Impact factor 6.15

Eurosurveillance

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

Vol. 18 | Weekly issue 16 | 18 April 2013

Rapid communications

Surveillance and outbreak reports

News

ecdc EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

www.eurosurveillance.org

Guiding outbreak management by the use of influenza A(H7Nx) virus sequence analysis

M Jonges (marcel.jonges@rivm.nl)[1,2], A Meijer[1], R A Fouchier[2], G Koch[3], J Li[4], J C Pan[4], H Chen[5], Y L Shu[6], M P Koopmans[1,2]

1. Department of Virology, Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
2. Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
3. Central Veterinary Institute, Wageningen University and Research Center, Lelystad, the Netherlands
4. Hangzhou Center for Disease Control and Prevention, Hangzhou, China
5. National Avian Influenza Reference Laboratory, Harbin Veterinary Research Institute, Harbin, China
6. National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

Citation style for this article:
Jonges M, Meijer A, Fouchier RA, Koch G, Li J, Pan JC, Chen H, Shu YL, Koopmans MP. Guiding outbreak management by the use of influenza A(H7Nx) virus sequence analysis. Euro Surveill. 2013;18(16):pii=20460. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20460

Article submitted on 15 April 2013 / published on 18 April 2013

The recently identified human infections with avian influenza A(H7N9) viruses in China raise important questions regarding possible source and risk to humans. Sequence comparison with an influenza A(H7N7) outbreak in the Netherlands in 2003 and an A(H7N1) epidemic in Italy in 1999–2000 suggests that widespread circulation of A(H7N9) viruses must have occurred in China. The emergence of human adaptation marker PB2 E627K in human A(H7N9) cases parallels that of the fatal A(H7N7) human case in the Netherlands.

Background

Since 31 March 2013, Chinese health authorities have been reporting human cases of avian influenza A(H7N9) virus infections. This novel reassortant influenza virus, carrying six internal gene segments of poultry A(H9N2) viruses, supplemented with a haemagglutinin (HA) subtype 7 and a neuraminidase (NA) subtype 9 originating from wild birds [1,2], has caused infections in at least 82 persons, of whom 17 have died, as of 17 April 2013. The human infections occurred in eastern China in four provinces (Henan, Anhui, Jiangsu, and Zhejiang) and two municipalities (Shanghai and Beijing). Currently, the source of the human infections is unclear. However, in response to the detection of the influenza A(H7N9) virus among chickens, pigeons, ducks and environmental samples from some animal markets, as reported to the World Organisation for Animal Health (OIE), Chinese authorities have suspended live poultry trade and implemented the immediate closure of poultry markets, launched road inspections for transport of poultry, and have culled birds in an effort to deal with the issue. The outbreak raises important questions regarding possible source and risk to humans, and these will be addressed through case investigations. Here, we compare some findings from the first two weeks of the outbreak with those from a large highly pathogenic avian influenza (HPAI) A(H7N7) virus outbreak in the Netherlands in 2003 and from a low pathogenic avian influenza (LPAI) A(H7N1) epidemic in Italy in 1999–2000 [3-5] and discuss issues related to diagnosis and the use of molecular surveillance to monitor the outbreak.

Influenza A(H7N7) outbreak in the Netherlands in 2003

Exactly 10 years ago, the Netherlands was struck by an HPAI A(H7N7) virus outbreak that resulted in the infection of poultry on 255 farms and the subsequent culling of about 30 million chickens. A total of 453 exposed persons had mild symptoms and were investigated, of whom 89 were laboratory-confirmed as having an A(H7N7) virus infection [6,7].

Diagnosis of influenza A(H7Nx) virus infection

During the HPAI A(H7N7) virus outbreak in the Netherlands, almost all human cases had mild symptoms, particularly conjunctivitis, but one veterinarian died after an episode of severe influenza-like illness complicated by acute respiratory distress syndrome (ARDS) [7] . Diagnosis was based on virus detection by reverse transcription polymerase chain reaction (RT-PCR) from eye swabs, or combined nose and throat swabs. An important observation was that the sensitivity of eye swab-based diagnostics was much higher than that of diagnostics based on combined nose and throat swabs [6,7]. Similarly, in later sporadic infections of humans with H7 influenza A viruses, ocular symptoms were observed, probably caused by a preference of H7 influenza viruses for receptors in the eye [8]. Studies have shown that H7 influenza viruses may use the ocular mucosa as portal of entry for systemic infection and that this is strain dependent [9,10]. Such symptoms have not been described for the cases of A(H7N9) virus infection in China in 2013, but it may be important to actively monitor for conjunctivitis in the outbreak investigation, as it may increase the success of case finding, particularly for mild cases.

Table, panel A

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI439488	PB2	China	2013-Jan-01	A/Shanghai/1/2013		WHO Chinese National Influenza Center	
EPI439486	HA	China	2013-Jan-01	A/Shanghai/1/2013		WHO Chinese National Influenza Center	
EPI439487	NA	China	2013-Jan-01	A/Shanghai/1/2013		WHO Chinese National Influenza Center	
EPI439495	PB2	China	2013-Jan-01	A/Shanghai/2/2013		WHO Chinese National Influenza Center	
EPI439500	NA	China	2013-Jan-01	A/Shanghai/2/2013		WHO Chinese National Influenza Center	
EPI439502	HA	China	2013-Jan-01	A/Shanghai/2/2013		WHO Chinese National Influenza Center	
EPI439504	PB2	China	2013-Jan-01	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI439507	HA	China	2013-Jan-01	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI439509	NA	China	2013-Jan-01	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI441601	PB2	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li,J; Pan,JC; Pu,XY; Yu,XF; Kou,Y; Zhou,YY
EPI440095	HA	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li,J; Pan,JC; Pu,XY; Yu,XF; Kou,Y; Zhou,YY
EPI440096	NA	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li,J; Pan,JC; Pu,XY; Yu,XF; Kou,Y; Zhou,YY
EPI440682	PB2	China	2013-Apr-03	A/Chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440684	NA	China	2013-Apr-03	A/Chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440685	HA	China	2013-Apr-03	A/Chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440690	PB2	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440692	NA	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440693	HA	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440698	PB2	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440700	NA	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440701	HA	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database, on which this research is based.

Table, panel B

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI238407	HA	Italy	1999-Jan-01	A/chicken/Italy/1067/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238409	NA	Italy	1999-Jan-01	A/chicken/Italy/1067/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238414	PB2	Italy	1999-Jan-01	A/chicken/Italy/1067/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI63315	HA	Italy	1999-Jan-01	A/chicken/Italy/1082/1999	Other Database Import	
EPI63320	NA	Italy	1999-Jan-01	A/chicken/Italy/1082/1999	Other Database Import	
EPI63332	PB2	Italy	1999-Jan-01	A/chicken/Italy/1082/1999	Other Database Import	
EPI63258	HA	Italy	1999-Dec-21	A/chicken/Italy/4746/1999	Other Database Import	
EPI63263	NA	Italy	1999-Dec-21	A/chicken/Italy/4746/1999	Other Database Import	
EPI63275	PB2	Italy	1999-Dec-21	A/chicken/Italy/4746/1999	Other Database Import	
EPI69063	HA	Italy	1999-Dec-22	A/chicken/Italy/4789/1999	Other Database Import	
EPI69068	NA	Italy	1999-Dec-22	A/chicken/Italy/4789/1999	Other Database Import	
EPI69080	PB2	Italy	1999-Dec-22	A/chicken/Italy/4789/1999	Other Database Import	
EPI107331	PB2	Italy	1999-Jan-01	A/chicken/Italy/5093/99	Other Database Import	
EPI107338	HA	Italy	1999-Jan-01	A/chicken/Italy/5093/99	Other Database Import	
EPI107342	NA	Italy	1999-Jan-01	A/chicken/Italy/5093/99	Other Database Import	
EPI69044	HA	Italy	1999-Dec-30	A/quail/Italy/4992/1999	Other Database Import	
EPI69049	NA	Italy	1999-Dec-30	A/quail/Italy/4992/1999	Other Database Import	
EPI69061	PB2	Italy	1999-Dec-30	A/quail/Italy/4992/1999	Other Database Import	
EPI69215	HA	Italy	1999-Apr-10	A/turkey/Italy/1265/99	Other Database Import	
EPI69220	NA	Italy	1999-Apr-10	A/turkey/Italy/1265/99	Other Database Import	
EPI69232	PB2	Italy	1999-Apr-10	A/turkey/Italy/1265/99	Other Database Import	
EPI69177	HA	Italy	1999-Jul-12	A/turkey/Italy/2715/99	Other Database Import	
EPI69182	NA	Italy	1999-Jul-12	A/turkey/Italy/2715/99	Other Database Import	
EPI69194	PB2	Italy	1999-Jul-12	A/turkey/Italy/2715/99	Other Database Import	
EPI238618	HA	Italy	1999-Jan-01	A/turkey/Italy/2732/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238619	NA	Italy	1999-Jan-01	A/turkey/Italy/2732/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238624	PB2	Italy	1999-Jan-01	A/turkey/Italy/2732/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI68484	HA	Italy	1999-Sep-03	A/turkey/Italy/3185/99	Other Database Import	
EPI68489	NA	Italy	1999-Sep-03	A/turkey/Italy/3185/99	Other Database Import	
EPI68501	PB2	Italy	1999-Sep-03	A/turkey/Italy/3185/99	Other Database Import	
EPI238625	HA	Italy	1999-Jan-01	A/turkey/Italy/3283/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238627	NA	Italy	1999-Jan-01	A/turkey/Italy/3283/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238632	PB2	Italy	1999-Jan-01	A/turkey/Italy/3283/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database, on which this research is based.

Table, panel C

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI68503	HA	Italy	1999-Sep-23	A/turkey/Italy/3488/1999	Other Database Import	
EPI68508	NA	Italy	1999-Sep-23	A/turkey/Italy/3488/1999	Other Database Import	
EPI68520	PB2	Italy	1999-Sep-23	A/turkey/Italy/3488/1999	Other Database Import	
EPI69139	HA	Italy	1999-Sep-23	A/turkey/Italy/3489/99	Other Database Import	
EPI69144	NA	Italy	1999-Sep-23	A/turkey/Italy/3489/99	Other Database Import	
EPI69156	PB2	Italy	1999-Sep-23	A/turkey/Italy/3489/99	Other Database Import	
EPI69158	HA	Italy	1999-Sep-27	A/turkey/Italy/3560/99	Other Database Import	
EPI69163	NA	Italy	1999-Sep-27	A/turkey/Italy/3560/99	Other Database Import	
EPI69175	PB2	Italy	1999-Sep-27	A/turkey/Italy/3560/99	Other Database Import	
EPI238633	HA	Italy	1999-Jan-01	A/turkey/Italy/3675/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238635	NA	Italy	1999-Jan-01	A/turkey/Italy/3675/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238640	PB2	Italy	1999-Jan-01	A/turkey/Italy/3675/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI42171	PB2	Italy	1999-Jan-01	A/turkey/Italy/4169/99	Other Database Import	
EPI89837	NA	Italy	1999-Jan-01	A/turkey/Italy/4169/99	Other Database Import	
EPI90388	HA	Italy	1999-Jan-01	A/turkey/Italy/4169/99	Other Database Import	
EPI69082	HA	Italy	1999-Nov-22	A/turkey/Italy/4294/99	Other Database Import	
EPI69087	NA	Italy	1999-Nov-22	A/turkey/Italy/4294/99	Other Database Import	
EPI69099	PB2	Italy	1999-Nov-22	A/turkey/Italy/4294/99	Other Database Import	
EPI68465	HA	Italy	1999-Nov-22	A/turkey/Italy/4295/1999	Other Database Import	
EPI68470	NA	Italy	1999-Nov-22	A/turkey/Italy/4295/1999	Other Database Import	
EPI68482	PB2	Italy	1999-Nov-22	A/turkey/Italy/4295/1999	Other Database Import	
EPI69125	NA	Italy	1999-Nov-22	A/turkey/Italy/4301/1999	Other Database Import	
EPI69137	PB2	Italy	1999-Nov-22	A/turkey/Italy/4301/1999	Other Database Import	
EPI69120	HA	Italy	1999-Nov-22	A/turkey/Italy/4301/1999	Other Database Import	
EPI238648	HA	Italy	1999-Jan-01	A/turkey/Italy/4482/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238650	NA	Italy	1999-Jan-01	A/turkey/Italy/4482/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238655	PB2	Italy	1999-Jan-01	A/turkey/Italy/4482/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238656	HA	Italy	1999-Jan-01	A/turkey/Italy/4580/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238658	NA	Italy	1999-Jan-01	A/turkey/Italy/4580/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238663	PB2	Italy	1999-Jan-01	A/turkey/Italy/4580/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI69101	HA	Italy	1999-Dec-14	A/turkey/Italy/4617/1999	Other Database Import	
EPI69106	NA	Italy	1999-Dec-14	A/turkey/Italy/4617/1999	Other Database Import	
EPI69118	PB2	Italy	1999-Dec-14	A/turkey/Italy/4617/1999	Other Database Import	

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database, on which this research is based.

TABLE, PANEL D

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI69025	HA	Italy	1999-Dec-16	A/turkey/Italy/4644/99	Other Database Import	
EPI69030	NA	Italy	1999-Dec-16	A/turkey/Italy/4644/99	Other Database Import	
EPI69042	PB2	Italy	1999-Dec-16	A/turkey/Italy/4644/99	Other Database Import	
EPI68427	HA	Italy	1999-Dec-21	A/turkey/Italy/4708/1999	Other Database Import	
EPI68432	NA	Italy	1999-Dec-21	A/turkey/Italy/4708/1999	Other Database Import	
EPI68444	PB2	Italy	1999-Dec-21	A/turkey/Italy/4708/1999	Other Database Import	
EPI238664	HA	Italy	1999-Jan-01	A/turkey/Italy/977/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238666	NA	Italy	1999-Jan-01	A/turkey/Italy/977/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.
EPI238671	PB2	Italy	1999-Jan-01	A/turkey/Italy/977/1999	Other Database Import	Kim,L.M.; Scott,M.A.; Suarez,D.L.; Spackman,E.; Swayne,D.E.; Afonso,C.L.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database, on which this research is based.

Serological surveillance is important to rule out infection in patients sampled too late for direct virus detection and to assess the extent of transmission. This may be a problem since serological responses in persons with confirmed influenza A(H7Nx) virus infection have been difficult to detect, making assessment of A(H7N9) virus exposure using serosurveys challenging [11,12]. However, determining the kinetics of the antibody response in confirmed cases of influenza A(H7N9) virus infection will provide important information that can inform public health action.

Comparative analysis based on virus sequencing

Detecting the novel virus in animals is challenging as the A(H7N9) virus is a LPAI virus that is expected to cause few or no signs of disease in poultry, allowing silent spread among poultry flocks. The sharing of influenza A(H7N9) virus sequence data by both Chinese veterinary and public health institutes through the Global Initiative on Sharing All Influenza Data (GISAID) allows comparison with the sequences obtained during the Dutch outbreak. We therefore performed a comparative analysis using HA, NA and PB2 (subunit of the influenza virus RNA polymerase complex) fragment sequences from Chinese A(H7N9) viruses in 2013, Dutch A(H7N7) viruses in 2003 and sequences from a well-described LPAI A(H7N1) epidemic in Italy in 1999–2000 [5]. Providers of sequences downloaded from GISAID, listed with accession numbers, are acknowledged in the Table.

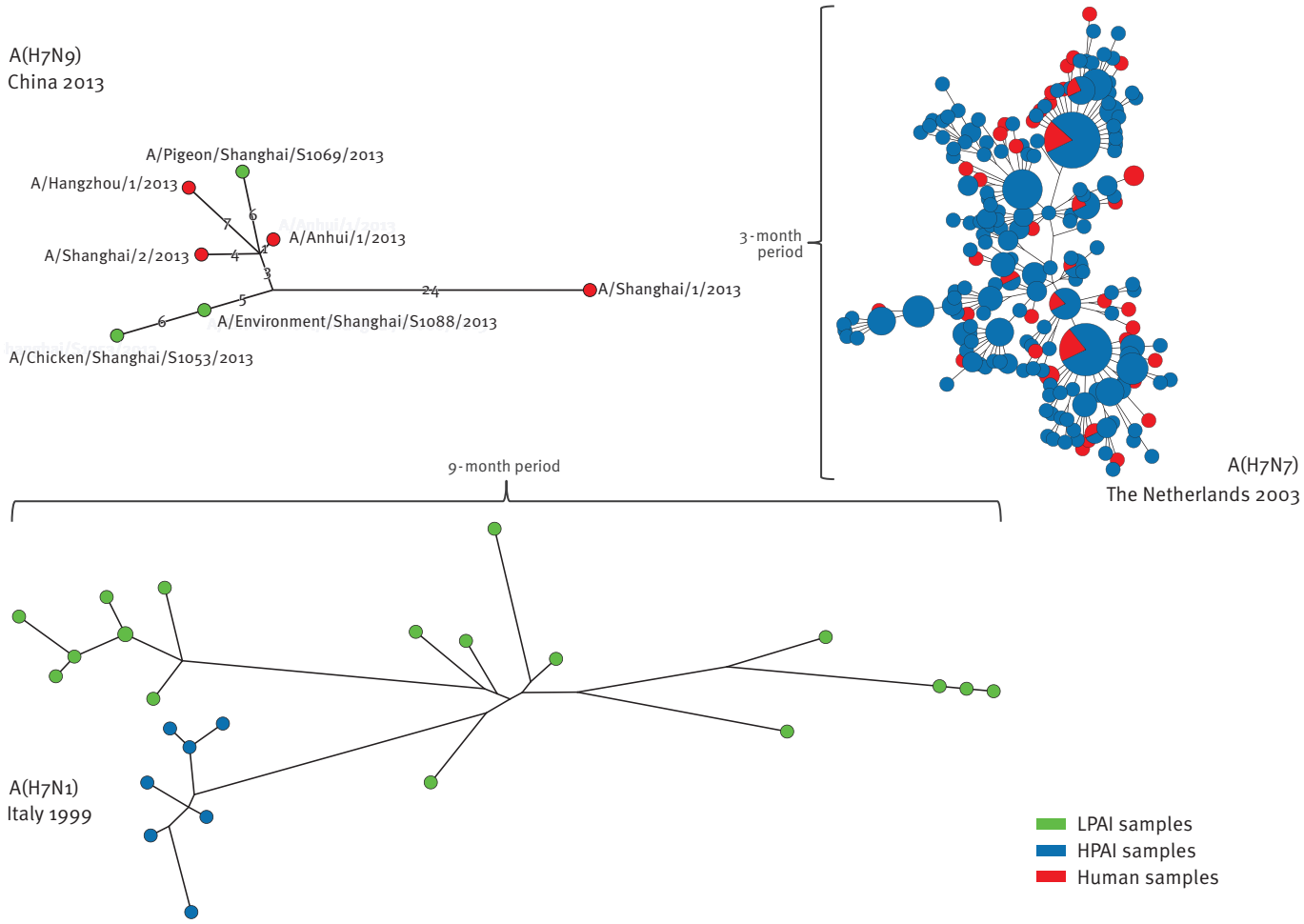
Sequence analysis of the Dutch viruses detected in poultry and in humans showed rapid diversification of the outbreak strain into multiple lineages (Figure). On the basis of the combined epidemiological and laboratory analyses, we demonstrated that sequences from humans were positioned mostly at ends of the branches of minimal spanning trees, confirming that humans were probably not involved in onward transmission [3].

In the current study, we compared the sequence diversity observed during the Dutch A(H7N7) outbreak and Italian A(H7N1) epidemic with the initial A(H7N9) virus sequences from the current outbreak in China. The maximum genetic distance generated during the three months of the Dutch HPAI A(H7N7) outbreak in concatenated HA, NA and PB2 segments of A(H7N7) viruses was 25 nucleotide substitutions. For the Italian LPAI A(H7N1) epidemic, the distance generated during a nine-month period was 66 nucleotide substitutions. For the A(H7N9) outbreak strains, this genetic distance is 35 substitutions, or 21 substitutions when the outlier strain A/Shanghai/1/2013 is ignored (Figure).

All (n=7) NA sequences of the A(H7N9) viruses are characterised by a deletion in the stalk region, associated with adaptation to gallinaceous hosts [1,2,13]. Similar deletions in the NA stalk were also observed during the A(H7N7) outbreak in the Netherlands and the A(H7N1) epidemic in Italy [5]. Given the degree of

Figure

Genetic diversity of three influenza A(H7Nx) virus outbreaks expressed by minimum spanning trees



HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza.

The minimum spanning trees were constructed using concatenated haemagglutinin, neuraminidase and PB2 (subunit of the influenza virus RNA polymerase complex) nucleotide sequences in BioNumerics software version 6.6.4. The scaling of the branches, representing nucleotide substitutions, is equal for the three outbreaks.

sequence diversity present in initial A(H7N9) virus sequences, compared with that of the Dutch HPAI A(H7N7) and Italian LPAI A(H7N1) outbreak strains, and the large geographical area affected, the data are suggestive of (silent) spread and adaptation in domestic animals before the novel A(H7N9) virus was identified in humans.

Human adaptation markers

The majority of the Dutch human cases of A(H7N7) virus infection had mild symptoms, with the exception of one fatal case who was diagnosed with an A(H7N7) virus with the mammalian adaptation marker PB2 E627K. This mutation most probably occurred during infection of this case and was associated with high virulence [14]. Remarkably, the PB2 segments of the four available human virus genome sequences from China all carry this E627K substitution, which is absent in the virus isolates obtained from birds and the environment [2]. In addition, three of the four infections with the virus with PB2 E627K were fatal. There are two plausible explanations for this observation:

1. the mammalian adaptation markers are selected during replication in humans following exposure to viruses that do not have this mutation, which are circulating in animals;

2. the mammalian adaptation markers result from virus replication in animals from which humans become infected.

The relatively protracted disease course in the current outbreak of A(H7N9) virus infection, with relatively mild symptoms at first, followed by exacerbation in the course of a week or longer, is suggestive of the first hypothesis, similar to the outbreak in the Netherlands. In this scenario, an important difference in the A(H7N7) observations from the Netherlands is the frequency of finding the PB2 E627K mutation in humans (4/4 A(H7N9) sequenced patient strains compared with 1/61 sequenced A(H7N7) patient strains). Therefore, an outstanding question is whether the A(H7N9) viruses are more readily mutating in humans or milder cases are being missed. Contact investigations have found no mild cases and only one asymptomatic case), but in order to address this issue, more enhanced testing of persons exposed to a similar source is needed, using the most sensitive tests available on the optimal clinical specimen type obtained at the right time.

Although human infections with H7 influenza viruses have occurred repeatedly over the last decades without evidence of sustained human-to-human transmission, the absence of sustained human-to-human transmission of A(H7N9) viruses does not come with any guarantee. Five of seven A(H7N9) virus strains obtained from humans (n=2), birds (n=2) and the environment (n=1) have a mutation in HA, Q226L, that is associated with binding to alpha(2,6)-linked sialic acids, the virus receptors in the human upper respiratory tract [2]. This Q226L substitution in combination with G228S has been associated with human receptor preference for influenza viruses that caused the pandemics of 1957 and 1968 and with airborne transmission of A(H5N1) virus [15,16]. For H7 viruses, it has recently been demonstrated that these mutations also increased human receptor-binding affinity [17]. In combination with the PB2 E627K mutation, the A(H7N9) virus thus contains two well-known mammalian adaptation markers.

Conclusion

Comparative analysis of the first virological findings from the current outbreak of influenza A(H7N9) virus infection in China with those from other influenza A(H7Nx) outbreaks suggests that widespread circulation must have occurred, resulting in major genetic diversification. Such diversification is of concern, given that several markers associated with increased risk for public health are already present. Enhanced monitoring of avian and mammalian animal reservoirs is of utmost importance as the public health risk of these A(H7N9) viruses may change following limited additional modification.

Acknowledgements

This work was supported by the Dutch Ministry of Economic Affairs, Agriculture, and Innovation, Castellum Project.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database (www.gisaid.org), on which this research is based.

Authors' contributions

Yue-Long Shu, Hualan Chen, Jun Li, Jing-Cao Pan, Ron A.M. Fouchier and Guus Koch improved the manuscript following writing by Marcel Jonges, Adam Meijer and Marion Koopmans. All authors were directly involved in the generation, sharing and analysis of influenza sequence data.

Conflict of interest

None declared.

References

3. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013 Apr 11. [Epub ahead of print].
4. Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, et al. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. . Euro Surveill. 2013;18(15):pii=20453. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20453
5. Jonges M, Bataille A, Enserink R, Meijer A, Fouchier RA, Stegeman A, et al. Comparative analysis of avian influenza virus diversity in poultry and humans during a highly pathogenic avian influenza A (H7N7) virus outbreak. J Virol. 2011;85(20):10598-604. http://dx.doi.org/10.1128/JVI.05369-11 PMid:21849451 PMCid:3187520
6. Bataille A, van der Meer F, Stegeman A, Koch G. Evolutionary analysis of inter-farm transmission dynamics in a highly pathogenic avian influenza epidemic. PLoS Pathog. 2011;7(6):e1002094.
7. Capua I, Mutinelli F, Pozza MD, Donatelli I, Puzelli S, Cancellotti FM. The 1999-2000 avian influenza (H7N1) epidemic in Italy: veterinary and human health implications. Acta Trop. 2002;83(1):7-11. http://dx.doi.org/10.1016/S0001-706X(02)00057-8
8. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, et al. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet. 2004;363(9409):587-93. http://dx.doi.org/10.1016/S0140-6736(04)15589-X
9. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A. 2004;101(5):1356-61. http://dx.doi.org/10.1073/pnas.0308352100 PMid:14745020 PMCid:337057
10. Olofsson S, Kumlin U, Dimock K, Arnberg N. Avian influenza and sialic acid receptors: more than meets the eye? Lancet Infect Dis. 2005;5(3):184-8.
11. Belser JA, Wadford DA, Xu J, Katz JM, Tumpey TM. Ocular infection of mice with influenza A (H7) viruses: a site of primary replication and spread to the respiratory tract. J Virol. 2009;83(14):7075-84. http://dx.doi.org/10.1128/JVI.00535-09 PMid:19458003 PMCid:2704783
12. Belser JA, Davis CT, Balish A, Edwards LE, Zeng H, Maines TR, et al. Pathogenesis, transmissibility, and ocular tropism of a highly pathogenic avian influenza A (H7N3) virus associated with human conjunctivitis. J Virol. 2013 Mar 13. [Epub ahead of print].
13. Meijer A, Bosman A, van de Kamp EE, Wilbrink B, Du Ry van Beest Holle M, Koopmans M. Measurement of antibodies to avian influenza virus A(H7N7) in humans by hemagglutination inhibition test. J Virol Methods. 2006;132(1-2):113-20.
14. Min JY, Vogel L, Matsuoka Y, Lu B, Swayne D, Jin H, et al. A live attenuated H7N7 candidate vaccine virus induces neutralizing antibody that confers protection from challenge in mice, ferrets, and monkeys. J Virol. 2010;84(22):11950-60. http://dx.doi.org/10.1128/JVI.01305-10 PMid:20810733 PMCid:2977864
15. Li J, Zu Dohna H, Cardona CJ, Miller J, Carpenter TE. Emergence and genetic variation of neuraminidase stalk deletions in avian influenza viruses. PLoS One. 2011;6(2):e14722.
16. de Wit E, Munster VJ, van Riel D, Beyer WE, Rimmelzwaan GF, Kuiken T, et al. Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. J Virol. 2010;84(3):1597-606. http://dx.doi.org/10.1128/JVI.01783-09 PMid:19939933 PMCid:2812334
17. Pappas C, Viswanathan K, Chandrasekaran A, Raman R, Katz JM, Sasisekharan R, et al. Receptor specificity and transmission of H2N2 subtype viruses isolated from the pandemic of 1957. PLoS One. 2010;5(6):e11158.
18. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336(6088):1534-41. http://dx.doi.org/10.1126/science.1213362 PMid:22723413
19. Srinivasan K, Raman R, Jayaraman A, Viswanathan K, Sasisekharan R. Quantitative description of glycan-receptor binding of influenza A virus H7 hemagglutinin. PLoS One. 2013;8(2):e49597.

Specific detection by real-time reverse-transcription PCR assays of a novel avian influenza A(H7N9) strain associated with human spillover infections in China

V M Corman[1], M Eickmann[2], O Landt[3], T Bleicker[1], S Brünink[1], M Eschbach-Bludau[1], M Matrosovich[2], S Becker[2], C Drosten (drosten@virology-bonn.de)[1]
1. Institute of Virology, University of Bonn Medical Centre, Bonn, Germany
2. Institute for Virology, University of Marburg, Marburg, Germany
3. TibMolbiol, Berlin, Germany

Citation style for this article:
Corman VM, Eickmann M, Landt O, Bleicker T, Brünink S, Eschbach-Bludau M, Matrosovich M, Becker S, Drosten C. Specific detection by real-time reverse-transcription reaction assays of a novel avian influenza A(H7N9) strain associated with human spillover infections in China. Euro Surveill. 2013;18(16):pii=20461. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20461

Article submitted on 12 April 2013 / published on 18 April 2013

In response to a recent outbreak in China, detection assays for a novel avian influenza A(H7N9) virus need to be implemented in a large number of public health laboratories. Here we present real-time reverse-transcription polymerase chain reaction (RT-PCR) assays for specific detection of this virus, along with clinical validation data and biologically-safe positive controls.

Background

An avian influenza A(H7N9) virus has emerged in south eastern China in March 2013 [1]. As of 16 April 2013, the Chinese authorities have reported 63 laboratory-confirmed human cases, 14 of whom have died [2]. While epidemiological data suggest no direct human-to-human transmission, there is huge concern that the presence of mutations typical for mammalian-adapted influenza A viruses such as E627K in the polymerase basic protein 2 (PB2) gene might indicate a certain propensity of the virus to further adapt to humans [1,3]. Even in absence of proven human-to-human transmission, the emergence of the avian influenza A(H7N9) virus in humans constitutes a test scenario for pandemic preparedness.

The rapid deployment of diagnostic methodology is among the top priorities in laboratory-based pandemic response. While capacities and responsibilities are in place in many countries, the actual provision of test technology involves major challenges, including the necessity to provide validation data for new test protocols, as well as the need for qualified and safe biological materials suitable as positive controls. In particular, positive controls based on in-vitro transcribed RNA containing only small fragments of the viral genome can be shipped without biosafety concerns. We already started using this option for the wide distribution of diagnostic tests during the severe acute respiratory syndrome (SARS) epidemic in 2003, and made use of it several times thereafter [4-6]. In response to the emergence of HCoV-EMC in 2012 we provided validated protocols along with positive controls through a European Union (EU) research network. This strategy enabled implementation of diagnostic capacity across the EU within only a few weeks [7,8]. In this report we present diagnostic methods for detection of the emerging influenza A(H7N9) virus from clinical specimens.

Methods

Clinical samples and influenza cell culture supernatants

Respiratory swabs, sputum, and endotracheal aspirates were obtained during 2012 and 2013 from hospitalised patients of the University of Bonn Medical Centre and the University of Marburg Medical Centre. Cell culture supernatants from typed influenza viruses were obtained from the German Society for Promotion of Quality Assurance in Medical Laboratories (INSTAND) proficiency testing panels. RNA was extracted from the samples as described earlier by using a viral RNA mini kit (Qiagen) [8].

Template for design of assays

The first three published genome sequences of the 2013 influenza A(H7N9) epidemic from the GISAID EpiFlu database, as listed in Table 1, served as the template for assay design. An influenza A/Mallard/Sweden/91/2002 (H7N9) strain [9], provided by Ron Fouchier, Rotterdam, to author M.M. was used for initial validation experiments.

Real-time reverse-transcription polymerase chain reaction targets

In order to design highly specific real-time reverse-transcription polymerase chain reaction (RT-PCR) targets that would not cross-react with human influenza viruses, we chose the haemagglutinin (HA) and neuraminidase (NA) genes of avian influenza A(H7N9) as targets for amplification.

Table 1

Origin of the haemagglutinin and neuraminidase sequences of emerging influenza A(H7N9) virus used for assay design, April 2013

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Submitter/author
EPI439507	HA	China	2013	A/Anhui/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439486	HA	China	2013	A/Shanghai/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439502	HA	China	2013	A/Shanghai/2/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439509	NA	China	2013	A/Anhui/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439487	NA	China	2013	A/Shanghai/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439500	NA	China	2013	A/Shanghai/2/2013	WHO Chinese National Influenza Center	Lei Yang

HA: haemagglutinin; ID: identity; NA: neuraminidase; WHO: World Health Organization.

We gratefully acknowledge the authors and laboratories for originating and submitting these sequences to the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID); these sequences were the basis for the research presented here.

All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

Because no isolates of the emerging influenza A(H7N9) lineage were available from China, we selected an influenza A/Mallard/Sweden/91/2002 (H7N9) strain whose HA and NA genes were closely related [1,9]. The finding of annealing sites for primers and probes was guided by an alignment of three available sequences from the 2013 emerging influenza A(H7N9) lineage, and the influenza A/Mallard/Sweden/91/2002 (H7N9) sequence. Thermodynamically suitable primers and probes were selected to minimise the number of nucleotide mismatches at their binding sites to the emerging A(H7N9) sequences as well as the A/Mallard/Sweden/91/2002 (H7N9) sequence. The NA gene fragment of A/Mallard/Sweden/91/2002 (A7N9) had to be sequenced for this purpose.

The final test layout included two adjacent regions in the HA gene, termed HA(I) and HA(II), which were respectively targeted by primers and probes of two RT-PCR assays. The two HA regions were included in one control RNA construct derived from the influenza A/Mallard/Sweden/91/2002 (H7N9) strain (Figure 1A). A region was also chosen for amplification of the NA gene, constituting the target of a third RT-PCR assay (NA(I)). A respective control RNA for this NA gene region, derived from the influenza A/Mallard/Sweden/91/2002 (H7N9) strain was also constructed (Figure 1B). In each of the three regions targeted by the RT-PCR assays, mutations in the oligonucleotide binding sites between the emerging influenza A(H7N9) lineage sequences and the influenza A/Mallard/Sweden/91/2002(H7N9) strain sequence were minimal, enabling the use of influenza A/Mallard/Sweden/91/2002 (H7N9)-derived RNAs as positive controls for all RT-PCR assays (Figure 1C and D).

Real-time reverse-transcription polymerase chain reaction

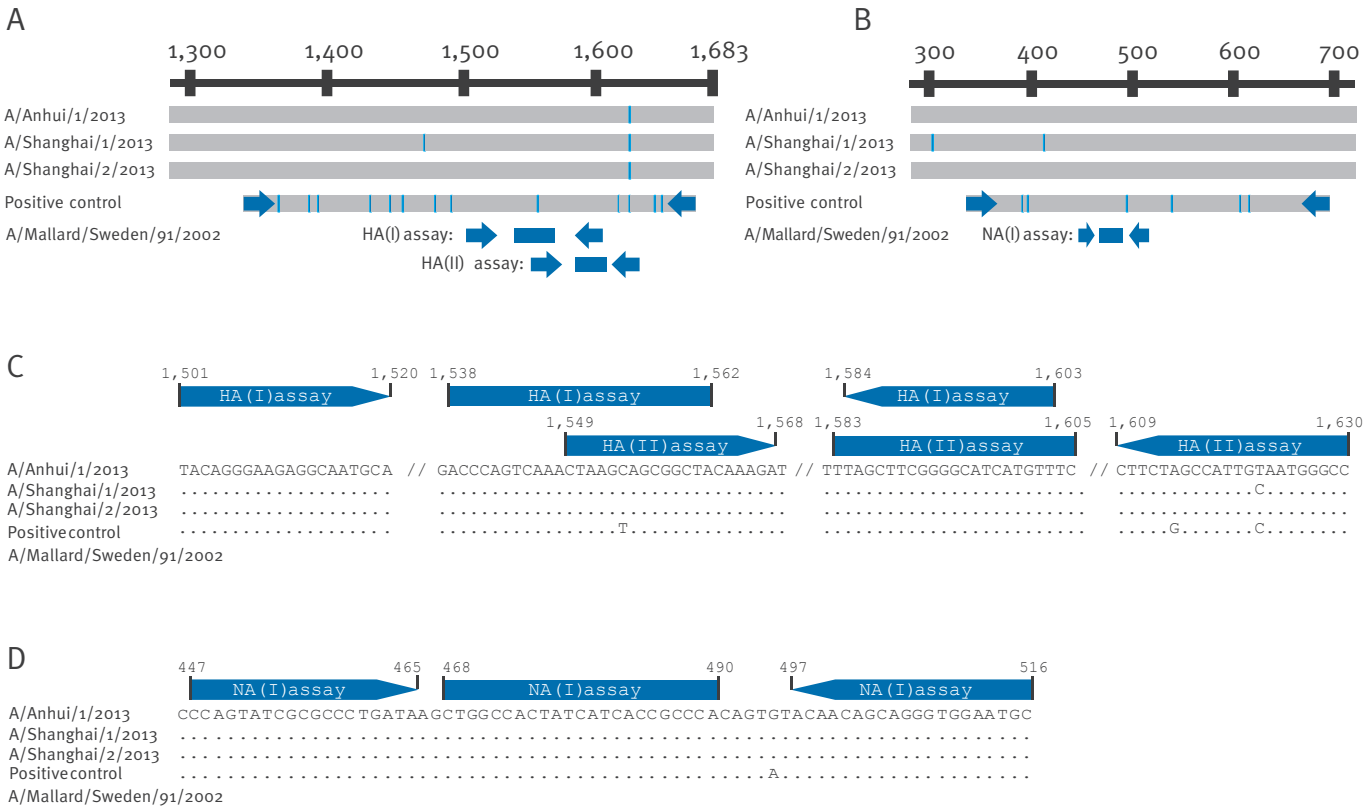
All three assays had the same conditions but the primer and probe sequences varied (Table 2). A 25-µl reaction was set up containing 5 µl of RNA, 12.5 µl of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each deoxyribonucleotide triphosphates (dNTP) and 3.2 mM magnesium sulfate), 1 µl of reverse transcriptase/Taq mixture from the kit, 0.4 µl of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), 1 µg of non-acetylated bovine serum albumin (Roche), 400 nM concentrations of each of the primers, as well as 200 nM of the probe. All oligonucleotides were synthesised and provided by Tib-Molbiol, Berlin, where stock solutions from the original synthesis lots are kept. Thermal cycling consisted of 55°C for 15 min, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 25 s.

In-vitro transcribed RNA controls

Using influenza A/Mallard/Sweden/91/2002 (H7N9) strain RNA as a template, a reverse-transcription PCR fragment encompassing both HA regions respectively targeted by the two HA(I) and (II)assays as well as additional flanking nucleotides was generated using primers IVT_HA-FWD and IVT_HA-REV. Likewise a reverse-transcription PCR fragment comprising the region of the NA gene targeted by the NA(I) assay was amplified with primers IVT_NA-FWD and IVT_NA-REV (Table 2). The HA and NA reverse-transcription PCR

Figure 1

Target sequence regions used for real-time reverse-transcription polymerase chain reaction assays to detect the emerging influenza A(H7N9) virus, March 2013



RT-PCR: real-time reverse-transcription polymerase chain reaction.

Each panel of the Figure shows partial alignments of three available sequences of the emerging influenza A(H7N9) lineage, which are designated as A/Anhui/1/2013, A/Shanghai/1/2013 and A/Shanghai/2/2013. Also aligned are the corresponding partial sequences of the influenza A/Mallard/Sweden/91/2002 (H7N9) strain, which serve to generate positive control templates for the RT-PCR assays. The Figure shows the regions of the haemagglutinin (HA) (panel A and C) and the neuraminidase (NA) genetic sequences (panel B and D) targeted by primers, represented by blue arrows, and probes, as blue bars, of the different PCR assays. Two regions are targeted for the HA gene, resulting in two separate RT-PCR assays, HA(I) and HA(II). One region of the NA gene is targeted by one NA(I) RT-PCR assay. Numbers in panels A to D represent genome positions according to the A/Anhui/1/2013 genome sequence. Grey horizontal bars in panels A and B represent the sequences, while blue vertical lines represent sequence variations between any of the listed strains in the alignment. Panels C and D show detailed alignments of target sequences of the three assays. In Panel C, nucleotides in the aligned sequences between the binding sites were omitted (marked with //). In the alignments, dots represent identity to the A/Anhui/1/2013 sequence, and all nucleotide substitutions are specified.

fragments are thereafter referred to as 'peri-amplicon fragments'. These PCR products were ligated into pCR 4 plasmid vectors and cloned in *Escherichia coli* by means of a pCR 4-TOPO TA cloning reagent set (Invitrogen). Plasmids were examined for correct orientation of inserts by PCR, purified, and re-amplified with plasmid-specific primers from the reagent set to reduce the plasmid background in subsequent in vitro transcription. Products were transcribed into RNA with the MegaScript T7 in vitro transcription reagent set (Ambion). After DNase I digestion, RNA transcripts were purified with Qiagen RNeasy columns and quantified photometrically. The RNAs derived from the peri-amplicon fragments were used as positive control for the performance of the RT-PCR assays (Figure 1).All transcript dilutions were carried out in nuclease-free water containing 10 µg/mL carrier RNA (Qiagen).

Results

Sensitivity of the real-time reverse-transcription polymerase chain reaction assays

Sensitivity tests employed quantified, in-vitro transcribed RNA derived from the peri-amplicon fragments of the combined HA(I/II) assays, as well as the NA(I) assay. Transcripts were generated and tested in serial tenfold dilution experiments. To obtain a statistically robust assessment of limits of detection (LODs), transcripts were tested in multiple parallel reactions containing RNA copy numbers above and below the pre-determined end point dilution detection limits of

Table 2

Primers and probes for assays used to screen for the emerging influenza A(H7N9) virus, April 2013

Assay/target	Oligonucleotide ID[a]	Sequence (5'–3')[b,c]	Polarity
RT-PCR/HA(I)	HA7_1_2013rtF	TACAGGGAAGAGGCAATGCA	+
	HA7_1-2013rtP	FAM-ACCCAGTCAAACTAAGCAGCGGCTA-TAMRA	+
	HA7_1_2013rtR	AACATGATGCCCCGAAGCTA	-
RT-PCR/HA(II)	HA7_2_2013rtF	CTGAGCAGCGGCTACAAAGA	+
	HA7_2_2013rtP	FAM-TTAGCTTCGGGGCATCATGTTTC-BBQ	+
	HA7_2_2013rtR	GKCCCATTRCAATGGCTAGAAG	-
RT-PCR/NA(I)	NA9_2013rtF	CCAGTATCGCGCCCTGATA	+
	NA9_2013rtP	FAM-CTGGCCACTATCATCACCGCCCA-TAMRA	+
	NA9_2013rtR	GCATTCCACCCTGCTGTTGT	-
Sequencing/HA-IVT	IVT_HA-FWD	CAATTGATCTGGCTGATTCAGA	+
	IVT_HA-REV	GTGCACCGCATGTTTCCATTC	-
Sequencing/NA-IVT	IVT_NA-FWD	CAAGAGAACCCTATGTTTCATGC	+
	IVT_NA-REV	GTTGTGGCATACACATTCAGATTC	-
Synthetic control	Fragment I	AGTATCACATCTTTGTAGCCGCTGCTTAGTTTGACT GGGTCAATCTGTATTCTATTTTGCATTGCCTCTTCCCTGTATTTGCTGTGA	-
	Fragment II	ACAAAGATGTGATACTTTGGTTTAGCTTCGGGGCA TCATGTTTCATACTTCTAGCCATTGTAATGGGC	+
	Primer F	TCACAGCAAATACAGGGAAGAG	+

BBQ: blackberry quencher; FAM: 6-carboxyfluorescein; HA: haemagglutinin gene; ID: identity; NA: neuraminidase gene; RT-PCR: real-time reverse-transcription polymerase chain reaction; TAMRA: 6-carboxy-N,N,N,N´-tetramethylrhodamine.

HA(I) and (II) correspond to two regions of the HA gene targeted by two respective RT-PCR assays. HA-IVT is a sequence encompassing both of the HA(I) and HA(II) regions, as well as additional flanking nucleotides. NA(I) corresponds to a region of the NA gene targeted by another RT-PCR assay. The NA-IVT sequence comprises the region of the NA gene targeted by the NA (I) RT-PCR assay, as well as flanking nucleotides. The synthetic control is a nucleotide sequence construct presenting 100% identity to a region of the HA sequence of an emerging influenza A(H7N9) virus strain, encompassing HA(I) and HA(II). It is synthesised by PCR fusion of Fragments I and II oligonucleotides using primers F and HA7_2_2013rtR.

[a] For the RT-PCR assays, the last letter of the oligonucleotide ID, is either 'F' for forward primer, 'P' for probe, or 'R' for reverse primer. For the sequencing assays, 'FWD' indicates the forward primer and 'REV' the reverse primer.

[b] When present, dye labels are indicated.

[c] Within the oligonucleotide sequences, a degenerate site with G/T is designated as a K and a site with G/A is designated as R.

each assay. The results in terms of the fractions of positive reactions at each concentration were subjected to probit regression analysis.

Detection probabilities of >95% were achieved at RNA concentrations 7.0 and 7.8 copies per reaction with the HA(I) and NA(I) assays, respectively (Figure 2). Probit analysis is not shown for the HA(II) assay because this assay is not proposed as a first line test; however, sensitivity of this assay was highly comparable to that of HA(I).

Because the peri-amplicon HA(I) and HA(II) oligonucleotide binding sites each presented with a small number of mismatches to the primers and probes designed for the RT-PCR assays (Figure 1), the sequence of the combined peri-amplicon region of the assays was synthesised in-vitro by PCR fusion of oligonucleotides fragment I and II using primers F and HA7_2_2013rtR to match the A/Anhui/1/2013 sequence (region 1491–1629) 100% (Table 1). The fragment was cloned in *E. coli* (GenExpress) and transcribed into RNA to be used for parallel testing of the HA assays. For both assays a concentration of five copies of RNA per reaction returned positive in nine of 10 replicates (none were positive with 0 copies per reaction, and all with 50 copies).

Specificity of the assays

To exclude non-specific reactivity of oligonucleotides among each other, all formulations were tested 45 times in parallel with assays containing water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected. Cross-reactivity with known heterospecific human influenza A viruses as well as other human repiratory viruses was excluded by testing virus positive clinical specimens and high-titre cell culture materials as summarised in Table 3.

To obtain a clinically relevant figure of assay specificity, all assays were applied on original clinical samples in which other respiratory viruses had already been detected during routine screening at Bonn and Marburg University Medical Centers (Table 3). These samples were prepared using the Qiagen Viral RNA kit, a formulation widely used to extract RNA in clinical laboratories. Of note, the tested panel included samples containing human influenza A viruses. In total, none of the 121 original clinical samples containing a wide range of respiratory viruses gave any detection signal with either assay, while positive controls were detected. It was concluded that the assay could be applied reliably for clinical samples.

During our validation studies, Word Health Organization (WHO) released RT-PCR protocols targeting other regions of the HA and NA genes (http://www.who.int/influenza/gisrs_laboratory/a_h7n9/en/ on 9 April 2013). Due to the lack of sequence agreement between the A/Anhui/1/2013 (H7N9) and A/Mallard/Sweden/91/2002 (H7N9) we were not able to evaluate the sensitivity of those assays. However, we included them for specificity testing running a panel of clinical samples as listed in Table 3. No false-positive amplifications were encountered while a full validation of these assays would require access to the A/Anhui1/2013 (H7N9) viral RNA or to generate a longer synthetic gene.

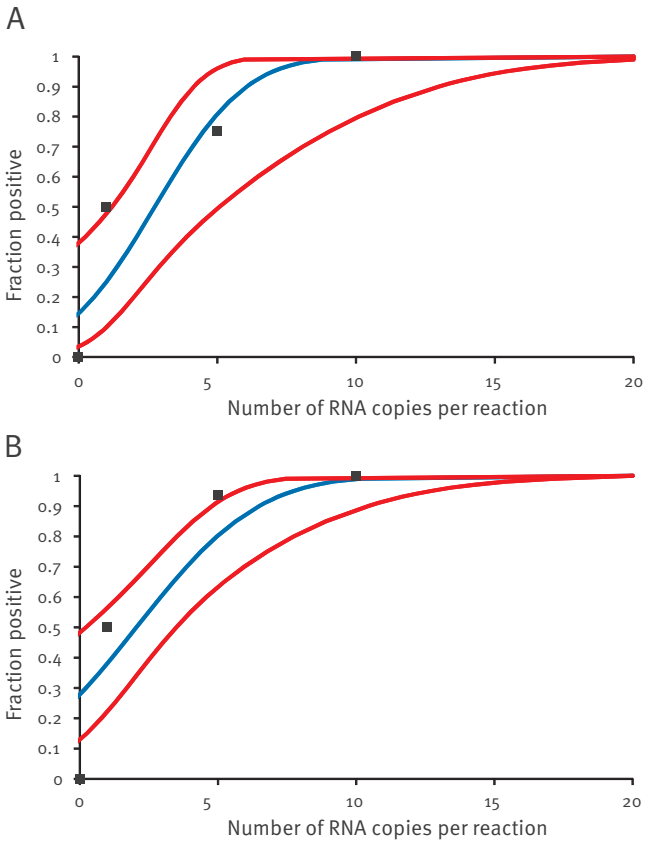
Conclusions

Medical laboratories often use conserved target genes such as the matrix gene for the detection of influenza. In cases of suspected human infection with the emerging influenza A (H7N9) strain, however, laboratories need to make sure their diagnostics do not return false positive results due to cross-reactivity with ubiquitous human influenza A viruses. Such cross-reactivity is likely to occur with matrix gene assays, and will thus pose a risk of misleading interpretations of test data. The here-provided protocols provide high specificity for influenza A(H7N9) while detecting minute quantities of virus due to high analytical sensitivity.

In cases of positive detection of influenza A(H7N9), laboratories would want to achieve confirmation by sequence analysis of the amplified fragment. The two primer pairs IVT_HA-FWD, IVT_HA-REV and IVT_NA-FWD, IVT_NA-REV enable sequence confirmation in the HA and NA genes, respectively. It is important to note that the provided in-vitro transcribed RNA controls contain mutations to be discriminated from the emerging influenza A(H7N9) lineage RNA, making it possible to discriminate true virus detections from possible laboratory contaminations. Control material is available from the authors through the European Virus Archive (www.european-virus-archive.com).

Figure 2

Probit regression analyses to determine the sensitivity of the real-time reverse-transcription polymerase chain reaction assays developed to detect the emerging influenza A(H7N9) virus, April 2013



HA: haemagglutinin; LOD: limit of detection; NA: neuraminidase.

The y-axis shows fractional hit-rates (positive reactions per reactions performed), the x-axis shows input RNA copies per reaction. Squares are experimental data points resulting from replicate testing of given concentrations in parallels assays. The blue regression line is a probit curve (dose-response rule). The outer red lines are 95% confidence intervals.

A. HA (I) assay; technical LOD = 7.013 RNA copies/reaction, at 95% hit rate; 95% CI: 4.812–15.41 RNA copies/reaction.

B. NA (I) assay; technical LOD = 7.754 RNA copies/reaction, at 95% hit rate; 95% CI: 5.741–12.739 RNA copies/reaction.

Table 3

Known respiratory viruses used for testing the specificity of the assays developed to detect the emerging influenza A(H7N9) virus, April 2013

Virus	Number of samples tested in the assays (HA(I); HA(II), NA)[a]	Number of samples tested in the WHO assays (H7 and N9)[b]
Clinical samples with known virus		
Pandemic influenza A(H1N1)pdm09	12	10
Influenza A(H3N2)	17	13
Influenza B	21	16
Human coronavirus		
hCoV-HKU1	3	-
hCoV-OC43	4	-
hCoV-NL63	4	-
hCoV-229E	4	-
hCoV-EMC	1	-
Human rhinovirus	5	4
Human respiratory syncytial virus	16	15
Human parainfluenza virus		
Parainfluenza 1 virus	1	1
Parainfluenza 2 virus	3	2
Parainfluenza 3 virus	4	2
Parainfluenza 4 virus	3	-
Human metapneumovirus	4	2
Human enterovirus	2	-
Human adenovirus	4	-
Human parechovirus	2	-
Subtotal	**110**	**65**
Cell culture supernatants		
Influenza A(H1N1) (older than 2009)	2	2
Influenza A(H5N1)	6	6
Influenza A(H3N2)	3	3
Subtotal	**11**	**11**
Total	**121**	**76**

WHO: World Health Organization.

[a] HA(I); HA(II) and NA(I) were respective target regions of the haemagglutinin and neuraminidase genes of the emerging influenza influenza A(H7N9) virus for real-time reverse-transcription polymerase chain reaction assays developed in this study.

[b] Assays published online on 9 April 2013 at http://www.who.int/influenza/gisrs_laboratory/a_h7n9/en/.

Oligonucleotides as well as the synthetic positive plasmid control (DNA) can be ordered from stock at Tib-Molbiol, Berlin (www.tib-molbiol.de). In-vitro transcribed control RNA for the HA(I), HA(II) and NA(I) assays can be acquired from author C. D. through the European Virus Archive platform (www.european-virus-archive.com), Further information and assay updates can be retrieved at www.virology-bonn.de.

Acknowledgements

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database used for assay design (www.gisaid.org).

The development and provision of these assays was done by a European research project on emerging diseases detection and response, EMPERIE, http://www.emperie.eu/emp/, contract No 223498 and the European Union Seventh Framework Programme [FP7/2007-2013] under Grant Agreement n°278433-PREDEMICS (http://predemics.biomedtrain.eu/cms/).

Authors C.D., M.M., M.E. and S.B. has received support from the German Centre for Infection Research (DZIF) that included full funding of the position of author V.M.C. at the Institute of Virology, Bonn. The European Virus Archive platform (www.european-virus-archive.com), through which in-vitro transcribed control RNA for the HA(I), HA(II) and NA(I) assays can be acquired from author C. D is funded by the European Commission under contract number 228292.

Conflict of interest

None declared.

Authors' contributions

Authors VMC, ME, OL, MM, S Becker and CD designed the study and analysed data. VMC, ME, OL, TB, S Brünink, and MEB did experiments. VMC, ME, OL, MM, S Becker and CD wrote and revised the article.

References

1. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human Infection with a Novel Avian-Origin Influenza A (H7N9) Virus. N Engl J Med. 2013 Apr 11. http://dx.doi.org/10.1056/NEJMoa1304459. PMid:23577628.
2. World Health Organization (WHO). Human infection with influenza A(H7N9) virus in China – update. Geneva: WHO; 16 April 2013. Available from: http://www.who.int/csr/don/2013_04_16/en/index.html
3. Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, et al. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. Euro Surveill. 2013;18(15):pii=20453. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20453
4. Abbott A. SARS testing: First past the post. Nature. 2003;423(6936):114. http://dx.doi.org/10.1038/423114a. PMid:12736651.
5. Panning M, Eickmann M, Landt O, Monazahian M, Olschläger S, Baumgarte S, et al. Detection of influenza A(H1N1)v virus by real-time RT-PCR. Euro Surveill. 2009;14(36):pii=19329. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19329. PMid:19758541.
6. Panning M, Charrel RN, Donoso Mantke O, Landt O, Niedrig M, Drosten C. Coordinated implementation of chikungunya virus reverse transcription-PCR. Emerg Infect Dis. 2009;15(3):469-71. http://dx.doi.org/10.3201/eid1503.081104. PMid:19239767. PMCid:2681123.
7. Palm D, Pereyaslov D, Vaz J, Broberg E, Zeller H, Gross D, et al. Laboratory capability for molecular detection and confirmation of novel coronavirus in Europe, November 2012. Euro Surveill. 2012;17(49): pii=20335. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20335
8. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17(39): pii=20285. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20285
9. Munster VJ, Wallensten A, Baas C, Rimmelzwaan GF, Schutten M, Olsen B, et al. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis. 2005;11(10):1545-51. http://dx.doi.org/10.3201/eid1110.050546. PMid:16318694 PMCid:3366752.

Investigation and management of an outbreak of *Salmonella* Typhimurium DT8 associated with duck eggs, Ireland 2009 to 2011

P Garvey (patricia.garvey@hse.ie)[1], P McKeown[1], P Kelly[2], M Cormican[3], W Anderson[4], A Flack[2], S Barron[2], N De Lappe[3], J Buckley[5], C Cosgrove[6], D Molloy[6], J O'Connor[3], P O'Sullivan[7], J Matthews[4], M Ward[8], A Breslin[9], M B O'Sullivan[10], K Kelleher[11], A McNamara[12], C Foley-Nolan[13], H Pelly[14], F Cloak[1], Outbreak control team[15]

1. Health Protection Surveillance Centre, Dublin, Ireland
2. Irish Department of Agriculture, Food and the Marine, Dublin, Ireland
3. National Salmonella, Shigella and Listeria Reference Laboratory, Medical Microbiology Department, University Hospital Galway, Galway, Ireland
4. Food Safety Authority of Ireland, Dublin, Ireland
5. Veterinary Department, Cork County Council, Cork, Ireland
6. Health Service Executive, Communicable Disease Unit of the Environmental Health Service, Blanchardstown Dublin, Ireland
7. Department of Public Health, Health Service Executive Mid-West, Department of Public Health, HSE West, Limerick, Ireland
8. Department of Public Health, Health Service Executive East, Dr Steeven's Hospital, Dublin, Irelan
9. Department of Public Health Medicine, Health Service Executive North West, Sligo, Ireland
10. Department of Public Health, Health Service Executive South, Wilton, Cork, Ireland
11. National Office for Health Protection, Health Service Executive Offices, Limerick, Ireland
12. Department of Public Health, Health Service Executive Dublin/Mid-Leinster, Tullamore Co. Offaly, Ireland
13. Food Safety Promotion Board, Cork, Ireland
14. Department of Public Health, Health Service Executive West, Merlin Park Hospital, Galway, Ireland
15. The remaining members of the team are listed at the end of the article

Citation style for this article:
Garvey P, McKeown P, Kelly P, Cormican M, Anderson W, Flack A, Barron S, De Lappe N, Buckley J, Cosgrove C, Molloy D, O'Connor J, O'Sullivan P, Matthews J, Ward M, Breslin A, O'Sullivan MB, Kelleher K, McNamara A, Foley-Nolan C, Pelly H, Cloak F, Outbreak control team. Investigation and management of an outbreak of Salmonella Typhimurium DT8 associated with duck eggs, Ireland 2009 to 2011. Euro Surveill. 2013;18(16):pii=20454. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20454

Article submitted on 07 May 2012 / published on 18 April 2013

Salmonella Typhimurium DT8 was a very rare cause of human illness in Ireland between 2000 and 2008, with only four human isolates from three patients being identified. Over a 19-month period between August 2009 and February 2011, 34 confirmed cases and one probable case of *Salmonella* Typhimurium DT8 were detected, all of which had an MLVA pattern 2-10-NA-12-212 or a closely related pattern. The epidemiological investigations strongly supported a link between illness and exposure to duck eggs. Moreover, *S.* Typhimurium with an MLVA pattern indistinguishable (or closely related) to the isolates from human cases, was identified in 22 commercial and backyard duck flocks, twelve of which were linked with known human cases. A range of control measures were taken at farm level, and advice was provided to consumers on the hygienic handling and cooking of duck eggs. Although no definitive link was established with a concurrent duck egg-related outbreak of *S.* Typhimurium DT8 in the United Kingdom, it seems likely that the two events were related. It may be appropriate for other countries with a tradition of consuming duck eggs to consider the need for measures to reduce the risk of similar outbreaks.

Introduction

Salmonella enterica is the second most common cause of bacterial gastroenteritis in most European Union (EU) Member States, including Ireland [1]. The reported annual crude incidence rate for human salmonellosis in Ireland has ranged from 8.1 to 10.8 per 100,000 between 2004 and 2010 (8.4 per 100,000 in 2010). Although *S.* Typhimurium was the most common serotype among indigenous cases of salmonellosis from 2008 and 2010 [2], the DT8 phage type was a very rare cause of illness, with only four isolates from three patients identified between 2000 and 2008. The related *S.* Typhimurium phage type DT30 is also very rare.

S. Typhimurium DT8 is also uncommon in the rest of Europe [1], but was associated with a small salmonellosis outbreak in Scotland in late 2009 [3] and implicated in sporadic cases of salmonellosis associated with consumption of duck eggs in Germany during the 1990s [4]. Contaminated duck eggs have been recognised as a vehicle of *Salmonella* Typhimurium infection as far back as 1949, when 40 nurses in a London hospital fell ill after eating a lightly cooked pudding prepared with duck eggs [5]. Here we report on the investigation of an outbreak of S. Typhimurium DT8 which occurred in Ireland between August 2009 and May 2011.

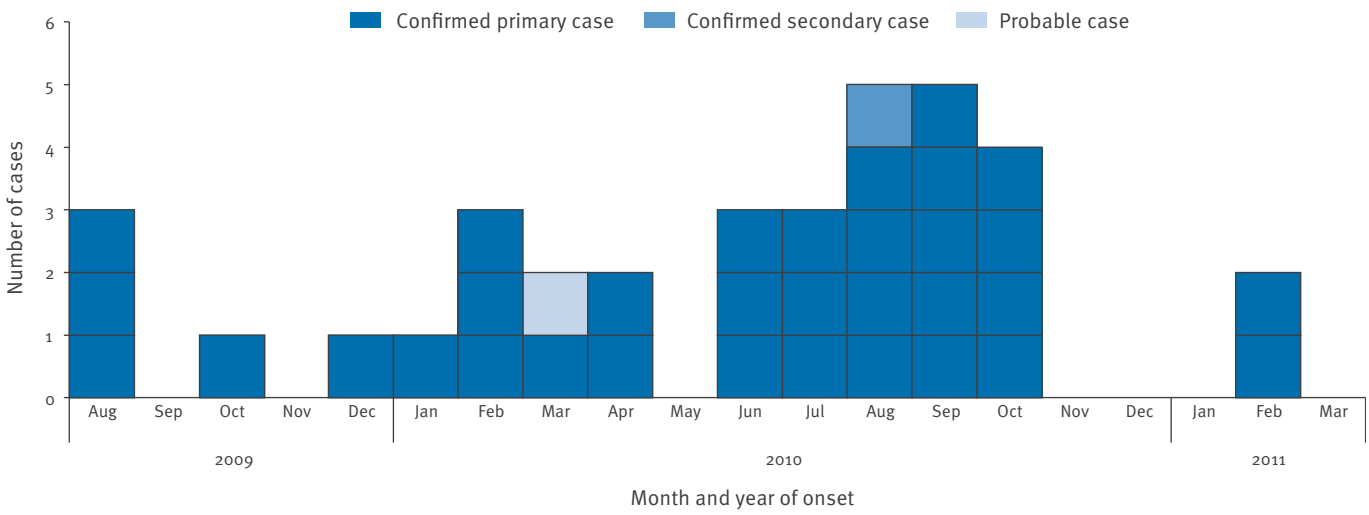
Methods

Outbreak case definition and case finding

In this outbreak, a confirmed case was defined as a person with laboratory-confirmed infection with

Figure

Salmonella Typhimurium DT8 cases by month and year of onset, Ireland, 1 August 2009–24 May 2011 (n=35)



Salmonella Typhimurium DT8 (MLVA pattern 2-10-NA-12-212 or a closed related pattern) and a date of onset or sample date between 1 August 2009 and 24 May 2011. A closely related pattern was one that differed at one locus or with minor differences at two loci. A probable case was defined as a person with diarrhoea, fever, abdominal pain or vomiting between 1 August 2009 and 24 May 2011 and an epidemiological link (household) to a laboratory-confirmed case. The definition was further refined to exclude one person who had spent their entire incubation period outside of Ireland, and to exclude a laboratory-acquired case.

Confirmed outbreak cases were identified from isolates submitted for typing. Referral of *Salmonella* isolates by clinical laboratories is voluntary but essentially complete (Martin Cormican NSSLRL, personal communication, April 2010), thus ascertainment of laboratory-confirmed cases is likely to have been comprehensive. Probable cases were identified among household contacts of confirmed cases during interviews.

Epidemiological investigations

Demographic, laboratory and exposure information on human cases was gathered. The Epidemic Intelligence Information System (EPIS) of the European Centre for Disease Prevention and Control (ECDC) was used to circulate a request for information on similar cases to colleagues in other EU Member States on 23 April 2010, when a hypothesis began to emerge.

Food and traceback investigations

Traceback investigations involved multiple agencies to establish the suppliers of duck eggs to those outlets where cases had bought/obtained duck eggs, followed by on-farm testing of environmental samples, including duck faeces, for *Salmonella*. Forward tracking of ducks from positive farms to secondary farms was also undertaken, with *Salmonella* testing at secondary farms. Duck farms were classified as 'large commercial' (≥100 birds) or 'backyard' (<100 birds) flocks. As the outbreak progressed, all large commercial duck farms were tested. In addition, hygiene and production practices at the duck farms were inspected and the duck egg distribution network was reviewed.

Laboratory typing methods

All isolates detected were confirmed as *S. enterica* serotype Typhimurium according to the Kauffmann-Whyte-Le Minor scheme using slide agglutination (Murex Biotech Ltd., Dartford, United Kingdom, and Sifin Institut, Berlin, Germany) [6,7]. Antimicrobial susceptibility testing to a range of antimicrobial agents (ampicillin, chloramphenicol, streptomycin, sulphonamides (8-1024), tetracycline, trimethoprim, naladixic acid, kanamycin, ciprofloxacin, cefpodoxime, gentamicin, ceftazadime and cefotaxime) was determined using the broth dilution method (Sensititre, TREK Diagnostic Systems, East Grinstead, United Kingdom) [8]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints were used for interpretation [9]. Multilocus variable-number tandem repeat analysis (MLVA) was performed according to method of Larrson et al. 2009 [10]. Pulsed-field gel electrophoreses (PFGE) was performed on selected isolates using the PulseNet protocol [11].

Table 1

Reported exposure to and source of duck eggs, *Salmonella* Typhimurium outbreak, by disease onset, Ireland, 1 August 2009–24 May 2011 (n=35)

Month and year	Duck egg exposure[a]				No duck egg exposure	Exposure history unknown	Total
	Large commercial flock	Backyard flock selling eggs for profit	Backyard flock with eggs for private use only	Unclassified source			
Aug 2009	0	0	1	1	0	1	**3**
Sep 2009	0	0	0	0	0	0	**0**
Oct 2009	0	0	0	0	0	1	**1**
Nov 2009	0	0	0	0	0	0	**0**
Dec 2009	0	0	0	1	0	0	**1**
Jan 2010	0	0	1	0	0	0	**1**
Feb 2010	2	0	0	1	0	0	**3**
Mar 2010	2	0	0	0	0	0	**2**
Apr 2010	1	0	0	0	1	0	**2**
May 2010	0	0	0	0	0	0	**0**
Jun 2010	0	0	0	0	3	0	**3**
Jul 2010	1	0	0	0	2	0	**3**
Aug 2010	0	0	4[b]	0	1	0	**5**
Sep 2010	1	2	1	0	0	1	**5**
Oct 2010	0	0	2	0	2	0	**4**
Nov 2010	0	0	0	0	0	0	**0**
Dec 2010	0	0	0	0	0	0	**0**
Jan 2011	0	0	0	0	0	0	**0**
Feb 2011	0	2	0	0	0	0	**2**
Total	**7**	**4**	**9**	**3**	**9**	**3**	**35**

[a] Duck flocks are classified either as large commercial flocks (≥100 birds) or backyard flocks (<100 birds).

[b] Secondary case included here along with case from whom they acquired their illness.

Results

Descriptive epidemiology

When the outbreak was declared over on 24 May 2011 because no new cases have appeared since the end of February, 34 laboratory-confirmed cases and one probable case have been identified. Cases were distributed across seven of the eight public health administrative areas (HSE areas) in Ireland. The earliest date of onset was 14 August 2009, while the last was 27 February 2011 (Figure). The shape of the outbreak curve is consistent with an intermittent-source outbreak. Notably, fewer cases were reported during winter periods.

Within the outbreak, there were five family clusters of two cases each. In one family, a young infant was considered a secondary case. The cases' ages ranged from less than one year to 88 years (median: 42 years) and male cases predominated (23 male versus 12 female cases).

The clinical symptoms of the cases were diarrhoea (28/29), fever (19/23), abdominal pain (19/25), and nausea (16/22), with less frequent reports of blood in stools (13/24), vomiting (14/26) and headache (4/14). Symptom duration ranged from one to 21 days (median: 7 days), based on information from 21 cases. Most cases (18/33) were admitted to hospital. One of them died, but the death was not attributed to salmonellosis.

Those EU Member States who responded to the initial inquiry in April 2010 indicated at the time that *Salmonella* Typhimurium DT8 was an uncommon cause of human illness and that they had no increase in case numbers. However, Norwegian colleagues reported that there were isolations of similar but not indistinguishable MLVA previously from larger birds, but there were no reports of recent isolations from ducks. In July 2010, colleagues from the former Health Protection Agency in the United Kingdom (UK) informed the Irish Health Protection Surveillance Centre (HPSC) of an excess of *S.* Typhimurium DT8 cases in England, Wales and Northern Ireland that began in the summer of 2010 [12].

Hypothesis generation

Under Infectious Diseases (Amendment) Regulations 2011 (S.I. No. 452 of 2011), human salmonellosis is notifiable in Ireland, and epidemiological investigation

of clusters is routine, the size threshold being lowest for uncommon strains. In August 2009, the reporting of three cases of a salmonella variant previously reported as rare in Ireland prompted an epidemiological investigation. Exposure data obtained on two of the three reported cases using a standard national *Salmonella* trawling questionnaire indicated that both had consumed duck eggs, although from different sources, including one backyard flock from which duck eggs were not sold to the public. The third case could not be contacted.

All three isolates available at that time, as well as two further human isolates identified later in 2009, had the MLVA pattern 2-10-NA-12-212 or a closely related pattern, suggesting a common source. The MLVA pattern of these isolates differed at multiple loci from that observed with the three previous human *S.* Typhimurium DT8 from Ireland isolated between 2000 and 2008.

On detection of a second cluster of cases beginning late January 2010, national salmonella trawling questionnaires were again applied. Of seven confirmed and one probable case with onset dates between January and April 2010, seven reported consumption of/exposure to duck eggs. Although comparison of consumption data for the population as a whole was not possible, this exposure was believed to be relatively uncommon in the general population. Therefore ducks eggs were considered the likely source of the outbreak.

Exposure history

When the outbreak was declared over, duck egg exposure was reported for 22 of 31 primary cases with known exposure histories. An additional case classified as a secondary case was linked to a confirmed case who had consumed duck eggs. Cases reported obtaining duck eggs from multiple sources including non-commercial sources, suggesting that infection was probably not related to a single duck flock. Earlier cases were exposed to duck eggs from both larger commercial and backyard sources, while later cases were more likely to have consumed duck eggs from backyard flocks (Table 1).

Traceback investigations

The duck egg trade proved to be highly complex. The eggs are often supplied from one flock to several retailers, and retailers often source duck eggs from multiple suppliers. This necessitated forward- as well as back-tracing investigations when *S.* Typhimurium was detected on farms. Unlike all hen eggs sold at retail level in Ireland, which must be sourced from an egg packing centre registered with the Irish Department of Agriculture, Food and the Marine (DAFM), retailers source duck eggs directly from producers. In addition, eggs from backyard flocks were not marked, making it difficult to trace back to source.

Table 2

Duck flocks tested positive for *Salmonella* Typhimurium during the outbreak investigation Ireland, 1 August 2009–24 May 2011 (n=25)

	Large commercial flock	Backyard flock selling eggs for profit	Backyard flock with eggs for private use only
Flocks associated with human cases	6	3	3
Flocks not associated with human cases but tested as part of investigation	12[a]	1	0
Total	**18[a]**	**4**	**3**

All *S.* Typhimurium were either DT8 or DT30 unless indicated otherwise by footnotes.

[a] includes one flock each positive for DT56var, DT41 and DT120(low).

Initial investigations focused on larger commercial duck farms directly or indirectly linked with outbreak cases, but were expanded to include all large commercial duck farms and a number of backyard flocks selling for profit. Between March 2010 and May 2011, 64 farms had been inspected and sampled, with *S.* Typhimurium isolated from 22 of these (Table 2). Of these 22, nine farms were linked to eleven human cases. In addition, three backyard flocks (linked with another four human cases) were also found positive for salmonella (Table 2), making it 25 positive flocks in total. Isolates from 22 of these 25 *S.* Typhimurium-positive duck egg farms were typed as DT8 or DT30 with MLVA pattern 2-10-NA-12-212 or a related pattern. A further three commercial flocks not linked to human DT8 cases were positive for *S.* Typhimurium phage types DT56var, DT41 or DT120(low) (Table 2).

One of two duckling hatcheries in Ireland was positive for *S.* Typhimurium (DT56var) but not for the outbreak strain. This hatchery supplied three of the farms positive for *Salmonella*, but two of those farms also received chicks from other sources. Duck farm samples taken for testing did not include duck eggs; in one instance, eggs were tested from a supplier, but no eggs were left over for testing from case households. While poultry feed was not specifically sampled during this outbreak, feed from licensed poultry feed mills is analysed routinely by DAFM as part of their procedures.

Strain typing

MLVA analyses of isolates from the 34 laboratory-confirmed human cases within the outbreak showed that 20 had the MLVA pattern 2-10-NA-12-212. The remainder had patterns that differed at one locus or showed minor differences at two loci (Table 3). All but two isolates were susceptible to all antimicrobial agents

tested. Similarly, all DT8/DT30 isolates recovered during the investigation of egg-laying duck farms had the MLVA pattern 2-10-NA-12-212 (or a pattern which differed at a single locus).

PFGE was performed on 18 isolates (selected to represent different sources (human/animal, MLVA profiles and antibiograms). In all, 13 human and two duck DT8 isolates were examined. These were indistinguishable from each other and from a pattern previously designated as STYMXB.0330 on the PulseNet Europe PFGE database..

Control measures

A variety of control measures were applied at different points during the investigation, as evidence for likely sources of infection became available. Some were aimed at farms and others at consumers.

Farm level control measures

Movement of birds was immediately restricted and duck egg sales suspended on all farms from which *S.* Typhimurium was isolated. Most flocks were culled voluntarily. Farms were advised that duck houses should be cleansed and disinfected, and lime applied to pasture. Environmental swabs were recommended to confirm absence of *Salmonella* after cleansing and disinfection of the infected premises to ensure that no infection remained before new stock was introduced. Sourcing of new birds from parent flocks with documented negative tests for *Salmonella* was also recommended.

In summer 2010, a code of practice was issued to commercial duck egg producers incorporating recommendations on traceability, disease control, biosecurity and testing [13]. In October 2010, additional guidelines were devised for backyard producers of small quantities of duck eggs and circulated to more than 3,000 people registered as keeping ducks [14]. In November 2010, new legislation (S.I. No. 565 of 2010), the 'Diseases of Animals Act 1966 (Control of *Salmonella* in Ducks) Order 2010' [15] set down a legal basis for the control of salmonellosis in ducks and duck eggs in Ireland. This legislation applies to all duck flocks, regardless of size, from which duck eggs are traded for profit [15].

Advice to consumers

A series of press releases was issued to the public by the Food Safety Authority of Ireland (FSAI) and HPSC (on 23 April 2010, 27 April 2010, 29 July 2010 and 14 September 2010) advising consumers that duck eggs should be thoroughly cooked and that good hygiene practices should be followed to avoid cross contamination from duck eggs [16-19]. The risk from eating raw cake batter containing duck eggs was highlighted [19]. In April 2010, Environmental Health Officers distributed a point-of-sale warning notice for public display to all duck egg retailers [20]. Similar notices were distributed to nursing homes and hospitals. In November 2010, a public information campaign (including an information postcard for distribution at point of sale) was undertaken by *Safe*food, an agency with responsibility for promotion of food safety messages to consumers [21].

Table 3

Multilocus variable-number tandem repeat analysis and antimicrobial agent sensitivity patterns of isolates for confirmed human outbreak cases (n=34)

MLVA pattern	Resistance to antimicrobial agents			
	None	A	ASuTm	Total
2-10-NA-12-212	21	0	1	22
2-10-NA-12-NA	1	0	0	1
2-12-NA-12-212	1	0	0	1
2-9-NA-11-212	3	0	0	3
2-9-NA-12-212	3	1	0	4
2-9-NA-13-212	2	0	0	2
3-10-NA-12-212	1	0	0	1
Total	**32**	**1**	**1**	**34**

A: ampicillin; MLVA: multilocus variable-number tandem repeat analysis; Su: sulfadiazine; Tm: trimethoprim.

EUCAST clinical breakpoints were used.

Discussion

This outbreak-associated strain of *S.* Typhimurium represented more than 12% (28/225) of the confirmed salmonellosis cases in Ireland in 2010 that were not travel-related. The link between illness and duck eggs was supported by descriptive epidemiology and by microbiological evidence. Given the strength of evidence, an analytical epidemiological study was deemed unnecessary.

The PFGE pattern was indistinguishable from the strain implicated in the summer 2010 outbreak in England and Northern Ireland, and from an isolate identified in Scotland in 2009 [3,12]. Colleagues at the Centers for Disease Control and |Prevention in the United States (Peter Gerner-Smidt, personal communication, September 2010) reported that the outbreak PFGE profile (known as JPXX01.1689 in the Pulsenet USA database) was rare in the United States and had not been seen since August 2008. This PFGE profile was also identified previously in China (known there as JPXX01.CN0030) but was not identified among human cases there in 2010 (Biao Kan, personal communication, September 2010).

The variety of typing methods used facilitated investigation and management of the outbreak. Serotyping and phage typing allowed for rapid preliminary identification of outbreak-related cases. MLVA showed that the initial isolates were indistinguishable from

each other but distinguishable from older isolates and helped to confirm that we were dealing with a new phenomenon. That some diversity in MLVA types was observed as transmission continued is not surprising in an extended outbreak, given the mutability of MLVA loci. The fact that all outbreak isolates were indistinguishable by PFGE is consistent with the greater stability of PFGE patterns over time and helped to support our view that the degree of tolerance of MLVA variation that was accepted within the definition of the outbreak strain was appropriate.

In Ireland, the duck egg industry is very small compared with the chicken egg industry. It is comprised of 30 to 40 larger commercial businesses and an estimated 3,000 backyard flocks, some of whom sell duck eggs to the public. Production is largely outdoors, and duck eggs represent less than 1% of table egg sales (Patricia Kelly DAFM, personal communication, April 2010). Although duck eggs are a traditional food in Ireland, no association with human salmonellosis had been documented before 2009. There is no reason to believe that there was an abrupt increase in duck egg consumption or change in the pattern of consumption or methods of preparation in recent years.

S. Typhimurium DT8 has been reported elsewhere as associated with ducks for decades [4]. We are not aware of studies of *Salmonella* in egg-laying duck flocks in Ireland that predate this outbreak and can therefore not say that laying duck flocks were free of *Salmonella* before this outbreak. However, given no apparent change in consumption patterns, it seems likely that the cause of this outbreak may be related to recent introduction into Ireland of a *Salmonella* strain with considerable pathogenic potential. The similarity of the isolates by MLVA (which is discriminatory for DT8 isolates) also suggests recent and rapid dissemination from a common source.

The source of contamination has not been definitively established. Introduction through contact with wild birds is possible. However, the published description of a similar outbreak of *S.* Typhimurium DT8 that occurred in England and Northern Ireland around the same time, and the documented trade of ducklings from UK hatcheries to Ireland, means that a UK source of contamination related to trade in ducklings is plausible [12].

There are few reported outbreaks associated with *S.* Typhimurium DT8, although in Germany sporadic infections with *S.* Typhimurium DT8 have been associated with the consumption of raw and undercooked duck eggs [3,4]. During the early 1990s, *S.* Typhimurium DT8 was also the most common *S.* Typhimurium phage type associated with disease in ducks in Germany, and it was suggested that DT8 (and another *S.* Typhimurium phage type DT46) might be host-adapted to ducks [4]. In the UK, the Department for Environment, Food and Rural Affairs has reported that, while *S.* Typhimurium accounted for a minority of isolates from ducks during 2005 and 2006, most of these were DT8 [22]. In France, a study by Lailler et al. also reported a close association between this phage type and ducks [23].

Only 22 of 31 of primary cases within the outbreak could be explained by documented consumption of/exposure to duck eggs. It is not uncommon in foodborne outbreaks to be unable to link all cases with the source identified. This may be explained by failure to recall exposure, unknowing consumption of duck egg, unknowing contact with an infected person, or by some of the cases being caused by alternative sources.

Because faecal contamination of duck eggs can occur, it is the practice to wash the duck eggs prior to sale (Patricia Kelly DAFM, personal communication, July 2010). Examination of a batch of implicated duck eggs after disinfection of the shell failed to detect *S.* Tyhimurium inside the eggs. This is consistent with the suggestion that *S.* Typhimurium is associated primarily with the shell, although it has been proposed that washing of chicken eggs can lead to *Salmonella* entering the egg via the pores in the shell [24].

From January to May 2011, there were only two new human cases of DT8 infection, a dramatic decrease from the 2010 levels. We declared the outbreak over in May 2011 assuming the period of high intensity transmission had passed, however we anticipated that occasional cases might still be reported for a prolonged period if the strain persisted in backyard flocks. In fact only six further cases occurred in the remaining seven months of 2011, and investigation of duck egg sources associated with these cases continued through 2011. The legislation and good practice guidance introduced in connection with this outbreak was key to the ongoing control of the risk of salmonellosis associated with consumption of duck eggs. As there has been only a single non-travel-related human isolate of *S.* Typhimurium DT8 to date in 2012 (MLVA 2-9-NA-12-212), it appears that the measures taken have had a sustained impact in protecting public health.

From an international perspective, this outbreak highlights the vulnerability of animal food production systems that are outside the scope of regulatory control to the emergence of new pathogenic variants, and the effectiveness of a vigorous multi-agency response to the protection of human health. It may be appropriate for other countries with a tradition of consumption of duck eggs to consider the need for measures to reduce the risk of similar outbreaks and for the EU to consider whether any changes are required to EU regulations in this regard.

Acknowledgements

The authors wish to acknowledge the contribution of the Food and Waterborne Disease (FWD) team at ECDC, colleagues in the ECDC FWD Network, Consultant Microbiologists, Medical Scientists, Public Health Physicians, Surveillance Scientists, and Environmental Health Officers around Ireland, other staff at HPSC, DAFM, NSSLRLG, FSAI, Cork County Council Veterinary Food Safety Laboratory, and the Central Veterinary Research Laboratory, during the course of the investigation. We also wish to thank the former Health Protection Agency in the UK for sharing information on the concurrent *S.* Typhimurium DT8 outbreak in the UK, and Health Protection Scotland colleagues for sharing *Salmonella* strains.

Conflict of interests

None declared

Members of the cutbreak control team

S Jackson, D O Mahony, D Guina-Dornan, D Crowley, L Thornton, A Reilly, D Igoe, F O'Neill, A Sheehan, M Mulcahy, G McGrane.

References

1. European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA). The Community summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. Stockholm, Parma: ECDC, EFSA; 2010. Available from: http://www.efsa.europa.eu/en/efsajournal/doc/1496.pdf
2. Health Protection Surveillance Centre (HPSC). Annual report 2009. Dublin: HPSC; 2010. ISSN 1649-0436. Available from: http://www.hpsc.ie/hpsc/AboutHPSC/AnnualReports/File,4751,en.pdf
3. Smith-Palmer A, Brownlie S, Cowden J. Gastro-intestinal and foodborne infections General outbreaks of infectious intestinal disease reported to HPS during the third quarter of 2008. HPS Weekly Report. 2009;43(2009/49):460-1. Available from: http://www.hps.scot.nhs.uk/documents/ewr/pdf2009/0949.pdf PMid:11953356 PMCid:127920
4. Rabsch W, Andrews HL, Kingsley RA, Prager R, Tschäpe H, Adams LG, et al. Salmonella enterica serotype Typhimurium and its host-adapted variants. Infect Immun. 2002;70(5):2249-55. http://dx.doi.org/10.1128/IAI.70.5.2249-2255.2002 PMid:11953356 PMCid:127920
5. Garrod LP, McIlroy MB. Hospital outbreak of enteritis due to duck eggs. Br Med J. 1949;2(4639):1259-61. http://dx.doi.org/10.1136/bmj.2.4639.1259
6. Grimont PA, Weill FX. Antigenic formulae of the Salmonella serovars. 9th edition. Paris: World Health Organization Collaborating Centre for Reference and Research on Salmonella, Pasteur Institute; 2007. Available from: http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089
7. Anderson ES, Ward LR, Saxe MJ, de Sa JD. Bacteriophage-typing designations of Salmonella Typhimurium. J Hyg (Lond). 1977;78(2):297-300. http://dx.doi.org/10.1017/S0022172400056187
8. Wikler MA, Cockerill FR, Bush K, Dudley MN, Eliopoulos GM, Hardy DJ, et al. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard. 8th Edition. Clinical and Laboratory Standards Institute (CLSI) document M07-A8 (ISBN 1-56238-689-1). Wayne: CLSI; 2009. Available from: http://www.scribd.com/doc/89642226/CSLI-2009
9. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 2.0. Basel: European Society of Clinical Microbiology and Infectious Diseases; 2012. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2.0_120221.pdf
10. Larsson JT, Torpdahl M, Petersen RF, Sørensen G, Lindstedt BA, Nielsen EM. Development of a new nomenclature for Salmonella Typhimurium multilocus variable number of tandem repeats analysis (MLVA). Euro Surveill. 14(15):pii=19174. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19174 PMid:16602980 PMid:21470442
11. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet. Foodborne Pathog Dis. 2006;3(1):59-67. http://dx.doi.org/10.1089/fpd.2006.3.59 PMid:16602980
12. Noble DJ, Lane C, Little CL, Davies R, De Pinna E, Larkin L, et al. Revival of an old problem: an increase in Salmonella enterica serovar Typhimurium definitive phage type 8 infections in 2010 in England and Northern Ireland linked to duck eggs. Epidemiol Infect. 2012;140(1):146-9. http://dx.doi.org/10.1017/S0950268811000586 PMid:21470442
13. Department of Agriculture, Food and the Marine (DAFM). Code of practice for duck table egg producers. Dublin: DAFM; 2010. Available from: http://www.agriculture.gov.ie/media/migration/farmingsectors/poultry/DuckEggsCOPAug151010.pdf
14. Department of Agriculture, Food and the Marine (DAFM). Guidelines for producers of small quantities of duck eggs (backyard flocks). Dublin: DAFM; 2010. Available from: http://www.fsai.ie/uploadedFiles/Food_Businesses/Topics_of_Interest_to_Food_Businesses/Guidance%20for%20producers%20of%20small%20quantities%20of%20duck%20eggs%20%20Final%20%2013%20Sept%2010.pdf
15. Department of Agriculture, Food and the Marine (DAFM). The diseases of animals act 1966 (Control of Salmonella in ducks) order 2010 (S.I. No. 565 of 2010). Dublin: DAFM; 2010. Available from: http://www.agriculture.gov.ie/media/migration/legislation/statutoryinstruments2010/SI5652010.pdf
16. Food Safety Authority of Ireland (FSAI). FSAI investigating Salmonella outbreak linked to duck eggs. Dublin: FSAI; 2010. Available from: http://www.fsai.ie/news_centre/press_releases/23042010.html
17. Food Safety Authority of Ireland (FSAI). FSAI reiterates advice on safe consumption of duck eggs. Dublin: FSAI; 2010. Available from: http://www.fsai.ie/news_centre/press_releases/29072010.html
18. Food Safety Authority of Ireland (FSAI). Largest outbreak in recent years linked to Salmonella in duck eggs. Dublin: FSAI; 2010. Available from: http://www.fsai.ie/14092010.html
19. Health Protection Surveillance Centre (HPSC). 'Don't lick spoon' when cooking or baking with duck eggs warns HPSC as children fall ill with salmonella. Dublin: HPSC; 2010. Available from: http://www.hpsc.ie/hpsc/A-Z/Gastroenteric/Salmonellosis/News/MainBody,4289,en.html
20. Food Safety Authority of Ireland (FSAI). Consumer advice on the safe usage of duck eggs. Dublin: FSAI; 2010. Available from: http://www.fsai.ie/uploadedFiles/News_Centre/News/News_Listing/Duck%20eggs%20updated%20pointofsalenotice(1).pdf
21. Stay safe with duck eggs. Postcard. Available from: Cork: Safefood: 2010. http://www.safefood.eu/Publications/Consumer-info/Stay-safe-with-duck-eggs.aspx
22. Veterinary Laboratories Agency.Salmonella in Livestock Production in GB: 2008 Report accessed at Available from: http://www.defra.gov.uk/ahvla-en/category/publications/disease-surv/salmonella-live-prod/
23. Lailler R, Grimont F, Jones Y, Sanders P, Brisabois A. Subtyping of Salmonella typhimurium by pulsed-field gel electrophoresis and comparisons with phage types and resistance types. Pathol Biol (Paris). 2002 Jul;50(6):361-8. http://dx.doi.org/10.1016/S0369-8114(02)00321-8
24. EFSA. Opinion of the Scientific Panel on Biological Hazards on the request from the Commission related to the Microbiological Risks on Washing of Table Eggs. The EFSA Journal (2005), 269, 1-39.

WHO revised definitions and reporting framework for tuberculosis

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

Citation style for this article:
Eurosurveillance editorial team. WHO revised definitions and reporting framework for tuberculosis. Euro Surveill. 2013;18(16):pii=20455. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20455

Article published on 18 April 2013

On 15 April 2013, the World Health Organization (WHO) launched the revision of the 'Definitions and reporting framework for tuberculosis' (2013 revision) [1]. This document updates the previous WHO standard case definitions for tuberculosis (TB) and drug-resistant TB, the categories used to assign outcomes and the standard reporting framework for TB [2-4].

The standardisation of definitions and reporting structures has permitted comparability of indicators of performance between national TB control programmes and over time. In the course of the 2013 revision, efforts were made to maintain continuity in the surveillance parameters while also ensuring that the new framework captures information about bacteriologically-confirmed TB cases and drug-resistant strains detected using novel, WHO-endorsed molecular testing methodologies. The treatment outcome definitions of 'cured' and 'treatment failed' for rifampicin-resistant TB (RR-TB) and multidrug-resistant TB (MDR-TB) patients needed simplification to allow their wider application to patients still on treatment. In addition, the judgemental terms 'defaulter' and 'TB suspect' have now been replaced by 'lost to follow-up' and 'presumptive TB' respectively.

The recording and reporting forms for paper-based systems were also updated to bring them in line with the revised case and treatment outcome definitions. Countries using electronic systems for TB recording and reporting are expected to adapt their software to incorporate the revised case and outcome definitions and to produce the indicator reports along the same lines. Nonetheless, the forms, registers and reports are intended to be illustrative rather than prescriptive and should serve to demonstrate how a minimum dataset for recording and reporting could be compiled. Each country will have its own particular requirements and will need to modify the forms, registers and reports to suit its needs. While the revised definitions are intended for immediate application, the process for their full adoption will need to factor in sufficient time to allow for effective communication, training, supervision, revision of procedures and policies, and the adaptation of information systems.

References

1. World Health Organization (WHO). Definitions and reporting framework for tuberculosis. Geneva: WHO. 15 Apr 2013. Available from: http://apps.who.int/iris/bitstream/10665/79199/1/9789241505345_eng.pdf
2. World Health Organization (WHO). Guidelines for treatment of tuberculosis, 4th ed. Geneva: WHO. 2009. Available from: http://whqlibdoc.who.int/publications/2010/9789241547833_eng.pdf
3. World Health Organization (WHO). Guidelines for the programmatic management of drug-resistant tuberculosis. Emergency update 2008. Geneva: WHO. 2008. Available from: http://whqlibdoc.who.int/publications/2008/9789241547581_eng.pdf
4. World Health Organization (WHO). Revised TB recording and reporting forms and registers - version 2006. Geneva: WHO. 2006. Available from: http://www.who.int/tb/dots/r_and_r_forms/en/