

Vol. 19 | Weekly issue 27 | 10 July 2014

Editorials	
Evidence and policy for influenza control by H Kelly, BJ Cowling	2
RESEARCH ARTICLES	
Effectiveness of trivalent seasonal influenza vaccine in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2012/13 end of season results by N Andrews, J McMenamin, H Durnall, J Ellis, A Lackenby, C Robertson, B von Wissmann, S Cottrell, B Smyth, C Moore, R Gunson, M Zambon, D Fleming, R Pebody	5
SURVEILLANCE AND OUTBREAK REPORTS	
Y155H amino acid substitution in influenza A(H1N1)pdm09 viruses does not confer a phenotype of reduced susceptibility to neuraminidase inhibitors by U Perez-Sautu, F Pozo, I Cuesta, S Monzon, A Calderon, M Gonzalez, M Molinero, I Lopez-Miragaya, S Rey, A Cañizares, G Rodriguez, C Gonzalez-Velasco, A Lackenby, I Casas	14
Till receipts - a new approach for investigating outbreaks? Evaluation during a large Salmonella Enteritidis phage type 14b outbreak in a north west London takeaway restaurant, September 2009	21

by D Zenner, J Zoellner, A Charlett, W Marmairis, C Lane, JY Chow



Evidence and policy for influenza control

H Kelly^{1,2}, B J Cowling (bcowling@hku.hk)³

- 1. Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia
- 2. National Centre for Epidemiology and Population Health, Australian National University, Canberra, Australia
- 3. Division of Epidemiology and Biostatistics, School of Public Health, Li Ka Shing Faculty of Medicine, the University of Hong Kong, Hong Kong Special Administrative Region, China

Citation style for this article:

Kelly H, Cowling BJ. Evidence and policy for influenza control. Euro Surveill. 2014;19(27):pii=20850. Available online: http://www.eurosurveillance.org/ViewArticle. aspx?Articleld=20850

Article submitted on 04 July 2014/ published on 10 July 2014

This edition of the journal includes two studies related to the control of influenza, one on neuraminidase inhibitor (NI) resistance [1] and the other on the effectiveness of trivalent influenza vaccine in the United Kingdom in 2012/13 [2]. Neuraminidase inhibitors (NIs) are the mainstay in influenza treatment and vaccination is the mainstay of prevention. It is thus important to monitor the effectiveness of both interventions over time. The current NI study demonstrates that mutations which may have had clinical significance for previously circulating seasonal influenza A(H1N1) viruses may not be clinically significant for influenza A(H1N1) pdmo9 viruses, highlighting the importance of continued monitoring of NI resistance. Of equal importance is the continued monitoring of NI effectiveness [3].

Influenza is a common disease with the annual risk of influenza virus infection exceeding 20% in some years [4,5]. However the great majority of influenza virus infections do not present as the classical triad of fever, cough and fatigue [6-8], and a substantial proportion of infections, perhaps even more than half, are asymptomatic [4,5]. Even symptomatic illnesses are generally self-limiting. However a small proportion of persons with influenza virus infections will require admission to hospital, intensive care and a smaller proportion will die [9]. These outcomes are uncommon and are influenced by age, with increased risk at the two extremes of life, and the presence of co-morbidities [10]. For instance, unadjusted annual risk estimates of laboratory-confirmed influenza hospitalisation in hospitals from the Emerging Infections Program in the United States between 2005 and 2011 ranged from 20 to 72 per 100,000 for children up to the age of four years, from 16 to 76 per 100,000 for adults aged at least 65 years, but only from 5 to 14 per 100,000 for adults aged 20 to 64 years, although higher in the first year after influenza A(H1N1)pdmo9 emerged [11]. About 10–30% of people hospitalised with influenza will require intensive care [12-14], and about 3-10% of patients hospitalised with laboratory confirmed influenza will die [13-15].

Because serious outcomes are relatively rare, randomised controlled trials (RCTs) in ambulatory settings for the treatment of influenza with NIs or the prevention of influenza by vaccination have not been designed with sufficient power to examine these outcomes. RCTs of antiviral drugs [16] and vaccines [17] and have shown efficacy against suspected and laboratory-confirmed influenza acquired and managed in the community but there are no RCTs investigating outcomes of hospitalisation or death due to laboratory-confirmed influenza.

It is generally acknowledged that when outcomes are rare, the RCT is not necessarily the study design of choice. The classic case-control study, in which cases and controls are ascertained retrospectively, has often been the preferred alternative design. A variation of the classic design has become increasingly popular for studying vaccine effectiveness (VE) against specific outcomes. In what is referred to as the case-testnegative design, patients with respiratory symptoms are ascertained prospectively, and vaccine coverage is compared between those who test positive and those who test negative for influenza, adjusting for potential confounders [18]. The second study of influenza in this issue of the Eurosurveillance uses the case-test-negative design in pooled community-based studies from the United Kingdom to estimate influenza VE against medically-attended respiratory disease confirmed as influenza. It reports point estimates of 73%, 26% and 51% against influenza A(H1N1), A(H3N2) and B, respectively [2]. These results confirm a number of other findings of low VE against influenza A(H₃N₂) in recent years [19-21], attributed to mismatch between the vaccine and circuiting strains [19]. They also highlight the importance of monitoring not only the antigenic match, as determined by serological assays, but also the genetic relatedness of circulating and vaccine viruses.

The case-test-negative design is also being increasingly used for studies of hospitalised patients, using PCR-confirmed influenza as an outcome. These studies suggest that inactivated influenza vaccines decrease the risk of hospital admission for laboratory-confirmed influenza by about half [22,23], although lower estimates have been reported for the protection against influenza A(H₃N₂) in the elderly [24] and higher estimates for protection against influenza A(H₁N₁)pdmo9 [25]. A 50% decrease in risk is similar to effectiveness estimates from community observational studies using the same design [26,27], and efficacy estimates from meta-analyses of community-based trials [17].

For information on the effectiveness of NIs among hospitalised patients, we likewise need to rely on observational studies. A recent review critically examined published cohort studies assessing oseltamivir treatment for laboratory-confirmed influenza and found evidence suggesting protection against mortality in four studies, all of which were judged by the review to be of reasonable quality, and between which there was no statistical heterogeneity [28].

Even the best designed observational studies may be subject to residual bias, suggesting the need for RCTs. However RCTs of NIs in outpatients with increased risk of complications, and in patients hospitalised soon after onset of symptoms may no longer be feasible because oseltamivir is the accepted front-line treatment in groups of patients with suspected or confirmed influenza [29-31] and such trials may no longer be granted ethical approval. The same argument applies to influenza vaccination for people aged 65 years and over. For these reasons, better quality data are unlikely to be derived from RCTs, so that observational studies might do well to follow published quality guidelines in an effort to improve VE estimates [32].

Doubt has been cast on the efficacy of influenza vaccines against serious outcomes in the elderly because of the absence of trial data [33]. Similar discussions are occurring about the efficacy of anti-viral medication [3,16]. At the same time, it is being increasingly recognised that influenza infection in the community is common and that infections are associated with a wide clinical spectrum, but the serious consequences of infection are generally uncommon, and often rare, in healthy young people [5]. Improved policies for the control of influenza virus infection should acknowledge the wide clinical spectrum resulting from infection, so that prevention or treatment of serious outcomes will be attempted when serious outcomes are more likely. Such policies should use data from observational studies where trial data are absent.

Conflict of interest

BJC has received research funding from MedImmune Inc. and Sanofi Pasteur, and consults for Crucell NV. HK has spoken at a clinical training session sponsored by Sanofi Pasteur.

References

- Perez-Sautu U, Pozo F, Cuesta I, Monzon S, Calderon A, Gonzalez M, et al. Y155H amino acid substitution in influenza A(H1N1)pdmo9 viruses does not confer a phenotype of reduced susceptibility to neuraminidase inhibitors. Euro Surveill. 2014;19(27):pii=20849.
- Andrews N, McMenamin J, Durnall H, Ellis J, Lackenby A, Robertson C, et al. Effectiveness of trivalent seasonal influenza vaccine in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2012/13 end of season results. Euro Surveill. 2014;19(27):pii=20851.
- Krumholz HM. Neuraminidase inhibitors for influenza. BMJ. 2014; 348:g2548. http://dx.doi.org/10.1136/bmj.g2548
- Monto AS, Koopman JS, Longini IM, Jr. Tecumseh study of illness. XIII. Influenza infection and disease, 1976-1981. Am J Epidemiol. 1985;121(6):811-22.
- Hayward AC, Fragaszy EB, Bermingham A, Wang L, Copas A, Edmunds WJ, et al. Comparative community burden and severity of seasonal and pandemic influenza: results of the Flu Watch cohort study. Lancet Respir Med. 2014;2(6):445-54. http://dx.doi.org/10.1016/S2213-2600(14)70034-7
- Thursky K, Cordova SP, Smith D, Kelly H. Working towards a simple case definition for influenza surveillance. J Clin Virol. 2003;27(2):170-9. http://dx.doi.org/10.1016/ S1386-6532(02)00172-5
- Call SA, Vollenweider MA, Hornung CA, Simel DL, McKinney WP. Does this patient have influenza? JAMA. 2005;293(8):987-97. http://dx.doi.org/10.1001/jama.293.8.987
- Lau LL, Nishiura H, Kelly H, Ip DK, Leung GM, Cowling BJ. Household transmission of 2009 pandemic influenza A (H1N1): a systematic review and meta-analysis. Epidemiology. 2012;23(4):531-42. http://dx.doi.org/10.1097/ EDE.obo13e31825588b8
- Wu JT, Ma ES, Lee CK, Chu DK, Ho PL, Shen AL, et al. The infection attack rate and severity of 2009 pandemic H1N1 influenza in Hong Kong. Clin Infect Dis. 2010;51(10):1184-91. http://dx.doi.org/10.1086/656740
- Uyeki TM. Preventing and controlling influenza with available interventions. N Engl J Med. 2014;370(9):789-91. http://dx.doi. org/10.1056/NEJMp1400034
- 11. Kostova D, Reed C, Finelli L, Cheng PY, Gargiullo PM, Shay DK, et al. Influenza Illness and Hospitalizations Averted by Influenza Vaccination in the United States, 2005-2011. PLoS One. 2013;8(6):e66312. http://dx.doi.org/10.1371/journal. pone.0066312
- 12. Campbell CN, Mytton OT, McLean EM, Rutter PD, Pebody RG, Sachedina N, et al. Hospitalization in two waves of pandemic influenza A(H1N1) in England. Epidemiol Infect. 2011;139(10):1560-9. http://dx.doi.org/10.1017/ S0950268810002657
- Louie JK, Acosta M, Winter K, Jean C, Gavali S, Schechter R, et al. Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California. JAMA. 2009;302(17):1896-902. http://dx.doi.org/10.1001/ jama.2009.1583
- 14. Bagdure D, Curtis DJ, Dobyns E, Glode MP, Dominguez SR. Hospitalized children with 2009 pandemic influenza A (H1N1): comparison to seasonal influenza and risk factors for admission to the ICU. PLoS One. 2010;5(12):e15173. http:// dx.doi.org/10.1371/journal.pone.0015173
- 15. Myles PR, Semple MG, Lim WS, Openshaw PJ, Gadd EM, Read RC, et al. Predictors of clinical outcome in a national hospitalised cohort across both waves of the influenza A/H1N1 pandemic 2009-2010 in the UK. Thorax. 2012;67(8):709-17. http://dx.doi.org/10.1136/thoraxjnl-2011-200266
- Jefferson T, Jones MA, Doshi P, Del Mar CB, Hama R, Thompson MJ, et al. Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. Cochrane Database Syst Rev. 2014;4:CD008965.
- 17. Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis. 2012;12(1):36-44. http:// dx.doi.org/10.1016/S1473-3099(11)70295-X
- Foppa IM, Haber M, Ferdinands JM, Shay DK. The case test-negative design for studies of the effectiveness of influenza vaccine. Vaccine. 2013;31(30):3104-9. http://dx.doi. org/10.1016/j.vaccine.2013.04.026
- Skowronski DM, Janjua NZ, De Serres G, Sabaiduc S, Eshaghi A, Dickinson JA, et al. Low 2012-13 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not antigenic drift in circulating viruses. PLoS One. 2014;9(3):e92153. http://dx.doi.org/10.1371/journal. pone.0092153
- 20. Sullivan SG, Komadina N, Grant K, Jelley L, Papadakis G, Kelly H. Influenza vaccine effectiveness during the 2012 influenza

season in Victoria, Australia: influences of waning immunity and vaccine match. J Med Virol. 2014;86(6):1017-25. http://dx.doi.org/10.1002/jmv.23847

- 21. Valenciano M, Kissling E, I-MOVE case-control study team. Early estimates of seasonal influenza vaccine effectiveness in Europe: results from the I-MOVE multicentre case-control study, 2012/13. Euro Surveill. 2013;18(7):pii=20400.
- 22. Puig-Barbera J, Arnedo-Pena A, Pardo-Serrano F, Tirado-Balaguer MD, Pérez-Vilar S, Silvestre-Silvestre E, et al. Effectiveness of seasonal 2008-2009, 2009-2010 and pandemic vaccines, to prevent influenza hospitalizations during the autumn 2009 influenza pandemic wave in Castellon, Spain. A test-negative, hospital-based, case-control study. Vaccine. 2010;28(47):7460-7. http://dx.doi.org/10.1016/j. vaccine.2010.09.042
- 23. Talbot HK, Griffin MR, Chen Q, Zhu Y, Williams JV, Edwards KM. Effectiveness of seasonal vaccine in preventing confirmed influenza-associated hospitalizations in community dwelling older adults. J Infect Dis. 2011;203(4):500-8. http://dx.doi. org/10.1093/infdis/jiq076
- 24. Kwong JC, Campitelli MA, Gubbay JB, Peci A, Winter AL, Olsha R, et al. Vaccine effectiveness against laboratory-confirmed influenza hospitalizations among elderly adults during the 2010-2011 season. Clin Infect Dis. 2013;57(6):820-7. http:// dx.doi.org/10.1093/cid/cit404
- 25. Castilla J, Godoy P, Domínguez A, Martínez-Baz I, Astray J, Martín V, et al. Influenza vaccine effectiveness in preventing outpatient, inpatient, and severe cases of laboratoryconfirmed influenza. Clin Infect Dis. 2013;57(2):167-75. http:// dx.doi.org/10.1093/cid/cit194
- 26. Kelly HA, Sullivan SG, Grant KA, Fielding JE. Moderate influenza vaccine effectiveness with variable effectiveness by match between circulating and vaccine strains in Australian adults aged 20-64 years, 2007-2011. Influenza Other Respir Viruses. 2013;7(5):729-37. http://dx.doi.org/10.1111/irv.12018
- 27. Ohmit SE, Petrie JG, Malosh RE, Cowling BJ, Thompson MG, Shay DK, et al. Influenza vaccine effectiveness in the community and the household. Clin Infect Dis. 2013;56(10):1363-9. http://dx.doi.org/10.1093/cid/cit060
- Freemantle N, Shallcross LJ, Kyte D, Rader T, Calvert MJ. Oseltamivir: the real world data. BMJ. 2014;348:g2371. http:// dx.doi.org/10.1136/bmj.g2371
- 29. Fiore AE, Fry A, Shay D, Gubareva L, Bresee JS, Uyeki TM, et al. Antiviral agents for the treatment and chemoprophylaxis of influenza --- recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2011;60(1):1-24.
- 30. World Health Organization (WHO). WHO Guidelines for Pharmacological Management of Pandemic Influenza A(H1N1) 2009 and Other Influenza Viruses. Geneva: WHO; 2010. Available from: http://www.who.int/csr/resources/ publications/swineflu/h1n1_use_antivirals_20090820/en/
- Public Health England (PHE). PHE guidance on use of antiviral agents for the treatment and prophylaxis of influenza. Version 4.1. London: PHE; 2014. Available from: http://www.hpa.org. uk/webc/HPAwebFile/HPAweb_C/1317140666783
- 32. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. PLoS Med. 2007;4(10):e296. http://dx.doi.org/10.1371/journal. pmed.0040296
- Doshi P. Influenza vaccines: time for a rethink. JAMA Intern Med. 2013;173(11):1014-6. http://dx.doi.org/10.1001/ jamainternmed.2013.490

RESEARCH ARTICLES

Effectiveness of trivalent seasonal influenza vaccine in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2012/13 end of season results

N Andrews (Nick.Andrews@phe.gov.uk)¹, J McMenamin², H Durnall³, J Ellis⁴, A Lackenby⁴, C Robertson^{2,5,6}, B von Wissmann², S Cottrell⁷, B Smyth⁸, C Moore⁷, R Gunson⁹, M Zambon⁴, D Fleming³, R Pebody¹ 1. Public Health England Health Protection Directorate, Colindale, London, United Kingdom

2. Health Protection Scotland, Glasgow, United Kingdom

3. Royal College of General Practitioners Research and Surveillance Centre, Birmingham, United Kingdom

4. Public Health England Operations Directorate, Microbiology Services, Colindale, London, United Kingdom

- University of Strathclyde, Glasgow, United Kingdom
 International Prevention Research Institute, Lyon, France
- Public Health Wales, Cardiff, United Kingdom
- 8. Public Health Agency Northern Ireland, Belfast, United Kingdom
- 9. West of Scotland Specialist Virology Centre, Glasgow, United Kingdom

Citation style for this article:

Andrews N, McMenamin J, Durnall H, Ellis J, Lackenby A, Robertson C, von Wissmann B, Cottrell S, Smyth B, Moore C, Gunson R, Zambon M, Fleming D, Pebody R. Effectiveness of trivalent seasonal influenza vaccine in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2012/13 end of season results. Euro Surveill. 2014;19(27):pii=20851. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20851

Article submitted on 25 September 2013 / published on 10 July 2014

The effectiveness of the 2012/13 trivalent seasonal influenza vaccine (TIV) was assessed using a testnegative case-control study of patients consulting primary care with influenza-like illness in the United Kingdom. Strain characterisation was undertaken on selected isolates. Vaccine effectiveness (VE) against confirmed influenza A(H3N2), A(H1N1) and B virus infection, adjusted for age, sex, surveillance scheme (i.e. setting) and month of sample collection was 26% (95% confidence interval (CI): -4 to 48), 73% (95% CI: 37 to 89) and 51% (95% CI: 34 to 63) respectively. There was an indication, although not significant, that VE declined by time since vaccination for influenza A(H₃N₂) (VE 50% within three months, 2% after three months, p=0.25). For influenza A(H3N2) this is the second season of low VE, contributing to the World Health Organization (WHO) recommendation that the 2013/14 influenza vaccine strain composition be changed to an A(H₃N₂) virus antigenically like cell-propagated prototype 2012/13 vaccine strain (A/Victoria/361/2011). The lower VE seen for type B is consistent with antigenic drift away from the 2012/13 vaccine strain. The majority of influenza B viruses analysed belong to the genetic clade 2 and were antigenically distinguishable from the 2012/13 vaccine virus B/Wisconsin/1/2010 clade 3. These findings supported the change to the WHO recommended influenza B vaccine component for 2013/14.

Introduction

The 2012/13 influenza season in the United Kingdom (UK) was unusually long with elevated levels of activity persisting from week 50 to 16. In England, Northern Ireland and Wales, the season was dominated initially by circulation of influenza B virus, with school outbreaks in the period before Christmas. This was followed by influenza A(H3N2) virus circulation particularly in the New Year and spring with influenza outbreaks in often highly vaccinated care home populations [1]. Scotland presented a different picture with influenza activity initially dominated by influenza A(H₃N₂) followed by influenza B virus circulation.

The occurrence of late season influenza A outbreaks in much of the UK some months after the completion of the 2012/13 influenza vaccine campaign led to guestions being raised about waning intra-seasonal vaccine protection. A similar observation of late season influenza A(H₃N₂) outbreaks in care home settings in 2011/12 was accompanied by the observation of significant intra-seasonal waning in protection for those vaccinated more than three months previously [2]. Trivalent seasonal influenza vaccine (TIV) in 2012/13 included an A/California/7/2009 (H1N1)pdm09-like virus, an A/Victoria/361/2011 (H3N2)-like virus and a B/Wisconsin/1/2010-like virus (Yamagata lineage). In 2012/13, vaccine uptake was 73.4% in those aged over 65 years and 51.3% in risk groups with individuals aged under 65 years in England [1].

Mid-season estimates from the UK and elsewhere were published in January 2013 with vaccine effectiveness (VE) against all influenza types ranging from 45% in Canada to 50% in a European study and 51% in the UK

TABLE 1

Reference influenza	B haemagglutinin 1	sequences used in	phylogenetic	analysis
	00	1	1 / 0	

Virus isolate	Segment	Sequence source	Segment ID/ Accession number	Country	Collection date (year-month- day)	Originating laboratory	Submitting laboratory
B/Wisconsin/01/2010	HA	GISAID EpiFlu	EPI271600	United States	2010-02-20	Wisconsin State Laboratory of Hygiene	Centers for Disease Control and Prevention
B/Estonia/55669/2011	HA	GISAID EpiFlu	EPI319345	Estonia	2011-03-14	Health Protection Inspectorate Estonia	National Institute for Medical Research
B/Odessa/3886/2010	HA	GISAID EpiFlu	EPI271913	Ukraine	2010-03-19	Ministry of Health of Ukraine	National Institute for Medical Research
B/Florida/4/06	HA	GenBank	CY073895	N/A	N/A	N/A	N/A
B/HongKong/330/2001	HA	GenBank	AJ783379	N/A	N/A	N/A	N/A
B/Victoria/2/87	HA	GenBank	CY018757	N/A	N/A	N/A	N/A
B/Yamagata/16/88	HA	GenBank	CY018765	N/A	N/A	N/A	N/A
B/Malaysia/2506/2004	HA	GenBank	Y038287	N/A	N/A	N/A	N/A
B/Victoria/304/2006	HA	GenBank	EU124261	N/A	N/A	N/A	N/A
B/Brisbane/60/2008	HA	GenBank	FJ766840	N/A	N/A	N/A	N/A

ID: identity; GISAID: Global Initiative on Sharing Avian Influenza Data; HA: haemagglutinin; N/A: not applicable.

[3-5]. This study presents the end-of-season VE for the 2012/13 seasonal TIV in preventing medically attended confirmed influenza A(H₃N₂), A(H₁N₁)pdmo9 and B virus infection using the established primary care sentinel swabbing surveillance schemes in the UK [2,3,6]. It also examines the protective effect of vaccination measured at different points during the season and by time since vaccination, to determine if there is any evidence of intra-seasonal waning protection.

Methods

Study population and period

Data were derived from five primary care influenza sentinel swabbing surveillance schemes in the UK from England (two schemes), Northern Ireland, Scotland, and Wales. Details of the Royal College of General Practitioners (RCGP), Public Health England (PHE) Specialist Microbiology Network (SMN), Public Health Agency (PHA) of Northern Ireland, Health Protection Scotland (HPS) and Public Health Wales swabbing schemes have been published previously [7].

The study period ran from 1 October 2012 to 24 April 2013. Patients were swabbed as part of clinical care, with verbal consent. Cases were defined, as persons presenting during the study period in a participating general practitioner (GP) practice with an acute influenza-like illness (ILI) who were swabbed and then tested positive for influenza A or B. ILI was defined as an individual presenting in primary care with an acute respiratory illness with physician-diagnosed fever or complaint of feverishness. Controls were individuals presenting with ILI in the same period who were swabbed and tested negative for influenza.

A standardised questionnaire was completed by the GP responsible for the patient during the consultation. Demographic, clinical and epidemiological information was collected from cases and controls, including date of birth, sex, defined underlying clinical risk group, date of onset of respiratory illness, date of specimen collection, and influenza vaccination status for the 2012/13 season with vaccination dates.

Laboratory methods

Laboratory confirmation was undertaken using realtime polymerase chain reaction (RT-PCR) assays capable of detecting circulating influenza A viruses, influenza B viruses and other respiratory viruses [8,9]. Samples in England were sent to the PHE Microbiology Services, Colindale (RCGP scheme) or one of the specialist PHE microbiology laboratories (SMN scheme). Samples in Northern Ireland were sent to the Regional Virus Laboratory, Belfast, in Scotland to the West of Scotland Specialist Virology Centre, Glasgow (HPS scheme), and in Wales to the Public Health Wales Specialist Virology Centre, Cardiff.

Further strain characterisation was also performed. Influenza viruses were isolated in Madin-Darby canine kidney (MDCK) or MDCK-SIAT1 cells from all RT-PCR positive samples from England as previously described [10]. Virus isolates with a haemagglutination titre \geq 40 were then characterised antigenically using post-infection ferret antisera in haemagglutination inhibition (HI) assays, with guinea pig (A(H₃N₂) viruses) or turkey (A(H₁N₁)pdmo9 and influenza B viruses) red blood cells [11]. Nucleotide sequencing of the HA₁ region of the haemagglutinin (HA) gene of a subset of influenza B viruses was performed (primer sequences available on request), and phylogenetic trees were constructed with a neighbour-joining algorithm available in the



Swabbing results in the United Kingdom, week 40 2012 to week 16 2013^a (n=4,649 individuals)

^a Corresponding to the period from 1 October 2012 to 24 April 2013.

Mega 4.0.1 software (http://www.megasoftware.net). Influenza B samples were selected for sequencing to be representative of the range of patient's age, date of sample collection, geographical location, and antigenic characterisation of the influenza B virus isolate, if performed. HA sequences from reference strains used in the phylogenetic analysis were obtained from the National Center for Biotechnology Information (NCBI) GenBank and EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID) (Table 1).

Statistical methods

Persons were defined as vaccinated if the date of vaccination with the 2012/13 TIV was 14 or more days before onset of illness. Those in whom the period between vaccination and onset of illness was less than 14 days were excluded from analysis. If the date of vaccination was missing, as the 2012/13 campaign occurred before influenza circulation was common, it was assumed that TIV vaccination was more than 14 days before onset date. Those with a missing date of onset or an onset date more than seven days before the swab was taken were excluded.

VE was estimated by the test-negative case-control (TNCC) design [12]. In this design VE is calculated as 1 - (odds ratio) obtained using multivariable logistic regression models with influenza A or influenza B PCR results as outcomes and seasonal vaccination status as the linear predictor. Influenza A results were also further

categorised for A(H₃N₂) and A(H₁N₁)pdmo9 viruses. In the analyses evaluating VE for a specific type or strain, those positive for other types were excluded. Age (coded into five standard age groups, <5, 5–14, 15–44, 45–64 and 65 years), sex, clinical risk group, surveillance scheme (RCGP, SMN, PHA of Northern Ireland, HPS, Public Health Wales) and date of sample collection (month) were investigated as potential confounding variables. To investigate whether the VE changed in relation to time since vaccination analyses stratifying VE by time since vaccination (<3 months - i.e. 91 days -, \geq 3 months) and by period (October to January, February to April) were undertaken. Where date of vaccination was not given, time since vaccination was estimated based on assuming vaccination occurred at the median vaccination date of 20 October 2012, and also treated as missing in a sensitivity analysis. To test for the significance of changes in VE with time since vaccination, the multivariable logistic regression was performed in vaccinated individuals with days since vaccination (between vaccination and symptom onset date) included as a continuous variable and with the binary variable of $\langle 3 \rangle$ months and $\geq 3 \rangle$ months since vaccination. VE was also assessed stratified by age and scheme with differences in VE tested by a likelihood ratio test between groups where numbers were not too low for a precise estimate. All statistical analyses were carried out in Stata version 12 (StataCorp, College Station, Texas).

Monthly influenza positivity rates by country, United Kingdom, 2012/13 influenza season (n=4,649 individuals)



Northern Ireland



Results

A total of 4,649 individuals were swabbed in primary care during the study period and had a laboratory result available. Figure 1 shows the numbers of swabs and swab results over the study period and Figure 2 shows positivity rates for England/Wales, Northern Ireland and Scotland during 2012/13, indicating the different timing of influenza A and B virus circulation in Scotland to the rest of the UK. For the VE analysis two individuals were excluded due to an inconclusive result, 350 due to a missing symptom onset date, 839 because they were swabbed more than seven days after symptom onset, 143 due to missing vaccination status and 29 because they were vaccinated within 14 days of onset. The details of the 3,286 individuals remaining in the study are given in Table 2 according to the swab result. Positivity rates differed significantly by all variables in this table.

Strain characterisation

Antigenic analysis by HI assay of influenza A(H₃N₂) viruses isolated from positive samples submitted through the RCGP scheme, demonstrated that these viruses were antigenically homogeneous, and closely related antigenically (fourfold differences in HI assays for all 89 tested) to the cell-propagated H₃N₂ vaccine strain, A/Victoria/361/2011 [1]. The relatively fewer influenza A(H₁N₁)pdmo9 viruses isolated in 2012/13 were closely related antigenically to the A(H₁N₁) pdmo9 2012/13 vaccine strain, A/California/7/2009, though six of thirty isolates did show reduced reactivity in antigenic characterisation assays with antiserum raised against influenza A/California/7/2009 (>fourfold difference in HI assays).

Of the 2012/13 UK influenza B viruses analysed, the majority (411/482, 85%) were characterised as belonging to the B/Yamagata/16/88-lineage, as was the 2012/13 influenza B vaccine strain, B/Wisconsin/1/2010 (Figure 3). Genetically, the HA genes of B-Yamagata 2012/13 strains fell within two HA genetic clades (clade 2 and 3). The majority of influenza B UK viruses analysed belonged to genetic clade 2 (Figure 3), and were antigenically distinguishable from the 2012/13 vaccine virus, B/Wisconsin/1/2010 clade 3. Of the 193 B/Yamagata-lineage viruses analysed antigenically, only 20 (10.3%) were antigenically similar to the B/ Wisconsin/1/2010 vaccine component. The majority showed reduced reactivity in antigenic characterisation assays with antiserum raised against influenza B/Wisconsin/1/2010, with 65 (33.7%) showing a fourfold difference and 108 (56.0%) a greater than fourfold difference.

Model fitting for vaccine effectiveness estimation

When estimating vaccine effects, age group, sex, time period (defined by month of sample collection) and surveillance scheme were adjusted for in a multivariable logistic regression model. Although all these variables, except sex, were significantly associated with having a positive swab, only age group was a confounder for the vaccine effects (changed the estimate by more than 5%). Information on risk group was missing for 182 of 3,286 samples (5.5%) and was therefore not included in the final model. If risk group was included, it was found not to be associated with being positive and the VE estimates remained similar.

Tables 3 and 4 show VE estimates against influenza A(H₃N₂), A(H₁N₁)pdmo9 and B according to vaccination status and time since vaccination and period.

Vaccine effectiveness against influenza A infection

The adjusted VE of TIV against influenza A was 35% (95% confidence interval (CI): 11 to 53), however this

differed for influenza A(H3N2) and A(H1N1)pdmo9. For influenza A(H1N1)pdmo9 overall VE was 73% (95% CI: 37 to 89) compared to 26% (95% CI: -4 to 48) for influenza A(H3N2).

For influenza A(H1N1)pdmo9, VE showed evidence of a decline by time since vaccination (Table 4). For influenza A(H3N2), there was also evidence of a decline in VE from 50% (95% Cl: 16 to 71) within three months of vaccination to 2% (95% Cl: -49 to 36) after three months. This decline was not, however statistically significant (p=0.25) and was less apparent when looking at VE by period (Table 3). Assessing time since vaccination with

those with a missing date of vaccination excluded gave similar results with a decline from 100% (95%Cl: 56 to 100) to 63% (95% Cl: -7 to 87) for influenza A(H1N1) pdmo9 and from 41% (95%Cl:o to 66) to 5% (95% Cl: -51 to 41) for influenza A(H3N2).

The adjusted age-specific estimate for influenza A protection was lower in the oldest age group (≥ 65 years compared to other ages) (Table 4), however, the observed differences were not significant. VE in the vaccine target group (aged ≥ 65 years or in a risk group) was 13% (95% CI:-44 to 67) due to the low VE estimate in those aged ≥ 65 years. VE also showed some

TABLE 2

Details for influenza A and B cases and controls, United Kingdom, October 2012-April 2013 (n=3,286 individuals)

	Total cases and controls ^a	Controls (N=1,956) n (%)	Influenza B cases (N=827) ^b n (%)	Influenza A cases (N=506) ^b n (%)	A(H1N1) pdm09 cases (N=127) n (%)	A(H3N2) cases (N=354) n (%)
Age group (years)			·			
<5	294	214 (72.8)	50 (17.0)	31 (10.5)	10 (3.4)	19 (6.5)
5-14	406	185 (45.6)	172 (42.4)	49 (12.1)	10 (2.5)	35 (8.6)
15-44	1,485	884 (59.5)	341 (23.0)	261 (17.6)	74 (5.0)	176 (11.9)
45-64	822	471 (57.3)	229 (27.9)	123 (15.0)	31 (3.8)	85 (10.3)
≥65	268	196 (73.1)	32 (11.9)	40 (14.9)	1 (0.4)	38 (14.2)
Missing	11	6 (54.5)	3 (27.3)	2 (18.2)	1 (9.1)	1 (9.1)
Sex			` 			
Male	1,321	748 (56.6)	355 (26.9)	219 (16.6)	50 (3.8)	157 (11.9)
Female	1,919	1,179 (61.4)	463 (24.1)	279 (14.5)	72 (3.8)	194 (10.1)
Missing	46	29 (63.0)	9 (19.6)	8 (17.4)	5 (10.9)	3 (6.5)
Surveillance scheme	·					
RCGP	1,535	920 (59.9)	397 (25.9)	219 (14.3)	72 (4.7)	147 (9.6)
SMN	408	274 (67.2)	69 (16.9)	65 (15.9)	16 (3.9)	30 (7.4)
HPS	1,086	653 (60.1)	265 (24.4)	170 (15.7)	32 (2.9)	132 (12.2)
Public Health Wales	87	31 (35.6)	39 (44.8)	17 (19.5)	3 (3.4)	14 (16.1)
PHA of Northern Ireland	170	78 (45.9)	57 (33.5)	35 (20.6)	4 (2.4)	31 (18.2)
Risk group						
No	2,488	1,432 (57.6)	669 (26.9)	388 (15.6)	108 (4.3)	261 (10.5)
Yes	616	419 (68.0)	115 (18.7)	84 (13.6)	9 (1.5)	70 (11.4)
Missing	182	105 (57.7)	43 (23.6)	34 (18.7)	10 (5.5)	23 (12.6)
Interval symptom onset-sample	(days)					
0-1	442	279 (63.1)	79 (17.9)	84 (19.0)	20 (4.5)	59 (13.3)
2-4	1,810	1,016 (56.1)	496 (27.4)	299 (16.5)	79 (4.4)	205 (11.3)
5-7	1,034	661 (63.9)	252 (24.4)	123 (11.9)	28 (2.7)	90 (8.7)
Vaccination status						
Unvaccinated	2,752	1,577 (57.3)	747 (27.1)	431 (15.7)	120 (4.4)	291 (10.6)
Vaccinated (14–91 days ago ^c)	292	226 (77.4)	43 (14.7)	23 (7.9)	o (o.o)	20 (6.8)
Vaccinated (>91 days ago ^c)	242	153 (63.2)	37 (15.3)	52 (21.5)	7 (2.9)	43 (17.8)

HPS: Health Protection Scotland; PHA: Public Health Agency; RCGP: Royal College of General Practitioners' surveillance scheme; SMN: PHE Specialist Microbiology Network.

Numbers and row percentages (to indicate positivity ratesa) are shown. For example of those 294 swabbed aged <5 years, 72.8% were negative, 17.0% had influenza B and 10.5% had influenza A.

Differences between cases and controls for all variables in this table were statistically significant, chi-squared test.

^a Two individuals positive for influenza A(H3N2) and B and one individual positive for A(H1N1)pdmo9 and B, are included in both the influenza B and influenza A columns. For the totals in this column, these individuals are only counted once.

^b 25 influenza A cases were of unknown strain, these are included in influenza A VE analysis but not the strain specific analyses. Also two individuals positive for influenza A(H₃N₂) and B and one individual positive for A(H₁N₁)pdmo9 and B are included in both the influenza B and influenza A columns, which is why the total adds to 3,289 for controls, influenza A and influenza B.

^c Where a date of vaccination was missing (n=150) this was estimated by assuming vaccination was on 20 October 2012, the median time of vaccination in controls with onset in 2013.

Phylogenetic analysis with sequences from reference viruses downloaded from NCBI GenBank and GISAID EpiFlu databases of influenza B sequences derived from patients in the United Kingdom, 2012/13 influenza season



GISAID: Global Initiative on Sharing Avian Influenza Data; NCBI: National Center for Biotechnology Information.

United Kingdom 2012/13 sequences are shown in bold; the 2012/13 vaccine strain is boxed. Branch lengths are drawn to scale. Amino acid changes characteristic of genetic clades are marked in the tree.

TABLE 3

Adjusted vaccine effectiveness estimates based on samples positive (cases, N=1,330) and negative (controls, N=1,956) for influenza according to vaccination status, United Kingdom, October 2012–April 2013

	Vaccination	Number of cases ^a and controls	Adjusted VE% (95% CI) by influenza type⁵				
Period	status	B:A:H1N1:H3N2:Con	Influenza B	Influenza A overall	Influenza A(H1N1)pdm09	Influenza A(H3N2)	
No		747:431:120:291:1,577				- ((, t- , 0)	
Oct 2012–Apr 2013	Yes	80:75:7:63:379	51 (34 to 63)	35 (11 to 53)	73 (37 to 89)	26 (-4 to 48)	
Octanda lan anda	No	518:245:58:170:1,092	49 (27 to 64)	44 (11 to 65)	82 (22 to 96)	38 (-4 to 63)	
Oct 2012–Jan 2013	Yes	49:29:2:24:248					
Eab ages Apr 2012	No	229:186:62:121:485	52 (22 to 72)	$24(40 \pm 0.17)$	68 (6 to 89)	23 (-31 to 54)	
Feb 2013–Apr 2013	Yes	31:46:5:39:131	53 (22 to 72)	31 (-10 10 4/)			

A: influenza A; B: influenza B; CI: confidence interval; con: control; H1N1: influenza A(H1N1)pdm09; H3N2: influenza A(H3N2); VE: vaccine effectiveness.

^a Because two individuals positive for influenza A(H₃N₂) and B, and one individual positive for A(H₁N₁)pdmo9 and B are included in both the influenza B and influenza A cases, summing up the cases presented in this column amounts to 1,333 instead of 1,330.

^b Adjusted for age group, sex, month of sample collection and surveillance scheme.

TABLE 4

Adjusted vaccine effectiveness estimates for influenza by age, surveillance scheme and by time since vaccination, United Kingdom, October 2012–April 2013

	Adjusted VEª% (95% Cl) by influenza type						
Factor	Influenza B	Influenza A overall	Influenza A(H1N1)pdm09	Influenza A(H3N2)			
Age (in years)							
<5	n too low ^b	n too low ^b	n too low ^b	n too low ^b			
5-14	74 (1 to 93)	n too low ^b	n too low ^b	n too low ^b			
15-44	68 (46 to 82)	54 (21 to 73)	83 (28 to 96)	40 (-7 to 66)			
45-64	34 (-1 to 57)	37 (-10 to 63)	90 (20 to 99)	32 (-27 to 63)			
All <65	50 (32 to 63)	43 (19 to 61)	76 (40 to 95)	35 (3 to 56)			
≥65	65 (18 to 85)	-19 (-217 to 55)	n too low ^b	-14 (-206 to -57)			
≥65 or in a risk group	46 (18 to 65)	13 (-44 to 47)	60 (-62 to 90)	11 (-53 to 49)			
Scheme							
RCGP	46 (14 to 64)	57 (25 to 75)	74 (15 to 92)	50 (8 to 73)			
SMN	49 (-48 to 82)	-22 (-246 to -56)	n too low ^b	n too low ^b			
HPS	44 (9 to 66)	32 (-16 to 60)	84 (-23 to 98)	16 (-47 to 52)			
Public Health Wales	94 (30 to 99)	n too low ^b	n too low ^b	n too low ^b			
PHA of Northern Ireland	81 (21 to 95)	n too low ^b	n too low ^b	n too low ^b			
Time since vaccination							
<3 months	57 (37 to 70)	56 (28 to 73)	100 (66 to 100) ^c	50 (16 to 71)			
≥3 months	42 (12 to 61)	15 (-25 to 42)	56 (-6 to 82)	2 (-49 to 36)			

CI: confidence interval; HPS: Health Protection Scotland; PHA: Public Health Agency; RCGP: Royal College of General Practitioners' surveillance scheme; SMN: Public Health England Specialist Microbiology Network, VE: vaccine effectiveness.

^a Adjusted for age group, sex, month of sample collection and surveillance scheme.

^b Number of vaccinated cases/controls too low to give an estimate with meaningful precision (95% CI lower end <-200 and upper end >80).
 ^c Unadjusted Cornfield 95% CI.

variability across the schemes although this difference was not significant.

Vaccine effectiveness against influenza B infection The adjusted VE of TIV against influenza B was 51% (95% Cl: 34 to 63). VE was 57% within three months of vaccination and non-significantly lower at 42% after three months (Table 4). VE did not vary by age group or scheme.

Discussion

In this study we found moderate effectiveness of 2012/13 TIV against laboratory-confirmed influenza B and good protection against influenza A(H1N1)pdmo9 infection. However, VE against influenza A(H₃N₂) infection was poor at only 26% (95% CI: -4 to 48). We also found a non-significant trend that effectiveness waned by time since vaccination for influenza A(H₃N₂), which is consistent with the waning seen against influenza $A(H_3N_2)$ for the 2011/12 TIV vaccine in the previous season where it reduced from 53% (95% CI: o to 78) within three months of vaccination to 12% (95% CI: -31 to 41) after three months [2]. The point estimate for 2011/12 was also similar for influenza A(H3N2) at 23% (95% CI: -10 to 47), although VE was higher in 2011/12 against influenza B at 92% (95% CI: 38 to 99) compared to 2012/13. The VE against A(H1N1)pdm09 is consistent with that seen with monovalent pandemic vaccine (adjuvanted) in 2009/10 and with TIV in 2010/11 [6,7].

Influenza vaccine strains are propagated in eggs during the vaccine manufacturing process. It has been reported that propagation of the A/Victoria/361/2011(H3N2)-like vaccine viruses for vaccine production resulted in antigenic changes in the virus resulting from adaptation to the growth in eggs, although circulating viruses were closely related antigenically to the cell-propagated influenza A(H₃N₂) vaccine strain, A/Victoria/361/2011 [13]. Following these observations the influenza A(H₃N₂) vaccine component for use in the 2013/14 season (northern hemisphere winter) has been updated to recommend an influenza A(H₃N₂) virus antigenically like the cell-propagated A/Victoria/361/2011 prototype strain (such as A/Texas/50/2012) [13]. The majority of influenza B virus isolates in 2012/13 were characterised as belonging to the B/Yamagata/16/88-lineage, as does the 2012/13 influenza B vaccine strain, B/ Wisconsin/1/2010. However, genetically, the HA genes of the majority of B-Yamagata strains fell within a genetic clade (clade 2), which in HI assays are antigenically distinguishable from B/Wisconsin/1/2010 (genetic clade 3)-like viruses. This provides an explanation for the lower VE observed against influenza B in 2012/13 compared to 2011/12. Consequently the influenza B vaccine component recommended for use in 2013/14 has also been updated, to a B/Massachusetts/2/2012 (clade2)-like virus [13].

When stratifying VE by age and scheme, VE is estimated with lower precision. There were no significant differences in VE by age or scheme although the point estimate for VE against influenza A was negative for the SMN scheme and also for the over 65 years age group. These differences are likely to be chance fluctuations due to small numbers and emphasise the need for large numbers of swabs for precise estimates for such subgroup analyses.

This is now the fourth season in which a pooled UK VE analysis has been performed using the TNCC design with mid-season estimates also produced for 2009/10, 2010/11 and 2012/13 [2,3,6,7,14]. Results from the RCGP scheme have also been published for 2005/06, 2006/07 and 2007/08 [15]. The results of each season have been consistent with those published from other countries and from pooled European analyses but often with greater precision in the UK due to the large numbers of swabs. The mid-season 2012/13 results gave VE against influenza A of 49% (95% CI: -2 to 75) and influenza B of 52% (95% CI: 23 to 70), which was similar to other early season results from Europe and Canada [4,5], and also similar to the end of season result of 51% (95% CI: 34 to 63) for influenza B, but higher than the end of season result of 35% (95% CI: 11 to 53) for influenza A. The TNCC design is now the most commonly used method for estimating the VE of influenza vaccines. This reflects the advantages of the method in terms of its simplicity and the fact that those that test negative form an excellent control group as they are well matched on propensity to consult a GP. Further discussion of the methodological issues have been published previously [12,16,17] and a recent paper has demonstrated the methods validity compared to placebo controlled clinical trial results [18].

The intra-seasonal waning of VE against influenza $A(H_3N_2)$ seen in 2011/12 in the UK is supported by the estimates seen this year, albeit non-significant. Carehome outbreaks late in the season both in 2011/12 and 2012/13 also support this observation [1,19], as did similar findings in other countries in 2011/12 [20]. With a new influenza $A(H_3N_2)$ and B strain recommended for 2013/14 and with the introduction of a trivalent live attenuated intranasal vaccine for all children aged 2-3 years and up to 10 years of age in parts of the UK [21] monitoring of VE remains an essential part of influenza surveillance.

Acknowledgements

We are grateful to the many primary care physicians in England, Wales, Northern Ireland and Scotland who supplied the clinical information on their patients; to the staff of the PHE Respiratory Virus Unit, the PHE Specialist Microbiology laboratories, Public Health Wales Specialist Virology Centre, the West of Scotland Specialist Virology Centre and the Regional Virus Laboratory, Belfast who undertook analysis of specimens. We thank the staff of the PHE, RCGP, Public Health Wales, Public Health Agency Northern Ireland and Health Protection Scotland teams who coordinate the GP schemes, in particular Joy Field from PHE; Richard Lewis and Hannah Evans from PHW; Catherine Frew, Alasdair MacLean & Celia Aitken from WoSSVC and Arlene Reynolds, Louise Primrose-Shaw and Karen Voy from HPS for overseeing data collection. Funding: The infrastructure and methodology to enable the work was developed as part of a European Centre for Disease Control and Prevention (ECDC) funded project "I-MOVE". The I-MOVE group continues to be co-ordinated by Epi-Concept (http://www.epiconcept.fr/).

We acknowledge the originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which some of the analyses are based (see Table 1). All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

Conflict of interest

D Fleming has received consultancy fees from influenza vaccine manufacturers (GSK, Sanofi and Medimmune). The Virus Reference Department of Public Health England receives funding from vaccine manufacturers (CSL, GSK, Novartis and Sanofi, Baxter).

Authors' contributions

Nick Andrews led the writing of the paper. All authors provided contribution to the paper and approved the final version. Nick Andrews and Chris Robertson performed statistical analyses of the 2012/13 influenza data. Joanna Ellis and Angie Lackenby did strain characterisation on RCGP data and Rory Gunson on Health Protection Scotland data. Richard Pebody, Nick Andrews, Douglas Fleming, Jim McMenamin and Chris Robertson were involved in the original methodological design but all other authors have had a role in modification of this design over the years.

References

- Public Health England (PHE). Surveillance of influenza and other respiratory viruses, including novel respiratory viruses, in the United Kingdom: Winter 2012/13. London: PHE; Jun 2013. Available from http://www.hpa.org.uk/Publications/ InfectiousDiseases/Influenza/1306influseasonalannualreport/
- Pebody R, Andrews N, McMenamin J, Durnall H, Ellis J, Thompson CI, et al. Vaccine effectiveness of 2011/12 trivalent seasonal influenza vaccine in preventing laboratoryconfirmed influenza in primary care in the United Kingdom: evidence of waning intra-seasonal protection. Euro Surveill. 2013;18(5):pii=20389.
- 3. McMenamin J, Andrews N, Robertson C, Fleming D, Durnall H, von Wissmann B, et al. Effectiveness of seasonal 2012/13 vaccine in preventing laboratory-confirmed influenza infection in primary care in the United Kingdom: mid-season analysis 2012/13. Euro Surveill. 2013;18(5):pii=20393.
- 4. Valenciano M, Kissling E; I-MOVE Case-Control Study Team. Early estimates of seasonal influenza vaccine effectiveness in Europe: results from the I-MOVE multicentre case-control study, 2012/13. Euro Surveill. 2013;18(7): pii=20400.
- Skowronski DM, Janjua NZ, De Serres G, Dickinson JA, Winter AL, Mahmud SM, et al. Interim estimates of influenza vaccine effectiveness in 2012/13 from Canada's sentinel surveillance network, January 2013. Euro Surveill. 2013;18(5):pii= 20394.
- Pebody RG, Andrews N, Fleming DM, McMenamin J, Cottrell S, Smyth B, et al. Age-specific vaccine effectiveness of seasonal 2010/2011 and pandemic influenza A(H1N1) 2009 vaccines in preventing influenza in the United Kingdom. Epidemiol Infect. 2013;141(3):620-30. http://dx.doi.org/10.1017/ S0950268812001148
- Hardelid P, Fleming DM, McMenamin J, Andrews NJ, Robertson C, Sebastianpillai P, et al. Effectiveness of pandemic and seasonal influenza vaccine in preventing pandemic influenza A(H1N1)2009 infection in England and Scotland 2009-2010. Euro Surveill. 2011;16(2):pii=19763.
- Ellis J, Iturriza M, Allen R, Bermingham A, Brown K, Gray J, et al. Evaluation of four real-time PCR assays for detection of influenza A(H1N1)v viruses. Euro Surveill. 2009;14(22):pii=19230.
- 9. Gunson R, Maclean A, Davies E, Bennett S, Miller R, Carman WF. Development of a multiplex real-time RT-PCR that allows universal detection of influenza A viruses and

simultaneous typing of influenza A/H1N1/2009 virus. J Virol Methods. 2010;163(2):258-61. http://dx.doi.org/10.1016/j. jviromet.2009.10.006

- Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD. Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. J Virol. 2003;77(15):8418-25. http://dx.doi. org/10.1128/JVI.77.15.8418-8425.2003
- Zambon M. Laboratory Diagnosis of Influenza. In: Nicholson K, Hay A, Webster RG, editors. Textbook of Influenza. Oxford: Blackwell Science; 1998. pp. 291–313.
- Orenstein EW, De Serres G, Haber MJ, Shay DK, Bridges CB, Gargiullo P, et al. Methodologic issues regarding the use of three observational study designs to assess influenza vaccine effectiveness. Int J Epidemiol. 2007;36(3):623-31. http:// dx.doi.org/10.1093/ije/dym021
- World Health Organization (WHO). Recommended composition of influenza virus vaccines for use in the 2013-2014 northern hemisphere influenza season. Geneva: WHO; 21 Feb 2013. Available from: http://www.who.int/influenza/vaccines/virus/ recommendations/
- 14. Pebody R, Hardelid P, Fleming D, McMenamin J, Andrews N, Robertson C, et al. Effectiveness of seasonal 2010/11 and pandemic influenza A(H1N1)2009 vaccines in preventing influenza infection in the United Kingdom: mid-season analysis 2010/11. Euro Surveill. 2011;16(6): pii=19791.
- Fleming DM, Andrews NJ, Ellis JS, Bermingham A, Sebastianpillai P, Elliot AJ, et al. Estimating influenza vaccine effectiveness using routinely collected laboratory data. J Epidemiology Community Health. 2010;64(12), 1062–7. http:// dx.doi.org/10.1136/jech.2009.093450
- Ferdinands JM, Belongia EA, Nwasike C, Shay DK. Influenza vaccination status is not associated with influenza testing among children: Implications for observational studies of vaccine effectiveness. Vaccine. 2011;29(10):1935-40. http:// dx.doi.org/10.1016/j.vaccine.2010.12.098
- Valenciano M, Kissling E, Ciancio BC, Moren A. Study designs for timely estimation of influenza vaccine effectiveness using European sentinel practitioner networks. Vaccine. 2010;28(46):7381-8. http://dx.doi.org/10.1016/j. vaccine.2010.09.010
- De Serres G, Skowronski D, Wu XW, Ambrose CS. The test-negative design: validity, accuracy and precision of vaccine efficacy estimates compared to the gold standard of randomised placebo-controlled clinical trials. Euro Surveill. 2013;18(37):pii=20585.
- Health Protection Agency (HPA). Surveillance of influenza and other respiratory pathogens in the UK, 2011/12. London: HPA; Jun 2012. Available from: http://www.hpa.org.uk/Publications/ InfectiousDiseases/Influenza/1206influannualreport/
- 20. Kissling E, Valenciano M, Larrauri A, Oroszi B, Cohen JM, Nunes B, et al. Low and decreasing vaccine effectiveness against influenza A(H3) in 2011/12 among vaccination target groups in Europe: results from the I-MOVE multicentre casecontrol study. Euro Surveill. 2013;18(5):pii=20390.
- 21. Hakin B, Coford P, Harvey F. Children's flu immunisation programme 2013-14. London: Department of Health and Publich Health England; 26 July 2013. Available from https://www.gov.uk/government/uploads/system/uploads/ attachment_data/file/225360/Children_s_flu_letter_2013.pdf

Y155H amino acid substitution in influenza A(H1N1) pdm09 viruses does not confer a phenotype of reduced susceptibility to neuraminidase inhibitors

U Perez-Sautu (uperez@isciii.es)¹, F Pozo¹, I Cuesta², S Monzon³, A Calderon¹, M Gonzalez¹, M Molinero¹, I Lopez-Miragaya⁴, S Rey⁴, A Cañizares⁵, G Rodriguez⁶, C Gonzalez-Velasco⁷, A Lackenby⁸, I Casas¹

- National Influenza Centre of Madrid, National Centre for Microbiology, Carlos III Health Institute, Majadahonda, Madrid, Spain
 Bioinformatics Unit, National Centre for Microbiology, Carlos III Health Institute, Majadahonda, Madrid, Spain
 Bioinformatics Unit. Institute for Research on Rare Diseases. CIBERER U758. Carlos III Health Institute, Majadahonda, Madrid,
- Spain
- 4. Microbiology Service, Meixoeiro Hospital, Vigo, Pontevedra, Spain
- 5. Microbiology Service, A Coruña University Hospital Complex, A Coruña, Galicia, Spain
- 6. Microbiology Service, San Pedro de Alcántara Hospital, Cáceres, Extremadura, Spain
- Don Benito-Villanueva de la Serena Hospital, Badajoz, Extremadura, Spain
- 8. Respiratory Virus Unit, Virus Reference Division, Public Health England, London, United Kingdom

Citation style for this article:

Perez-Sautu U, Pozo F, Cuesta I, Monzon S, Calderon A, Gonzalez M, Molinero M, Lopez-Miragaya I, Rey S, Cañizares A, Rodriguez G, Gonzalez-Velasco C, Lackenby A, Casas I. Y155H amino acid substitution in influenza A(H1N1)pdmo9 viruses does not confer a phenotype of reduced susceptibility to neuraminidase inhibitors. Euro Surveill. 2014;19(27):pii=20849. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20849

Article submitted on 01 September 2013 / published on 10 July 2014

The Y155H amino acid substitution in the neuraminidase gene (NA) has previously been associated with highly reduced inhibition by neuraminidase inhibitors in the seasonal H1N1 influenza A virus which circulated in humans before the 2009 pandemic. During the 2012/13 epidemic season in Spain, two A(H1N1) pdmo9 viruses bearing the specific Y155H substitution in the NA were detected and isolated from two patients diagnosed with severe respiratory syndrome and pneumonia requiring admission to the intensive care unit. Contrary to what was observed in the seasonal A(H1N1) viruses, neither of the Y155H A(H1N1) pdmo9 viruses described here showed a phenotype of reduced inhibition by NAIs as determined by the neuraminidase enzyme inhibition assay (MUNANA). Highthroughput sequencing of the NA of both Y155H viruses showed that they were composed to >99% of H155 variants. We believe that this report can contribute to a better understanding of the biological significance of amino acid substitutions in the neuraminidase protein with regard to susceptibility of influenza viruses to neuraminidase inhibitors. This is of critical importance for optimal management of influenza disease patients.

Introduction

Antiviral drugs are essential in the treatment of influenza disease, especially for severe cases, and can play a very important role in the response to the early phases of a pandemic, when a suitable vaccine may not be available and may take several months to develop [1-3]. Due to rapid emergence and spread of strains highly resistant to adamantanes, these first generation antiviral drugs targeting the influenza virus M2 protein, have been replaced by neuraminidase inhibitors (NAIs), oseltamivir and zanamivir [4]. Before their

introduction in 1999, analysis of over 1,000 clinical specimens confirmed the general lack of naturally occurring resistance to NAIs [5]. Reduced inhibition by NAIs without treatment pressure was rare in influenza viruses in 2002 [6], although reports of resistance to NAIs associated with treatment appeared subsequently [7,8]. In 2007/08, oseltamivir-resistant seasonal influenza A(H1N1) virus variants associated with the H275Y neuraminidase (NA) mutation (N1 numbering) and unrelated to drug use, emerged and spread globally. During 2008/09, these resistant variants became the predominant seasonal A(H1N1) viruses worldwide [9]. Since late 2009, A(H1N1) seasonal viruses have no longer been in circulation, having been replaced by the swine-origin A(H1N1)pdmo9 pandemic virus subtype. The influenza A(H1N1)pdmo9 viruses were susceptible to the NAIs oseltamivir and zanamivir, and carried an NA gene segment which belonged to the same genetic lineage as the NA in the Eurasian swine influenza viruses [10]. Since the beginning of the A(H1N1) pdmo9 pandemic, oseltamivir resistance has remained but with low prevalence. Several reports describe oseltamivir-resistant A(H1N1)pdmo9 viruses bearing the H275Y NA substitution, isolated from patients both treated and untreated with NAIs [11-16], as well as sporadic clusters of patients who acquired infection with these resistant viruses by human-to-human transmission [13,17-19]. These reports raise concerns that the prevalence of viruses exhibiting reduced inhibition by NAIs may increase in the future, and thus, monitoring for the emergence of such viruses is an essential part of national and international surveillance and prevention programmes.

Various amino acid substitutions in the viral NA have been associated with reduced inhibition by NAIs in both seasonal and pandemic A(H1N1) viruses, like the aforementioned H275Y or the E119G/V and I223M/V mutations among others (N1 numbering) [9]. The Y to H substitution in the residue number 155 of the viral NA (N1 numbering) was first described by Monto et al. in a seasonal influenza A(H1N1) virus (A/Hokkaido/15/02 strain) isolated during a study describing the global prevalence of resistance to NAIs during the first three years of their use [6]. Despite the fact that the Y155H substitution is uncommon and has not been included in the main surveillance programmes of the World Health Organization (WHO) [20], it has been listed as a mutation that should be routinely monitored, as recently published by the Antiviral Susceptibility Task Group of the European Influenza Surveillance Network (EISN), Community Network of Reference Laboratories for Human Influenza in Europe (CNRL), coordinated by the European Centre for Disease Control and Prevention (ECDC) [21]. Here we present what is, to the best of our knowledge, the first report of pandemic human influenza A(H1N1) viruses bearing the Y155H amino acid substitution in the NA protein and their susceptibility to NAIs.

Methods

Representative influenza virus isolates and positive respiratory clinical specimens gathered through the Spanish Influenza Surveillance System (SISS) during the 2012/13 influenza season in Spain were sent to our laboratory for complete characterisation. Briefly, the complete haemagglutinin (HA) and NA genes were amplified through RT-PCR and subsequently sequenced following previously described methods [22,23]. We then performed a phylogenetic analysis of the HA sequences to allocate each virus in the different genetic clusters [24]. The sequences of the NA (N1 numbering) were also analysed for the presence of mutations related with reduced inhibition by NAIs (V116A, I117V, E119G/A/V, Q136K, Y155H, D199G/N, I223M/V/K/R, S247N, K262R, H275Y and N295S) [23]. For the sake of clarity, N1 numbering will be used throughout. Selected isolates, including among others those bearing any of these mutations, specimens suspected to have reduced susceptibility to NAIs or from severe cases before start of oseltamivir treatment, were passaged on MDCK cells and their phenotype was analysed using the well described MUNANA assay as recommended in the WHO guidelines [25]. Resistant and sensitive virus controls distributed by the International Society for Influenza and Other Respiratory Viruses (ISIRV) were included in all assays.

The NA gene of the A(H1N1)pdmo9 viruses containing the Y155H mutation was amplified and subsequently sequenced with the Illumina MiSeq high-throughput sequencing platform. A control virus (A/StPetersburg/100/2011-like) previously characterised in our laboratory as bearing a tyrosine at position 155 was also included. A 1,000 bp NA gene fragment containing the position 155 was amplified through RT-PCR [22,23]. DNA libraries were prepared from the amplicons (starting DNA quantity: 1 ng from each) following the Nextera XT (Illumina) standard protocol. The libraries were sequenced in one Illumina MiSeq run of 2x150 (v2) format and represented 7% of the total of samples of the pool which was sequenced. The output was analysed with FastQC and NGStoolkit software for quality assurance. Alignment was performed with BWA alignment software (v.o.6.2) and the variant calling was performed with SAMtools mpileup software (vo.1.18) and a perl script specifically developed for this purpose.

In order to search for other Y155H A(H1N1)pdmo9 NA sequences, partial or entire A(H1N1)pdmo9 NA sets of nucleotide and amino acid sequences were downloaded from the GISAID EpiFlu (http://platform.gisaid. org) database (11,548 nucleotide and 11,871 amino acid sequences available on 2 December 2013). We gratefully acknowledge the originating and submitting laboratories who contributed sequences to that database. Sequences were aligned using Muscle software (v3.8.31). Sequences with a substitution in the residue Y155 were analysed and quantified with an R script specifically developed for this purpose.

Results

Haemagglutinin and neuraminidase genetic analysis

From the beginning of the 2012/13 influenza season until week 20 (2013), we genetically characterised a total of 227 influenza A virus isolates, which were classified according to the phylogenetic analysis of the HA gene sequence (data not shown). Among them, 175 were A(H1N1)pdmo9 viruses (143 A/StPetersburg/27/2011like and 32 A/StPetersburg/100/2011-like). NA gene analysis was performed on 47 A(H1N1)pdmo9 viruses, 45 of which did not have any mutations associated with reduced inhibition by NAIs. However, two viruses bearing the Y155H substitution were detected. These two viruses had been isolated from two severely ill hospitalised patients who had been diagnosed with severe respiratory syndrome and pneumonia and required admission to the intensive care unit (ICU) (Figure 1 and Table 1). Influenza A(H1N1)pdmo9 infection had been diagnosed in both cases at primary healthcare institutions based on nasopharyngeal aspirates obtained before the start of the oseltamivir treatment. These samples were sent to the regional laboratories for influenza surveillance for viral isolation. The resulting isolates (sample ID 13508 and 13752 from Patient 1 and Patient 2 respectively; Table 1) were then sent to our laboratory for further characterisation. Once we detected the Y155H substitution in both isolated viruses (A/Galicia/508/2013 and A/Extremadura/752/2013 from Patient 1 and Patient 2, respectively; Figure 1), RNA directly extracted from the corresponding nasopharyngeal aspirates was required to confirm the substitution in non-cultured viruses. Original RNA extract

Comparison of neuraminidase nucleotide and amino acid sequences from influenza A(H1N1)pdm09 virus isolates, Spain, 2012/13 (n=2)



Patient 1: A/Galicia/508/2013; Patient 2: A/Extremadura/752/2013. Reference strain: A/StPetersburg/100/2011.

The nucleotide change that leads to the Y155H substitution is highlighted with a red star.

from the direct sample specimen was only available from Patient 2 but not Patient 1. The Y155H substitution was confirmed in the NA sequence of the original specimen from Patient 2, presenting clear evidence that this substitution had not been generated during primary cell culture isolation.

MUNANA neuraminidase inhibition assay

In order to obtain a sufficiently high virus titre to perform the NA enzyme inhibition assay [25], virus isolates from Patient 1 and Patient 2 were passaged in MDCK cell cultures. The sequence of the NA gene of the new viral stocks was analysed again before performing the NA enzyme inhibition assays and the presence of Y155H substitutions was confirmed. NA enzyme inhibition assays were subsequently performed. In those, neither of the two Y155H pandemic viruses showed a phenotype of reduced inhibition by NAIs (Table 2). The IC_{50} values that were obtained for the Y155H viruses, were in the same range as the ones obtained for the other 21 sensitive viruses that were tested in our laboratory during the 2012/13 season (mean IC₅₀: 0.6±0.28, range: 0.17-1.60). In order to confirm these results, viral stocks of both cases were sent to the Respiratory Virus Unit at Public Health England (PHE) in London, United Kingdom. Phenotypic data from both laboratories closely correlated, confirming the absence of reduced inhibition by NAIs in both Y155H A(H1N1)pdmo9 viruses (Mean IC_{50} of 0.7 for oseltamivir and 0.4 for zanamivir in the case of A/Extremadura/752/2013, and of 0.3 for both NAIs in the case of A/Galicia/508/2013).

High-throughput sequencing of the neuraminidase gene of the Y155H viruses

Original virus isolates from Patient 1 and Patient 2, along with the RNA from the original clinical specimen from Patient 2 and a wild-type control virus (A/ StPetersburg/100/2011-like) were included in the highthroughput sequencing analysis. Around 300,000 high quality (70% of the bases with more than 20 Phred quality) reads per sample were obtained from the highthroughput sequencing run. Samples were sequenced to between 30,708 times and 64,154 times average depth of coverage. The first nucleotide of the triplet coding for NA residue 155 was read 35,488 times for A/Galicia/508/2013. Of those, 35,440 (99.86%) contained the codon CAT, coding for a histidine at amino acid position 155, and only 39 (0.11%) contained the wild-type codon TAT, coding for a tyrosine (Sample 508; Figure 2). In the case of A/Extremadura/752/2013, 20,136 reads were obtained for the first nucleotide of the codon of interest, of which 20,124 (99.94%) contained the codon CAT coding for a histidine and 10 (0.049%) contained the wild-type codon TAT (Sample 752; Figure 2). Similar results were observed in the RNA directly extracted from the clinical sample specimen from Patient 2 (Sample 948; Figure 2). In contrast, the wild-type A/StPetersburg/100/2011-like control virus (Sample Co77; Figure 2) contained the codon TAT coding for a tyrosine in 35,281 of 35,338 total reads (99.84%). In this sample, 53 (0.15%) reads with the Y155H substitution were also detected. These results correlated with those obtained in the Sanger sequencing (Figure 1) and confirmed that the Y155H viruses can be considered as pure H155 populations.

TABLE 1

Main clinical characteristics of the studied influenza A(H1N1)pdm09 patients, Spain, 2012/13 (n=2)

Sample ID	Sampling Date	Sex	Age (years)	NAIs Administration	ICU	Risk Factors	Complications	Outcome
13508	24/02/2013	Female	46	Oseltamivir	Yes	Chronic disease	None	Fully recovered
13752	20/03/2013	Male	49	Oseltamivir	Yes	OSAS	Multi-organ failure	Fully recovered

ICU: intensive care unit; ID: identification number; NAI: neuraminidase inhibitor; OSAS: obstructive sleep apnoea syndrome.

TABLE 2

Neuraminidase inhibition of influenza A(H1N1)pdm09 virus isolates, Spain, 2012/13 (n=2)

	Mean I	NA substitution	
Viral strain	Oseltamivir	Zanamivir	
A/Extremadura/752/2013 P2 Siat1	0.6	0.3	Y155H
A/Perth/265/09 P1 Siat1	1.1	0.9	WT
A/Perth/261/09 P4 Siat1	286.4	1.0	H275Y
A/Galicia/508/2013 P3 Siat1	0.5	0.4	Y155H
A/Perth/265/09 P4 Siat1	0.8	0.4	WT
A/Perth/261/09 P1 Siat1	269.4	0.4	H275Y

IC₅₀: half maximal inhibitory concentration; ISIRV: International Society for Influenza and Other Respiratory Viruses; NA: neuraminidase; P: passage number; Siat1: Siat1 cell line; WT: wildtype.

Data were obtained from two independent MUNANA assays perfomed at the National Influenza Centre of Madrid, against the ISIRV reference virus panel. Each virus was assayed in duplicate. Data are expressed as mean IC₅₀ values in nM. WT denotes isolates not containing any NA substitution related to reduced inhibition by NAIs.

Influenza A(H1N1)pdm09 viruses with Y155H mutation in the GISAID EpiFlu database

Among 11,871 NA amino acid sequences available at the GISAID EpiFlu database by 2 December 2013, we found 56 sequences with the Y to H substitution at position 155 of the NA. These sequences came from sample specimens collected from year 2009 to 2013 and from different countries in Africa, Asia, Australia, Europe and North America. Information on their phenotype of susceptibility to NAIs was only available for a five of these samples, which had all been reported as not showing reduced inhibition by NAIs in the GISAID EpiFlu database. We also found 10 sequences with a Y to F substitution at position 155, and one with a Y to C substitution.

Discussion

According to data obtained from the EISN network during the 2012/13 influenza season in Europe (up to week 20 in 2013), 11 of a total of 614 A(H1N1)pdm09 viruses tested for antiviral susceptibility carried the NA H275Y amino acid substitution [26]. These 11 viruses were detected mainly in oseltamivir-treated hospitalised patients, some of whom were immunocompromised [26]. Excluding the two isolates bearing the Y155H substitution described here, none of the remaining 601 A(H1N1)pdm09 viruses tested for neuraminidase inhibitor susceptibility showed genetic or phenotypic evidence of highly reduced or reduced inhibition [26]. To the best of our knowledge, this is the first report of the presence of the Y155H amino acid substitution in circulating pandemic A(H1N1) viruses. However, we found 56 NA amino acid sequences with the Y155H substitution among the 11,871 A(H1N1)pdmo9 NA records available at the GISAID EpiFlu database by the date of our analysis. These sequences had been reported to the database before and during the 2012/13 season from different countries in Africa, Asia, Australia, Europe and North America. This suggests that A(H1N1) pdmo9 viruses containing Y155H had already been circulating worldwide for several seasons, albeit at a very low prevalence.

It should be highlighted that the two influenza A(H1N1) pdmo9 viruses with the Y155H mutation were detected in samples obtained before the start of treatment with oseltamivir, and they are thus naturally occurring variants not associated with a selective pressure derived from the usage of NAIs. Monto et al. described for the first time the Y155H substitution in a Japanese seasonal influenza A(H1N1) virus strain (A/Hokkaido/15/o2) isolated three years after the licensing of oseltamivir and zanamivir [6]. This strain exhibited a 123- and a 555-fold increase in the mean IC₅₀ values for zanamivir and oseltamivir, respectively [6]. However, the two Y155H viruses described here behaved like the sensitive

control strains and did not show oseltamivir and zanamivir IC_{50} values in the highly reduced or reduced inhibition range (Table 2). These results are in accordance with the fact that the two patients fully recovered from disease under oseltamivir treatment.

The results of the high-throughput sequencing analysis of the NA of the Y155H A(H1N1)pdm o9 viruses revealed that they can be considered as pure H155 populations, as only a very low level of wild-type Y155 background was observed (<0.2% of the total reads). These results allow us to discard the possibility that the sensitive phenotype observed in the MUNANA assays for the

Y155H viruses could have been conferred by a wild type Y155 background with a higher NA activity, as it has been recently reported for the R292K substitution in H7N9 viruses [27,28].

Therefore, it can be concluded that, in contrast to what was observed for the Japanese seasonal influenza A(H1N1) A/Hokkaido/15/02 strain, a change from Y to H in the residue 155 of the NA does not seem to confer any reduced inhibition by NAIs in A(H1N1)pdmo9 viruses. This is somewhat unexpected, considering the significant impact that this substitution has on the A/Hokkaido/15/02 seasonal A(H1N1) virus, as shown by

FIGURE 2

Spectrum of single-nucleotide substitutions at the first nucleotide of the triplet coding for neuraminidase residue 155 of the influenza A(H1N1)pdm09 viruses with Y155H mutation, Spain, 2012/13 (n=2)



CAT: codon for histidine; TAT: codon for tyrosine.

508: A/Galicia/508/2013 Y155H virus; 752: A/Extremadura/752/2013 Y155H virus; 948: RNA extracted from the clinical specimen of Patient 2; C077: wild-type influenza A(H1N1)pdm09 virus (A/StPetersburg/100/2011-like) with a tyrosine at position 155 as obtained from previous Sanger sequencing.

Data are expressed in number of times a nucleotide has been sequenced by independent reads.

McKimm-Breschkin et al. in a recent publication [29]. McKimm-Breschkin et al. show that the Y155H mutation dramatically reduces susceptibility to all NAIs, and that it also reduces plaque size and affects the activity, stability, substrate affinity and pH profile of the NA [29]. However, the different behaviour of our A(H1N1) pdmo9 Y155H mutants in the inhibition assays could be explained by distinct structural features of their NA. Qing Li et al. show that the A(H1N1)pdmo9 NA does not have the 150-cavity characteristic of other group 1 neuraminidases, including seasonal A(H1N1) viruses [30]. Their results, together with other similar work related not only to NA but also to HA structures, show that the structures of both proteins in the 2009 pandemic virus are distinct from the corresponding structures in the seasonal virus [31,32].

In the case of severe influenza disease, a decision to use the current neuraminidase inhibitors must be made urgently. In these cases it is essential to rule out the presence of a substitution in the NA of the infecting virus which could compromise the effectiveness of the treatment. Our data imply that in a patient with severe influenza disease caused by an A(H1N1)pdmo9 virus carrying the Y155H substitution in the neuraminidase, oseltamivir and zanamivir can be chosen as the therapeutic tool. In conclusion, we believe that this report can contribute to a better understanding of the biological significance of amino acid substitutions in the influenza virus NA and HA in relation to susceptibility to NAIs.

GISAID EpiFlu database accession numbers

Sequences from the two A(H1N1)pdmo9 viruses described in this manuscript have been submitted to the EpiFlu Database publicly accessible in the GISAID Platform (http://platform. gisaid.org).

A/Extremadura/752/2013 (HA: EPI442926, NA: EPI466206) A/Galicia/508/2013 (HA: EPI439740, NA: EPI466205)

Acknowledgements

We would like to acknowledge Dr. Angel Zaballos Sanz at the Genomics Unit of the National Centre for Microbiology of the Carlos III Health Institute and the Next Generation Sequencing service at the Cantoblanco Genomics Unit of the Madrid Scientific Park for their support, and all the members of the Spanish Influenza Surveillance System working in the identification and declaration of patients during the 2012/13 influenza season. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID EpiFlu Database which have been included in this research. This work was supported by Instituto de Salud Carlos III (Programa especial de investigación sobre la gripe pandémica) GR09/0040. Sara Monzon received a research contract funding by the Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER U758). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflict of interest

None declared.

Authors' contributions

Unai Perez-Sautu, Francisco Pozo and Inmaculada Casas designed the study and performed the genotypic characterisations, the phenotypic neuraminidase enzyme inhibition assays (MUNANA) and the sample preparation for the high-throughput sequencing assay, as well as the interpretation of all data. Unai Perez-Sautu wrote the article, which was revised by Francisco Pozo, Inmaculada Casas and Angie Lackenby. Isabel Cuesta, Sara Monzon and Unai Perez-Sautu performed the high-throughput sequencing and bioinformatics data analysis. Ana Calderon, Monica Gonzalez and Mar Molinero provided technical assistance with the RT-PCR methods, sequencing and MUNANA assays. Angie Lackenby confirmed the MUNANA results at Public Health England, London, UK. Isabel Lopez-Miragaya, Sonia Rey, Angelina Cañizares, Guadalupe Rodriguez and Carmen Gonzalez-Velasco provided all the clinical samples and gathered and provided all the relevant clinical data.

References

- 1. Govorkova EA, Leneva IA, Goloubeva OG, Bush K, Webster RG. Comparison of Efficacies of RWJ-270201, Zanamivir, and Oseltamivir against H5N1, H9N2, and Other Avian Influenza Viruses. Antimicrob Agents Chemother. 2001;45(10):2723-32. http://dx.doi.org/10.1128/AAC.45.10.2723-2732.2001
- Monto AS. The role of antivirals in the control of influenza. Vaccine. 2003;21(16):1796-800. http://dx.doi.org/10.1016/ S0264-410X(03)00075-6
- 3. World Health Organization (WHO). WHO guidelines on the use of vaccines and antivirals during influenza pandemics. Geneva: WHO; 2004. Available from: http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_RMD_2004_8/en/index.html
- Bright RA, Medina Mj, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, et al. Incidence of adamantane resistance among influenza A (H₃N₂) viruses isolated worldwide from 1994 to 2005: a cause for concern. The Lancet. 2001;366(9492):1175-81. http:// dx.doi.org/10.1016/S0140-6736(05)67338-2
- McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, et al. Neuraminidase Sequence Analysis and Susceptibilities of Influenza Virus Clinical Isolates to Zanamivir and Oseltamivir. Antimicrob Agents Chemother.2003;47(7):2264-72. http://dx.doi.org/10.1128/ AAC.47.7.2264-2272.2003
- Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, et al. Detection of Influenza Viruses Resistant to Neuraminidase Inhibitors in Global Surveillance during the First 3 Years of Their Use. Antimicrob Agents Chemother. 2006;50(7):2395-402. http://dx.doi.org/10.1128/AAC.01339-05
- Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. The Lancet. 2004;364(9436):759-65. http://dx.doi.org/10.1016/ S0140-6736(04)16934-1
- Stephenson I, Democratis J, Lackenby A, McNally T, Smith J, Pareek M, et al. Neuraminidase Inhibitor Resistance after Oseltamivir Treatment of Acute Influenza A and B in Children. Clin Infect Dis. 2009;48(4):389-96. http://dx.doi. org/10.1086/596311
- Nguyen HT, Fry AM, Gubareva LV. Neuraminidase inhibitor resistance in influenza viruses and laboratory testing methods. Antivir Ther. 2012;17(1 Pt B):159-73. http://dx.doi.org/10.3851/ IMP2067
- Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans. Science. 2009;325(5937):197-201. http://dx.doi.org/10.1126/ science.1176225
- 11. Chen LF, Dailey NJM, Rao AK, Fleischauer AT, Greenwald I, Deyde VM, et al. Cluster of Oseltamivir-Resistant 2009 Pandemic Influenza A (H1N1) Virus Infections on a Hospital Ward among Immunocompromised Patients - North Carolina, 2009. J Infect Dis. 2011;203(6):838-46. http://dx.doi. org/10.1093/infdis/jiq124
- 12. European Centre for Disease Prevention and Control (ECDC). Rapid Risk Assessment: oseltamivir-resistant influenza A(H1N1)2009 cluster in Australia. Stockholm: ECDC; September 2011. Available from: http://www.ecdc.europa. eu/en/publications/Publications/110906_TER_Rapid_Risk_ Assessment_Oseltamivir-resistant%20influenza%20A.pdf

- Graitcer SB, Gubareva L, Kamimoto L, Doshi S, Vandermeer M, Louie J, et al. Characteristics of Patients with Oseltamivir-Resistant Pandemic (H1N1) 2009, United States. Emerg Infect Dis. 2011;17(2):255-7. http://dx.doi.org/10.3201/eid1702.101724
- 14. Lackenby A, Moran Gilad J, Pebody R, Miah S, Calatayud L, Bolotin S, et al. Continued emergence and changing epidemiology of oseltamivir-resistant influenza A(H1N1)2009 virus, United Kingdom, winter 2010/11. Euro Surveill. 2011;16(5):pii=19784.
- 15. Moore C, Galiano M, Lackenby A, Abdelrahman T, Barnes R, Evans MR, et al. Evidence of Person-to-Person Transmission of Oseltamivir-Resistant Pandemic Influenza A(H1N1) 2009 Virus in a Hematology Unit. J Infect Dis. 2011;203(1):18-24. http:// dx.doi.org/10.1093/infdis/jiq007
- 16. Wolfe C, Greenwald I, Chen L. Pandemic (H1N1) 2009 and Oseltamivir Resistance in Hematology/Oncology Patients. Emerg Infect Dis. 2010;16(11):1809-11. http://dx.doi. org/10.3201/eid1611.101053
- 17. Centers for Disease Control and Prevention (CDC). Oseltamivirresistant 2009 pandemic influenza A (H1N1) virus infection in two summer campers receiving prophylaxis - North Carolina, 2009. MMWR Morb Mortal Wkly Rep. 2009;58(35):969-72.
- Mai LQ, Wertheim HFL, Duong TN, van Doorn HR, Hien NT, Horby P. A Community Cluster of Oseltamivir-Resistant Cases of 2009 H1N1 Influenza. N Engl J Med. 2010;362(1):86-7. http:// dx.doi.org/10.1056/NEJMc0910448
- Hurt AC, Hardie K, Wilson NJ, Deng YM, Osbourn M, Gehrig N, et al. Community transmission of oseltamivir-resistant A(H1N1) pdmo9 influenza. N Engl J Med. 2011;365(26):2541-2. http:// dx.doi.org/10.1056/NEJMc1111078
- 20. World Health Organization (WHO). Laboratory methodologies for testing the antiviral susceptibility of influenza viruses: Neuraminidase inhibitor (NAI). Geneva: WHO; 2012. Available from: http://www.who.int/influenza/gisrs_laboratory/ antiviral_susceptibility/nai_overview/en/index.html
- 21. Pozo F, Lina B, Andrade HRd, Enouf V, Kossyvakis A, Broberg E, et al. Guidance for clinical and public health laboratories testing for influenza virus antiviral drug susceptibility in Europe. J Clin Virol. 2013;57(1):5-12. http://dx.doi. org/10.1016/j.jcv.2013.01.009
- 22. Coiras MT, Perez-Bre-a P, Garcia ML, Casas I. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. J Med Virol. 2003;69(1):132-44. http://dx.doi.org/10.1002/jmv.10255
- 23. Ruiz-Carrascoso G, Casas I, Pozo F, Perez-Gonzalez C, Reina J, Perez-Bre-a P. Development and implementation of influenza a virus subtyping and detection of genotypic resistance to neuraminidase inhibitors. J Med Virol. 2010;82(5):843-53. http://dx.doi.org/10.1002/jmv.21692
- 24. Ledesma J, Pozo F, Reina G, Blasco M, Rodriguez G, Montes M, et al. Genetic diversity of influenza A(H1N1)2009 virus circulating during the season 2010-2011 in Spain. J Clin Virol. 2012;53(1):16-21. http://dx.doi.org/10.1016/j.jcv.2011.09.032
- 25. World Health Organization (WHO) Global Influenza Surveillance Network. Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva: WHO; 2011. ISBN: 9789241548090. Available from: http://www.who.int/influenza/gisrs_laboratory/ manual_diagnosis_surveillance_influenza/en/
- 26. European Centre for Disease Prevention and Control (ECDC). Fortnightly influenza surveillance overview. Main surveillance developments in weeks 19–20/2013 (06–19 May 2013). Stockholm: ECDC; 24 May 2013. Available from: http://www. ecdc.europa.eu/en/publications/publications/influenzafortnightly-surveillance-overview-24-may-2013.pdf
- 27. Sleeman k, Guo Z, Barnes J, Shaw M, Stevens J, Gubareva LV. R292K Substitution and Drug Susceptibility of Influenza A(H7N9) Viruses. Emerg Infect Dis. 2013;19(9):1521-24. http:// dx.doi.org/10.3201/eid1909.130724
- 28. Yen HL, McKimm-Breschkin JL, Choy KT, Wong DDY, Cheung PPH, Zhou J, et al. Resistance to Neuraminidase Inhibitors Conferred by an R292K Mutation in a Human Influenza Virus H7N9 Isolate Can Be Masked by a Mixed R/K Viral Population. MBio. 2013;4(4):e00396-13. http://dx.doi.org/10.1128/ mBio.00396-13
- 29. McKimm-Breschkin JL, Williams J, Barrett S, Jachno K, McDonald M, Mohr PG, et al. Reduced susceptibility to all neuraminidase inhibitors of influenza H1N1 viruses with haemagglutinin mutations and mutations in non-conserved residues of the neuraminidase. J Antimicrob Chemother. 2013;68(10):2210-21.
- 30. Li Q, Qi J, Zhang W, Vavricka CJ, Shi Y, Wei J, et al. The 2009 pandemic H1N1 neuraminidase N1 lacks the 150-cavity in its active site. Nat Struct Mol Biol. 2010;17(10):1266-8. http:// dx.doi.org/10.1038/nsmb.1909

- 31. Xu R, Ekiert DC, Krause JC, Hai R, Crowe JE, Wilson IA. Structural Basis of Preexisting Immunity to the 2009 H1N1 Pandemic Influenza Virus. Science. 2010;328(5976):357-60. http://dx.doi.org/10.1126/science.1186430
- 32. Zhang W, Qi J, Shi Y, Li Q, Gao F, Sun Y, et al. Crystal structure of the swine-origin A (H1N1)-2009 influenza A virus hemagglutinin (HA) reveals similar antigenicity to that of the 1918 pandemic virus. Protein Cell. 2010;1(5):459-67. http://dx.doi.org/10.1007/S13238-010-0059-1

Till receipts - a new approach for investigating outbreaks? Evaluation during a large Salmonella Enteritidis phage type 14b outbreak in a north west London takeaway restaurant, September 2009

D Zenner (dominik.zenner@phe.gov.uk)^{1,2}, **J Zoellner**³, **A Charlett**¹, **W Marmairis**⁴, **C Lane**¹, **J Y Chow**⁴ 1. Centre for Infectious Disease Surveillance and Control (CIDSC), Public Health England, United Kingdom

- 2. University College London, United Kingdom
- 3. Imperial College London, United Kingdom
- 4. North West London Health Protection Team, Public Health England, United Kingdom

Citation style for this article:

Zenner D, Zoellner J, Charlett A, Marmairis W, Lane C, Chow JY. Till receipts - a new approach for investigating outbreaks? Evaluation during a large Salmonella Enteritidis phage type 14b outbreak in a north west London takeaway restaurant, September 2009. Euro Surveill. 2014;19(27):pii=20848. Available online: http:// www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20848

Article submitted on 17 April 2013 / published on 10 July 2014

Selecting suitable controls for outbreak investigations is often difficult and if done inappropriately will lead to biased inferences. Till receipts and other sales records are frequently available on food premises, but their applicability has not been fully explored. Using data from an investigation into a Salmonella outbreak affecting 66 individuals exposed in a London takeaway restaurant, this study aimed to evaluate the use of till receipts to assess associations between sales and illness. Cases identified through local case-finding were subjected to a standardised exposure questionnaire. Till receipts over the time period when cases arose were analysed. Estimated food exposures from sales were compared to case reported exposures and till receipts analysis showed strong association between illness and consumption of rotisserie chicken (odds ratio (OR): 2.75; confidence interval (CI): 1.7-4.5). Chicken sales immediately prior to food consumption for cases were compared to two control periods in an ecological case-crossover design. On average there was an estimated increase of 3.7 (CI: 2.2-5.2) extra chickens sold in the hour immediately prior to the consumption in the cases (p<0.0001) and the risk of becoming ill at busy times increased by 5% with each additional chicken quarter sold per hour (OR: 1.05; CI: 1.03-1.08). Microbiological and environmental investigations revealed Salmonella Enteritidis phage type (PT)14b in all available cases' stool samples, two environmental samples and leftover chicken from the takeaway. The feasibility of this novel approach to obtain exposure information in the population at risk has been demonstrated, and its limitations are discussed. Further validation is required, comparing results with those in a concurrent classic case-control study.

Introduction

This study is set in the context of a large outbreak in London of Salmonella Enteritidis phage type (PT)14b with antimicrobial resistance to nalidixic acid and reduced susceptibility to ciprofloxacin (SE PT14b NxCpL). On 16 September 2009, three large London hospitals informed the North West London Health Protection Unit (NWLHPU) of a cluster of patients presenting with diarrhoea and vomiting. All cases had consumed products from a takeaway restaurant, up to 55 hours before becoming ill. The first person with enteric illness presented to the hospital on 9 September. Analysis of stool samples from the initial patients identified S. Enteritidis PT14b. Immediate public health measures were taken on the day of reporting and the takeaway shop voluntarily closed on 16 September.

Food poisoning outbreaks related to non-typhoidal Salmonella can result in high attack rates [1] and serious complications with associated excess morbidity and mortality amongst those affected [1-3]. The investigation of these outbreaks requires microbiological and epidemiological evidence to ensure appropriate control measures and inform public health action [4].

When a cohort cannot be precisely identified, casecontrol studies are the method of choice. The feasibility of this study design is determined by available resources and the ability to recruit suitable controls from the population at risk. Greater social mobility and the use of mobile phones make traditional methods of control selection through random digit phone dialling more difficult and less valid, introducing sizable selection bias [5]. Case-nominated controls risk 'overmatching' on the causal exposure, and use of population registers is often not possible, as they do not always identify the population at risk in the outbreak.

Case definitions, outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, September 2009

A probable case was defined as a person developing diarrhoea or any two or more of the following symptoms: vomiting, fever (≥38°C) or abdominal pain within 72 hours of consumption of products from the implicated takeaway restaurant purchased between 6 and 16 September 2009.

A confirmed case was defined as a symptomatic person (see above) with a laboratory-confirmed isolate of *S*. Enteritidis phage type (PT)14b with resistance to nalidixic acid and with reduced susceptibility to ciprofloxacin from a blood or stool specimen.

Cases that travelled abroad in the seven days prior to symptom onset and cases with household contact to persons with diarrhoea or vomiting not associated with this outbreak were excluded.

Alternative study designs have been used, such as, case-crossover studies [6], case severity studies [1] and serotype case-case studies [5,7-9]. In addition, analyses-of-sales studies have been reported [10,11]. These were hypotheses-generating consumer record analyses [10], and a comparison of the probability of kebab consumption based on information from the kebab shop owner with exposure information from the cases [11]. However, many of these approaches are contextual and cannot be easily adapted for other outbreak situations.

Based on the initial report of this large cluster of cases with gastrointestinal illness with exposure to food from a north west London takeaway restaurant we sought to establish epidemiological and microbiological evidence for the source and vehicle of the outbreak. In addition, we explored the feasibility and validity of a new analytical approach, using till receipts from the restaurant to both estimate exposures in the population at risk, as well as sales volumes as an exposure in an ecological case-crossover approach.

Methods

Epidemiological investigation

The outbreak was described in time, person and place to generate hypotheses about the possible exposures and exposure mechanisms. Cases were defined as below (box). Following the initial notification, a casefinding exercise was conducted. This involved notification requests from local frontline clinicians (e.g. general practitioners, hospital doctors and microbiologists) for cases with an epidemiological link to the takeaway restaurant. Some reported cases also informed on known fellow diners that became ill. Additionally, national reference laboratory reports for all *S*. Enteritidis PT14b cases during an estimated period when cases may have been reported (6–30 September) were scrutinised for their plasmid profile and potential exposures. All *Salmonella* reports to the local unit during this period as well as some before and after this period were reviewed for an epidemiological link to the implicated source.

All reported cases who fulfilled the definition of a probable or confirmed case (n=66) were interviewed using a standardised questionnaire on food consumption and other risk exposures, which was tailored to this outbreak (based, for example, on the restaurant menu). The questionnaire was administered via telephone, or in person. The prototype had been developed, piloted and tested for validity in other outbreaks prior to this incident. All cases were interviewed within a week of exposure. The data were entered into a secure database, cleaned and cross-checked for inconsistencies. These case exposure data were used for descriptive analysis as well as the two analytical approaches described below.

Microbiology

Stool samples were available for all cases fulfilling the confirmed case definition (n=31) and from three of the four food handlers who worked in the takeaway restaurant. The food handlers were asymptomatic but were sampled to ensure that they were not the current or a continued source of the outbreak. Samples were initially cultured for *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* or *Shigella* spp. Positive stool samples for *Salmonella* ssp. were sent to the National Salmonella Reference Laboratory, Health Protection Agency (now Public Health England) Centre for Infections, for phagetyping [12] and plasmid profiling [13] and tested for resistance to a range of antimicrobials by a standard breakpoint method.

During the investigation by the environmental health team on 16 September, a range of food and environmental samples were obtained and submitted for testing. A total of 30 food samples were taken from the implicated takeaway restaurant, and all raw shell eggs (n=72) from the premises were tested for *Salmonella* spp. Twelve environmental samples at the premises as well as leftover food samples from the household of an affected individual were cultured and characterised as above.

Data analysis

All the takeaway restaurant sales during the period at risk of exposure were entered into a cash register machine – regardless of whether payment was by cash or card. This machine automatically registers time and date; the price and product specification were entered manually by staff at the restaurant. Complete till receipt print-outs for the entire period where customers could have been at risk of exposure from contaminated food at the shop (two days prior to the first case until shop closure, 7–16 September 2009, inclusive) were manually entered in an Excel spreadsheet, crosschecked and cleaned, and used for further analysis.

Till receipts are used for tax purposes and therefore precisely record the sales. Recording was accurate for date, time and price, but in this outlet, 25% of sales were recorded as miscellaneous. Information from the till receipts was used to reconstruct the population at risk, and the exposures in this population at risk for the comparative analysis and to generate sale volumes over time for the ecological case-crossover design.

Method 1 – a comparative approach

A theoretical cohort defining the population at risk. buying food from the restaurant between 7 and 16 September was constructed using sales information from the till receipts and estimated daily customer numbers from the restaurant owner. This was to establish a baseline sales' pattern by estimating the number of persons through the quantity and combination of individual food items bought. Individual food portions consumed by the cohort were calculated from sales, making assumptions about portion sizes and dish combinations. In principle the sale of one main dish (e.g. chicken kebab) or half a rotisserie chicken was equivalent to one portion. Side dishes (e.g. chips), sauces and sundries were counted as individual portions, if they were sold in the absence of a main dish. A person was assumed to consume one main dish (irrespective of an accompanying sundry) or one or more side dishes (in the absence of a main dish). It was assumed that joint food exposures in the cases were generalisable to the whole cohort. Miscellaneous food items were assumed to occur randomly by staff, person, and food product and have been excluded for the comparative analysis. The derived number of customers was compared with the information from the restaurant owner.

This data set with estimated numbers of individuals in the cohort consuming each food item was merged with the collected exposure information from the questionnaires administered to the cases. The theoretical exposures in the cohort were compared to the exposures in the cases providing estimated odds ratios and chi-squared tests of association. Each exposure was assessed only using single variable analysis. No multivariable analysis was attempted as the underlying assumptions preclude precise knowledge of joint exposure.

Method 2 – an ecological case-crossover analysis

The case-crossover design compares hypothesised risk exposures in the same individual in the interval before onset of illness to one or more intervals when the event does not occur [14]. It is particularly suitable for continuous common source outbreaks [15], where unbiased exposure information (e.g. a menu or purchase records) is available [6,15].

FIGURE 1

Epidemic curve obtained from investigating an outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, September 2009 (n=66 cases^a)



^a Cases include probable or laboratory-confirmed cases.

TABLE 1

Results of the comparative till receipt analysis, outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, September 2009

Menu items	Number of cases exposed ^a (percent of 66 total cases) n (%)	Number of portions consumed ^b (percent ^c of the total theoretical cohort of 2,390 exposed)	Odds ratio (95% Confidence interval)	P-value
Rotisserie chicken	41 (62.1)	894 (37.4)	2.75 (1.67–4.52)	<0.0001
Chips	32 (48.5)	508 (21.3)	3.52 (2.16–5.74)	<0.0001
Nan	20 (30.3)	269 (11.3)	3.43 (2.01–5.85)	<0.0001
Doner kebab	16 (24.2)	203 (8.5)	3.45 (1.94–6.13)	<0.0001
Pita	10 (15.2)	299 (12.5)	1.25 (0.64–2.45)	0.522
Chicken doner	8 (12.1)	157 (6.6)	1.96 (0.94–4.12)	0.075
Chicken shish	4 (6.1)	4 (0.2)	51.35 (12.57–209.57)	<0.0001
Mixed kebab	3 (4.6)	9 (0.4)	12.6 (3.61–44.26)	<0.0001
Cheese	3 (4.6)	90 (3.8)	1.22 (0.4–3.73)	0.743
Rice	3 (4.6)	20 (0.8)	5.65 (1.75–18.32)	0.002
Chicken tikka	1 (1.5)	20 (0.8)	1.82 (0-10.88)	0.555
Seekh kebab	1 (1.5)	99 (4.1)	0.36 (0-2.06)	0.287
Shahi special	o (o.o)	6 (0.3)	0 (0-23.46)	0.684
Paratha	0 (0.0)	14 (0.6)	0 (0-9.98)	0.533

The table is ordered by number of cases explainable by respective exposures (i.e. the numbers of cases who reported the particular exposure, column 1).

^a Based on observed data from the case questionnaires.

^b The sum of portions in this column exceeds the total number in the theoretical cohort (n=2,390) based on the till receipt data, as persons may choose more than one dish. The numbers of portions are calculated for the outbreak period from 7 to 16 September 2009.

^c These are estimated exposures in the theoretical cohort using till receipt data and assuming that joint food exposures in the cases were generalisable to the whole cohort.

This analysis was performed to assess the a priori hypotheses that rotisserie chicken was the causal food source. This hypothesis was developed from customer complaint information, site visits, descriptive analysis and microbiological findings in a left-over piece of rotisserie chicken.

The ecological case-crossover design utilises the complete time series during the study period of sale volumes of rotisserie chicken, whereby the volume of sales in a time interval of one hour before the time of consumption as declared by the case (exposure interval), is compared to the sale volumes in the same hour in the preceding and following day (control intervals). The difference in sales volumes between the exposure interval and the mean of the control intervals was assessed using a paired t-test.

In addition, a conditional logistic regression analysis was used to estimate the risk of illness per additional quarter chicken sold per hour.

All data were entered in EpiData 3.1 and statistical analysis was carried out using STATA SE 11.

Results

Descriptive epidemiology

We identified 72 epidemiologically-linked cases with enteric illness in total, however three of these did not fulfill the clinical case definition and for a further three, not enough information was available to determine whether they fulfilled the clinical case definition. The remaining 66 cases of enteric illness therefore fulfilled the definition for probable or confirmed cases. Thirty-five of the cases (53%) became ill on a single day (Figure 1). Most cases were male (41, 62%), and under 40 years of age (56, 85%). The mean age was 28 years (median: 26; range: 4–72). The majority of cases (42, 64%) resided near the takeaway restaurant.

The median incubation period was 16 hours (mean: 17; range: 2–55). In addition to diarrhoea, symptoms included abdominal pain (65, 98%), fever (61, 92%), headache (50/64, 78%), vomiting (46, 70%), bloody diarrhoea (10/63, 16%) and myalgia (13/64, 20%). Twenty-two cases (33%) were admitted to hospital with a median stay of four days (range: 1–7 days). None of these required admission to an intensive care unit.

Microbiology

Stool samples were available for 31 of the 66 cases; in all of them *S*. Enteritidis PT14b was isolated. Plasmid profiling was performed for seven of these isolates, and the profile was indistinguishable amongst them, but distinguishable by an additional 4 kb plasmid from profiles identified in other concurrent *S*. Enteritidis PT14b outbreaks in England and Wales.

All stool samples obtained from food handling staff at the venue (n=3) were negative for *Salmonella*. A food sample taken from a cooked leftover rotisserie chicken kept in the refrigerator of one of the cases and purchased at the implicated takeaway restaurant was

TABLE 2

Results of the case-crossover analysis, outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, September 2009

	Quarters of chicken sold	95% Confidence interval	P-value
Non-exposed times	19.75	17.67–21.83	-
Exposed times	34.59	27.07-42.11	-
Difference	14.84	8.71-20.97	<0.0001 ^a
	Odds Ratio	95% Confidence interval	P-value
Risk of exposure ^b	1.05	1.03-1.08	<0.0001

^a Differences between chicken sales in exposed and non-exposed times were compared with t tests.

^b The lower part shows the result of the conditional logistic regression model estimating the risk of illness per chicken quarter sold per hour.

positive for *S*. Enteritidis PT14b. Environmental swabs taken from the floor of the refrigerator in the food preparation area and from a light switch in the staff toilet were positive for *S*. Enteritidis PT14b. The plasmid profile of the food sample and the environmental samples was indistinguishable from the organism isolated from the stools and the antimicrobial resistance profile was identical in all tested samples. All other samples including 12 environmental, 30 food samples and 72 egg shells from the implicated takeaway were negative for *Salmonella*.

The inspection by the environmental health team raised concerns about food preparation processes, such as the proximity of raw and cooked foods. In addition, an insufficient recording of temperature control, particularly for refrigeration and the rotisserie operation was noted.

Epidemiological analysis

Comparative analysis

Individual portions were estimated from the number of sales. An estimated 2,390 portions were sold during the ten days of the study period (7–16 September), a mean of 239 portions per day (range: 129–287). This broadly agreed with information from the owner, who estimated around 200 customers per day. 25% of all sales were coded miscellaneous.

Table 1 provides an overview of the comparison between exposure information from the cases and estimated exposure in the theoretical cohort from the sales data, ordered by the number of explainable cases through the respective exposure. There was a significant association between consumption of rotisserie chicken and becoming a case in this analysis (odds ratio (OR): 2.75; confidence interval (CI): 1.67–4.52). Although similar or larger effect sizes were observed with other food items, consumption of rotisserie chicken can explain the majority of cases. It is possible that cross-contamination might explain some of these observations of large effect sizes, but which were affecting only few people.

Ecological case-crossover analysis

The times and volumes of rotisserie chicken sales corresponded well with the exposure times of cases (Figure 2), and based on this we formally analysed the correlation between rotisserie chicken sales and illness periods. On average, 34.59 quarters of chicken corresponding to 8.6 chickens (Cl: 6.8-10.5) were sold in the hour before the cases had consumed food from the takeaway. This compares with an average of 19.75 quarters, or 4.9 chickens (Cl: 4.4-5.5) sales, in the two reference periods. The difference of 14.89 quarters, i.e. 3.7 chickens (Cl: 2.2-5.2) is significantly different from zero (p <0.0001) (Table 2). The clustering of cases around the highest sales peaks, particularly on 10 September is shown in Figure 2.

The conditional logistic regression analysis found that the odds of becoming ill increases by 5% with each additional chicken quarter sold per hour (OR: 1.05; CI: 1.03–1.08). A locally weighted scatter plot smoothing (lowess) graph shows that the risk of becoming a case increases linearly, once a threshold of sales of 20 to 24 chicken quarters – i.e. about five to six chicken – per hour (stable 25% background risk) has been exceeded (Figure 3).

Figure 3. Lowess smoother graph denoting the risk of becoming a case per number of chicken quarters sold in the hour before exposure, outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, September 2009

Discussion

We present the results of the investigation into a large outbreak associated with a north west London takeaway restaurant, using till receipts as a feasible, new analytical approach to validate the hypothesised causal exposure in this outbreak. While acknowledging the limitations of sales data, our analyses of till receipts provided epidemiological evidence of an association between the consumption of rotisserie chicken and developing a gastrointestinal illness. These findings were supported by microbiological and environmental

Descriptive plot of the relationship between quantity of chicken quarters sold and number of cases, outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, 7–16 September 2009



The lines and left y axis denote the number of quarters of rotisserie chicken sold per time (5 minute intervals) and the red dots and right y axis denote the number of cases who were exposed to food from the takeaway restaurant at these times. The figure illustrates that most cases (red dots) occurred shortly after sales peaks.

findings. The ecological case-crossover study demonstrated that a linear relationship between chicken sales and risk of becoming a case occurred as soon as sales exceeded a certain amount. This may indicate that sales pressures led to substandard food handling practices, such as undercooking meat and cross-contamination. It was observed by some cases that raw chickens were being placed above roasted ones in the rotisserie and therefore potential cross-contamination through drippings could have occurred. Prior to serving, the chicken quarters were seared again on a hot grill before serving, in a rushed period this process may be inadequate, which may have caused the outbreak. This is useful information to change practices so as to prevent future outbreaks, reinforcing the need for scrupulous food hygiene practices at times of high demand.

Few published studies report the use of sales data in outbreak investigations. One of the reasons for this could be that access to sales data is not always granted to investigators, and our study benefits from the voluntary provision of complete till receipts by the restaurant owner. Supermarket bonus cards provide data on grocery shopping habits and have been used to generate hypotheses to inform subsequent analytical studies [10,16]. Purchase records from supermarkets have also been used in a virtual cohort approach [17]. The latter approach has some similarities to our study. Product purchase does not infer consumption, and in this study the unit of analysis was a group of people, which included a primary case (case-purchasing unit) [17].

Bonus card data allow identification of cases within a cohort, yield demographic information and thus allow combination with traditional methodologies. However, the use of bonus card schemes to investigate outbreaks is setting-specific, and limited by their popularity, representativeness and local data-sharing arrangements. The best approximation of this data type for a small caterer was the use of till receipts, which contrary to bonus cards contained all sales during the period at risk.

Synnott et al. calculated the probability that frequencies of observed case kebab consumption were within the expected range of owner-reported sales frequencies in support of their case-control investigation in a small kebab shop outbreak [11]. The analysis was carried out on a single food item, which all cases had been exposed to. In contrast, our study used a comparative approach for all possible exposures in the cohort, providing the exposures in the population at risk.

Our study benefits from extensive case-finding and good quality case exposure information acquired by applying a standardised exposure questionnaire in a standardised way. Till receipts contain readily available data, which are cheap, quick, accessible, and of good quality. Because till receipts are used for taxation purposes, data on price, date and time and food item (where entered) are likely to be accurate and unbiased. However, similar to other outbreak investigations, there remains potential for recall bias arising from the food exposure enquiry of the cases.

The main limitation of using till receipt data stems from the fact that sales data is not direct exposure/ consumption data, and that sales data do not allow an accurate calculation of a denominator. This is particularly relevant for the calculation of food exposures in

Lowess smoother graph denoting the risk of becoming a case per number of chicken quarters sold in the hour before exposure, outbreak of *Salmonella* Enteritidis phage type 14b related to a north west London takeaway restaurant, United Kingdom, September 2009



Number of chicken quarters sold in hour before exposure

The figure demonstrates that the risk of becoming a case was correlated with increasing rotisserie chicken sales in the hour before.

the cohort of takeaway restaurant customers for direct comparison. Assumptions on portion sizes and dish combinations were wide ranging, and sales descriptions were not available for 25% of the sales (miscellaneous category). Future studies using this approach may wish to include 'sensitivity' analyses and vary the underlying assumptions. No information on illness or demography of all the customers was available and cases were included within the cohort. A further limitation is the inability to provide 'adjusted' associations to account for potential confounders due to lack of knowledge of the joint food exposures of the total cohort. It is possible to extrapolate from the joint food exposures in the cases and in the situation here, where the outbreak is likely to have a single causal exposure, the assumption that joint food exposures in the cases are generalisable to the whole cohort is realistic.

The case-crossover study is less vulnerable to these assumptions, and using exposures occurring

immediately prior to when an individual case consumed food, and as this is compared to exposures at the same time of day, they act as their own controls, minimising the potential for selection bias [14,15]. Confounding by subject characteristics is controlled by design [15]. Information bias were largely avoided by use of purchase information [6], but there is a possibility that chicken sales were misclassified as 'miscellaneous' at busy times, although we were able to identify and recode a number of these on the basis of their typical price. While this would not change the direction of observed associations it could have led to an underestimate of the presented effect size.

However; this approach is more complex in terms of exposure data, potentially limiting the number of exposures that can be analysed. An a priori hypothesis is always required before commencing an analytical study, and is crucial in a case-crossover study. Results can be biased if control periods are not representative of the expected distribution of exposure for follow-up times that do not result in a case [6,14]. The latter is usually unknown, but it is unlikely that the exposure changed systematically during the risk time [6] (same chicken batch) and we minimised this potential by taking the mean of two control periods.

In conclusion we demonstrated that the use of till receipt data for analytical outbreak investigation was feasible and provided additional evidence of an association between the consumption of rotisserie chicken purchased in a specific London takeaway restaurant and subsequent development of gastrointestinal illness. Our approach will need validation and refinement; however, understanding its limitations, the use of till receipt data provides additional information in outbreaks investigated with traditional methods, and is a practical approach to estimate exposures in outbreak investigations, where classical study designs are not feasible due to practical difficulties.

Conflict of interest

None declared.

Authors' contributions

DZ, JZ, WM and YC initially investigated the outbreak, DZ wrote the manuscript, AC and DZ developed the statistical analysis with contributions of JZ, WM, CL and YC. YC is the sponsor and all authors have seen and extensively commented on the manuscript.

References

- Giraudon I, Cathcart S, Blomqvist S, Littleton A, Surman-Lee S, Mifsud A et al. Large outbreak of salmonella phage type 1 infection with high infection rate and severe illness associated with fast food premises. Public Health 2009;123(6):444-7. http://dx.doi.org/10.1016/j.puhe.2009.03.011
- Gordon MA. Salmonella infections in immunocompromised adults. J Infect. 2008;56(6):413-22. http://dx.doi.org/10.1016/j. jinf.2008.03.012
- Galofre J, Moreno A, Mensa J, Miro JM, Gatell JM, Almela M et al. Analysis of factors influencing the outcome and development of septic metastasis or relapse in Salmonella bacteremia. Clin Infect Dis. 1994;18(6):873-8. http://dx.doi. org/10.1093/clinids/18.6.873
- Palmer SR. Outbreak investigation: the need for 'quick and clean' epidemiology. Int J Epidemiol. 1995;24 Suppl 1:S34-8. http://dx.doi.org/10.1093/ije/24.Supplement_1.S34
- McCarthy N, Giesecke J. Case-case comparisons to study causation of common infectious diseases. Int J Epidemiol. 1999;28(4):764-8. http://dx.doi.org/10.1093/ije/28.4.764
- Haegebaert S, Duche L, Desenclos JC. The use of the casecrossover design in a continuous common source food-borne outbreak. Epidemiol Infect. 2003;131(2):809-13. http://dx.doi. org/10.1017/S0950268803001006
- Gillespie IA, O'Brien SJ, Frost JA, Adak GK, Horby P, Swan AV et al. A case-case comparison of Campylobacter coli and Campylobacter jejuni infection: a tool for generating hypotheses. Emerg Infect Dis. 2002;8(9):937-42. http://dx.doi. org/10.3201/eido809.010817
- 8. Krumkamp R, Reintjes R, Dirksen-Fischer M. Case-case study of a Salmonella outbreak: an epidemiologic method to analyse surveillance data. Int J Hyg Environ Health. 2008; 211(1-2):163-7. http://dx.doi.org/10.1016/j.ijheh.2007.02.006
- 9. Wilson N, Baker M, Edwards R, Simmons G. Case-case analysis of enteric diseases with routine surveillance data: Potential

use and example results. Epidemiol Perspect Innov. 2008;5:6. http://dx.doi.org/10.1186/1742-5573-5-6

- Ethelberg S, Smith B, Torpdahl M, Lisby M, Boel J, Jensen T et al. Outbreak of non-O157 Shiga toxin-producing Escherichia coli infection from consumption of beef sausage. Clin Infect Dis. 2009;48(8):e78-81. http://dx.doi.org/10.1086/597502
- Synnott M, Morse DL, Maguire H, Majid F, Plummer M, Leicester M et al. An outbreak of Salmonella mikawasima associated with doner kebabs. Epidemiol Infect. 1993; 111(3):473-81. http://dx.doi.org/10.1017/S0950268800057204
- 12. Ward LR, de Sa JD, Rowe B. A phage-typing scheme for Salmonella enteritidis. Epidemiol Infect. 1987;99(2):291-4. http://dx.doi.org/10.1017/S0950268800067765
- 13. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol. 1981;145(3):1365-73.
- 14. Redelmeier DA, Tibshirani RJ. Interpretation and bias in casecrossover studies. J Clin Epidemiol. 1997;50(11):1281-7. http:// dx.doi.org/10.1016/S0895-4356(97)00196-0
- Maclure M. The case-crossover design: a method for studying transient effects on the risk of acute events. Am J Epidemiol. 1991;133(2):144-53.
- 16. Centers for Disease Control and Prevention (CDC). Salmonella montevideo infections associated with salami products made with contaminated imported black and red pepper --- United States, July 2009-April 2010. MMWR Morb Mortal Wkly Rep. 2010; 59(50):1647-50.
- McCartney G, Cowden J, Murray S, Ahmed S. The use of a new virtual cohort study design to investigate an outbreak of E. coli 0157 linked to a supermarket delicatessen. Epidemiol Infect. 2010;138(10):1439-42. http://dx.doi.org/10.1017/ S0950268810000257