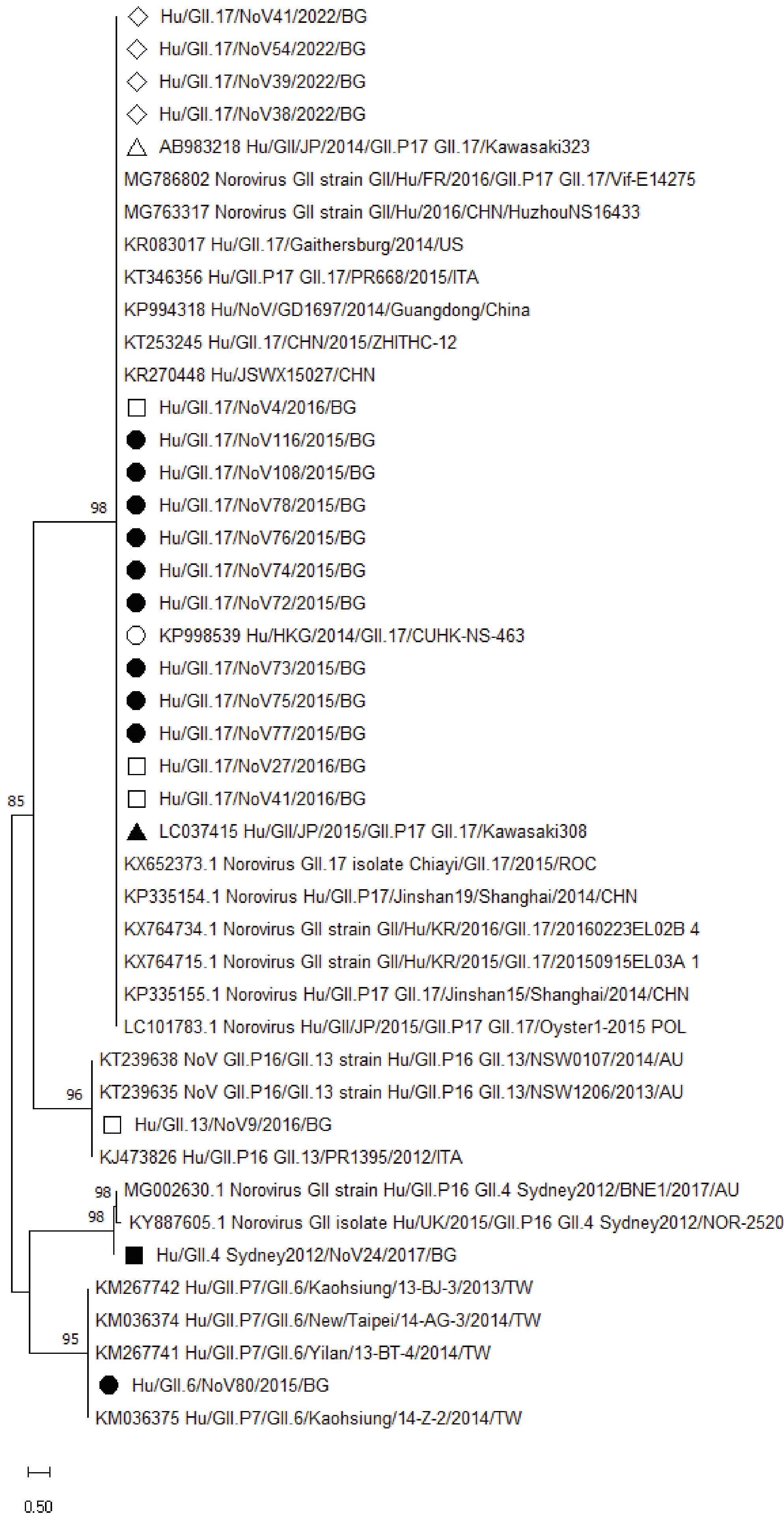

Figure 1. A phylogenetic tree showing the genetic relationships between the Bulgarian norovirus genotypes of genogroup GII and those distributed in Europe and the world regarding part of the VP1 gene encoding the capsid protein. Open circle marks the reference strain for genotype 17; open triangle - variant Kawasaki323, filled triangle - variant Kawasaki308, filled circles - the Bulgarian strains isolated in 2015, open squares – those from 2016, filled squares – from 2017 and 2022, and open diamond - from 2022.

Kawasaki323 proven in 2014 in Japan, as well as to those proven in the period 2014-2016 in the USA, China, Italy and France. Norovirus strains isolated in Pravets (2015) and from sporadic cases in our country (2016) were similar to the Kawasaki308 variant, proven in Japan in 2015, as well as in China, Poland and Korea (Figure 1).

These results conclusively demonstrated that noroviruses of genotype 17 were the cause of the acute gastroenteritis outbreak that occurred at the kindergarten.

DISCUSSION

Cases of VEI caused by rotaviruses are subject to monitoring and reporting in Bulgaria. Very rarely, other causes of acute gastroenteritis are sought. However, in case an epidemic outbreak of gastroenteritis involving the members of a closed collective (kindergarten, school, mass events with a common source of food and/or water) is suspected, samples for diagnosis of other enteric viruses are also sent to the reference laboratory.

During the hospitalization of two children attending the kindergarten in the village of Resilovo with characteristic symptoms, the infectious disease specialist of the hospital suspected a norovirus as the causative agent . Consequently, samples from children, as well as from some of the staff members were sent to NRL "Enteroviruses" for investigation.

Genogroup II noroviruses were detected in six samples (five samples from children and in one sample from staff) or in 27.3% (6/22) of the specimens. Four of the samples were successfully sequenced and the genotyping tool identified genotype 17 (GII.17).

Until recently, the dominant genotype causing epidemic outbreaks of AGE worldwide was GII.4, characterized by rapid evolution rates through mutations and recombinations. For this reason, a new variant capable of causing outbreaks appeared every 2-3 years.

For nearly 10 years, in several European and Asian countries, another genotype has been dominant in sporadic cases or epidemic outbreaks, i.e. GII.17.

NoV genotype 17 (GII.17) was first described in the late 1970s in French Guiana (15) and has periodically been associated with sporadic cases or outbreaks of AGE in Africa, Asia and Europe. During the 2014/2015 season, it replaced the dominant at that time GII.4 Sydney in several countries worldwide - China, Japan, Italy, Romania, and the Netherlands (16-21).

GII.17 was first detected in Bulgaria in 2015 as the causative agent of an AGE outbreak in a secondary school in the town of Pravets.

Subsequent phylogenetic analyses revealed that the Bulgarian strains from Resilovo were genetically related to GII.17, variant Kawasaki323 proven in 2014 in Japan, as well as to those proven in the period 2014-2016 in the USA, China, Italy and France. Norovirus strains from Pravets (2015) and sporadic cases (2016) in our country, showed similarity with the Kawasaki308 variant, proven in Japan in 2015, as well as in China, Poland and Korea.

In-depth studies, based on sequencing and phylogenetic analysis, show the specific genotype of the causative agent. The sufficiently large genetic similarity between the proven noroviruses, as well as the available epidemiological data, conclusively pointed out the cause of the epidemic outbreak.

To protect public health, continuous monitoring and targeted search for viral intestinal agents are essential, regardless of the transient and usually mild course of the disease. Also, continuous monitoring ensures the timely detection of new genotypes and/ or variants with serious epidemic potential .

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REFERENCES

1. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (accessed November 2nd, 2023)
2. https://www.cdc.gov/norovirus/burden.html
3. Jiang, X.; Wang, M.; Wang, K.; Estes, M.K. *Sequence and Genomic Organization of Norwalk Virus*. Virol 1993;195:51–61. http://doi.org/10.1006/viro.1993.1345
4. Thorne, L.G.; Goodfellow, I.G. *Norovirus Gene Expression and Replication*. J. Gen. Virol. 2014;95:278–291. http://doi.org/10.1099/vir.0.059634-0
5. Chhabra, P.; de Graaf, M.; Parra, G.I.; Chan, M.C.; Green, K.; Martella, V.; Wang, Q.; White, P.E.; Katayama, K.; Vennema, H.; et al. *Updated classification of norovirus genogroups and genotypes*. J. Gen. Virol. 2019;100:1393–1406. http://doi.org/10.1099/jgv.0.001318

6. Thongprachum A, Khamrin P, Pham NT, Takanashi S, Okitsu S, Shimizu H, Maneekarn N, Hayakawa S, Ushijima H. *Multiplex RT-PCR for rapid detection of viruses commonly causing diarrhea in pediatric patients* J Med Virol 2017;89: 818-824. http://doi.org/10.1002/jmv.24711
7. Yan H, Nguyen TA, Phan TG, Okitsu S, Li Y, Ushijima H. *Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotaviruses in diarrheal fecal specimens from children in China*. Kansenshogaku zasshi 2004;78: 699-709. http://doi.org/10.11150/kansenshogakuzasshi1970.78.699
8. Finkbeiner SR, Kirkwood CD, Wang D. *Complete genome sequence of a highly divergent astrovirus isolated from a child with acute diarrhea*. Virol J.2008;5: 117. http://doi.org/10.1186/1743-422X-5-117
9. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. *Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR*. J Virol Methods 2003;114: 37-44. http://doi.org/10.1016/j.jviromet.2003.08.009
10. http://www.mbio.ncsu.edu/BioEdit/bioedit.html
11. Kroneman A, Vennema H, Deforche K, v d Avoort H, Penaranda S, Oberste MS, et al. *An automated genotyping tool for enteroviruses and noroviruses*. J Clin Virol. 2011;51(2):121-125. http://doi.org/10.1016/j.jcv.2011.03.006 http://www.rivm.nl/mpf/norovirus/typingtool
12. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome
13. Tamura K, Stecher G, Kumar S. MEGA11: *Molecular Evolutionary Genetics Analysis version 11.0* Mol.Biol. Evol.2021;38(7):3022–3027. http://doi.org/10.1093/molbev/msab120
14. https://www.cdc.gov/norovirus/outbreaks/responding.html
15. Rackoff LA, Bok K, Green KY, Kapikian AZ. *Epidemiology and evolution of rotaviruses and noroviruses from an archival WHO Global Study in Children (1976-79) with implications for vaccine design*. PLoS One. 2013;8(3):e59394. http://doi.org/10.1371/journal.pone.0059394
16. Fu J, Ai J, Jin M, Jiang C, Zhang J, Shi C, et al. *Emergence of a new GII.17 norovirus variant in patients with acute gastroenteritis in Jiangsu, China, September 2014 to March 2015*. Euro Surveill. 2015;20(24):21157. http://doi.org/10.2807/1560-7917.es2015.20.24.21157
17. Lu J, Sun L, Fang L, Yang F, Mo Y, Lao J, et al. *Gastroenteritis Outbreaks Caused by Norovirus GII.17, Guangdong Province, China, 2014-2015*. Emerg Infect Dis. 2015;21(7):1240-1242. http://doi.org/10.3201/eid2107.150226
18. Matsushima Y, Ishikawa M, Shimizu T, Komane A, Kasuo S, Shinohara M, et al. *Genetic analyses of GII.17 norovirus strains in diarrheal disease outbreaks from December 2014 to March 2015 in Japan reveal a novel polymerase sequence and amino acid substitutions in the capsid region*. Euro Surveill. 2015;20(26):21173. http://doi.org/10.2807/1560-7917.es2015.20.26.21173
19. Medici MC, Tummolo F, Calderaro A, Chironna M, Giammanco GM, De Grazia S, et al. *Identification of the novel Kawasaki 2014 GII.17 human norovirus strain in Italy, 2015*. Euro Surveill. 2015;20(35):30010. http://doi.org/10.2807/1560-7917.ES.2015.20.35.30010
20. Dinu S, Nagy M, Negru D, Popovici E, Zota L, Oprișan G. *Molecular identification of emergent GII.P17-GII.17 norovirus genotype, Romania, 2015*. Euro Surveill. 2016;21(7):30141. http://doi.org/10.2807/1560-7917.ES.2016.21.7.30141
21. de Graaf M, van Beek J, Vennema H, Podkolzin AT, Hewitt J, Bucardo F, et al. *Emergence of a novel GII.17 norovirus – End of the GII.4 era?* Euro Surveill. 2015;20(26):21178. http://doi.org/10.2807/1560-7917.es2015.20.26.21178