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Monitoring influenza virus susceptibility to oseltamivir using a new rapid assay, iART

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A new rapid assay for detecting oseltamivir resistance in influenza virus, iART, was used to test 149 clinical specimens. Results were obtained for 132, with iART indicating 41 as 'resistant'. For these, sequence analysis found known and suspected markers of oseltamivir resistance, while no such markers were detected for the remaining 91 samples. Viruses isolated from the 41 specimens showed reduced or highly reduced inhibition by neuraminidase inhibition assay. iART may facilitate broader antiviral resistance testing.

Early detection of drug-resistant influenza viruses is needed for timely modification of policies and recommendations on the use of antivirals [1]. In many countries, neuraminidase (NA) inhibitor(s) are the medications of choice for treatment and prophylaxis of influenza infections, with oseltamivir being most commonly prescribed. The rapid, global spread of oseltamivir-resistant A(H1N1) viruses that emerged in Norway in 2008, necessitated close monitoring of oseltamivir resistance among circulating influenza viruses [2]. The emergence and subsequent seasonal circulation of the 2009 A(H1N1)pdm09 pandemic virus have further reinforced the need for enhanced surveillance. Moreover, there have been reports of locally transmitted oseltamivir-resistant A(H1N1)pdm09 viruses harbouring the NA amino acid (AA) substitution H275Y [3-5], the marker of clinically relevant resistance to oseltamivir [6,7]. Several genotypic methods (e.g. pyrosequencing) have been implemented by surveillance laboratories to screen clinical specimens for the presence of H275Y [8].

Assays to detect oseltamivir-resistant influenza viruses

Neuraminidase inhibition

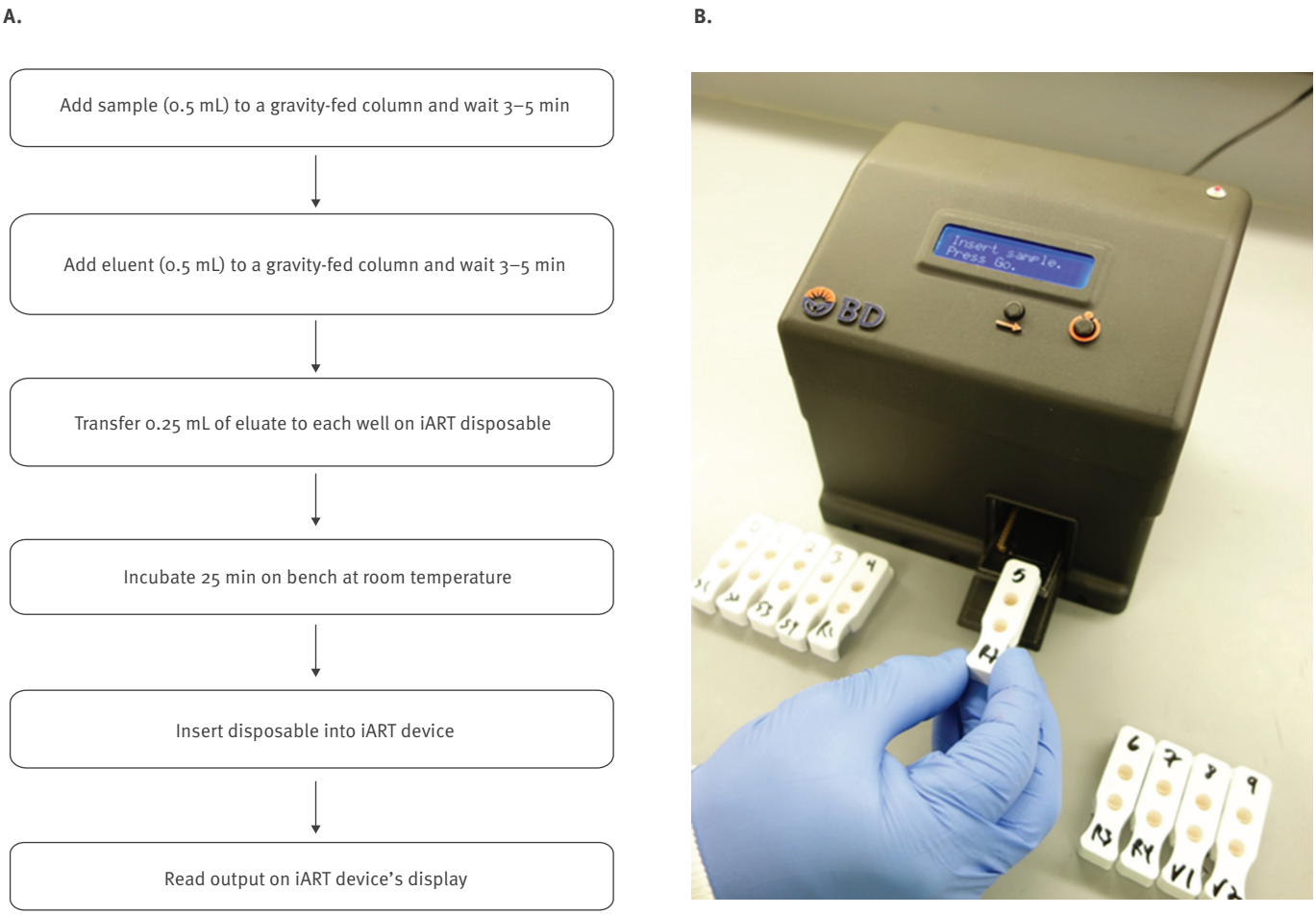
Unlike sequence-based assays, the NA inhibition (NAI) assay enables the detection of potentially drug-resistant viruses regardless of underlying genetic change(s). It is the gold standard method for assessing susceptibility to NA inhibitors [9,10]. Interpretation of the NAI assay is based on the determined IC50, a drug concentration needed to inhibit 50% of the NA enzyme activity. Depending on the fold increase of IC50 compared with a control, results are reported as normal (NI), reduced (RI) or highly reduced (HRI) inhibition. In the absence of established laboratory correlates of clinically-relevant oseltamivir resistance, all viruses displaying RI/HRI are considered to be potentially drug resistant and as such are monitored [9,10]. Although useful, this method is labour intensive, complex, and requires propagation of the viruses in cell culture. Additionally, the viral NA sequence from both the isolate and matching clinical specimen should be compared to rule out culturing artefacts [9-11]. Due to the complexity of the assay and data interpretation, testing is mainly performed by specialised surveillance laboratories [10,12,13].

New rapid prototype assay

In this study, we investigated whether the influenza Antiviral Resistance Test (iART), a rapid prototype assay developed by Becton Dickinson R and D for research use only, could be used to improve oseltamivir resistance surveillance by providing a simpler and faster testing method. iART utilises an advanced enzyme substrate that enables measurement of NA activity in virus isolates and in clinical specimens. Unlike the substrate used in the bioluminescence-based assay [14], the substrate used in iART is specific to influenza NA, making it more suitable for testing clinical specimens that may contain other pathogens. In this assay, the sample is divided between two wells of a disposable (Figure), one well containing substrate and the other well containing substrate and oseltamivir carboxylate. A simple device is used to measure the chemiluminescent signal generated from each well of the disposable. The

Figure

(A) Workflow of iART testing; (B) Prototype device and kit



iART: influenza Antiviral Resistance Test; VTM: viral transport medium.

Respiratory clinical specimens were stored in VTM before testing. The room temperature was monitored throughout the study and was consistently between 21 and 22°C.

built-in software calculates the ratio of signal intensity between the wells (R-factor), which appears on the device's display along with the final result: '*resistant*' or '*nonresistant*'. The threshold between *nonresistant* and *resistant* is different for type A and type B viruses, with R-factors of 0.7 and 2.2, respectively. If the NA activity is too low or absent, the message '*insufficient signal*' appears on the display.

Clinical specimens (n=149) were either applied to the gravity-fed column as is, or were diluted fivefold using viral transport medium (VTM). Virus isolates (n=76) were diluted 100- or 1,000-fold using VTM to meet the assay requirement (40,000‹signal‹6,000,000 luminescent units).

Testing viral isolates using the influenza Antiviral Resistance Test

International reference panel for neuraminidase inhibition assay

In the first experiment, the international reference panel for NAI assay was tested using iART and the United States Centers for Disease Control and Prevention (US CDC) standardised fluorescence-based NAI assay [13] (Table 1). Viruses identified as *resistant* by iART, displayed RI or HRI by NAI assay; viruses with NI were identified as *nonresistant*, indicating good agreement between the two assays (Table 1).

A(H1N1)pdm09 virus isolates carrying H275Y mutations

Monitoring the spread of A(H1N1)pdm09 viruses exhibiting HRI by oseltamivir and carrying H275Y is a priority for surveillance. To evaluate the ability of iART to detect oseltamivir-resistance conferred by H275Y, 13 virus isolates with this mutation, which had been collected between 2009 and 2016 were tested. All these H275Y viruses exhibited HRI by NAI assay and were also identified as *resistant* by iART with R-factor of 5.3 ± 0.76 (Table 2).

Virus isolates containing a mix of influenza viruses with and without H275Y mutations

In some instances, a sample may contain the drug-resistant and wild-type viruses (mix), but still be

Table 1

Results of testing viruses from the international reference panel, for resistance to oseltamivir, using the neuraminidase inhibition (NAI) and influenza Antiviral Resistance Test (iART) assays[a] (n=8)

Virus	Subtype or Lineage	NA amino acid substitution[b]		NAI assay[c]		iART	
		Straight numbering	N2 numbering	IC_{50}, nM (fold)[d]	Interpretation[e]	R-factor[f]	Result[g]
A/Mississippi/03/2001	H1N1	None	None	0.39±0.05 (1)	NI	0.13±0.04	*Nonresistant*
A/Mississippi/03/2001	H1N1	H275Y	H274Y	337.0±28.93 (876)	HRI	6.06±0.16	*Resistant*
A/Perth/265/2009	H1N1pdm09	None	None	0.25±0.03 (1)	NI	0.12±0.01	*Nonresistant*
A/Perth/261/2009	H1N1pdm09	H275Y	H274Y	171.81±20.66 (1,010)	HRI	4.83±0.35	*Resistant*
A/Fukui/20/2004	H3N2	None	None	0.12±0.02 (1)	NI	0.16±0.05	*Nonresistant*
A/Fukui/45/2004	H3N2	E119V	E119V	49.53±3.89 (450)	HRI	1.01±0.04	*Resistant*
B/Perth/211/2001	Yamagata	None	None	15.38±0.98 (1)	NI	1.63±0.14	*Nonresistant*
B/Perth/211/2001	Yamagata	D197E	D198E	98.08±20.21 (6)	RI	3.13±0.10	*Resistant*

IC50: inhibitory concentration 50%; NA: neuraminidase; R-factor: ratio of chemiluminescent signal intensity generated by viral NA activity on the substrate with and without inhibitor (i.e. oseltamivir carboxylate).

[a] International Society for Influenza and other Respiratory Virus Diseases Antiviral Group (ISIRV AVG) NAI susceptibility reference panel, a panel of sensitive, resistant and potentially resistant reference viruses to be used as controls for the harmonisation of NAI assays (https://isirv.org/site/index.php/reference-panel).

[b] NA amino acid substitution position is shown using both straight numbering (type/subtype specific) and N2 subtype numbering.

[c] Tested using the United States Centers for Disease Control and Prevention (CDC) standardised fluorescence-based NAI Assay [13].

[d] IC50, drug concentration required to inhibit 50% of NA activity; mean and standard deviation of at least three independent experiments; Fold, a fold increase in IC50 value compared with the control (IC50 value for the virus lacking the amino acid substitution).

[e] Criteria for reporting NAI assay results based on IC50 fold increase compared with the reference IC50 value (control virus): for influenza A, normal (<10-fold), reduced (10–100-fold) and highly reduced (>100-fold) inhibition, and for influenza B the same criteria, but using <5-fold, 5–50-fold and >50-fold increases [9]; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition.

[f] Mean and standard deviation of R-factors; results of at least three independent experiments.

[g] Output result as shown on the device's display; result is based on the pre-set cutoffs for influenza A (≥0.7) and B (≥2.2) viruses.

detected as normally inhibited in the NAI assay. To assess the ability of iART to detect oseltamivir resistance in such samples, we next tested samples with increasing proportions of H275Y (as determined by pyrosequencing [15]). Notably, isolates containing ≥24% of the H275Y variant were identified as *resistant* by iART, whereas NAI assay required ≥52% of the H275Y variant to detect RI, suggesting that iART was more efficient at this task (Table 3).

Influenza virus isolates with mutations other than H275Y

Next, we assessed iART ability to detect influenza viruses harbouring NA mutations other than H275Y and displaying RI/HRI against oseltamivir (Table 2): A(H1N1) pdm09 viruses that displayed RI by oseltamivir carrying the S247R (n=3) or I223R (n=1) were identified as *resistant* with high R-factors for S247R and an R-factor of 1.99±0.30 for I223R. One virus carrying I223K was detected as *nonresistant* with an R-factor substantially below the resistance threshold (0.42±0.03). The virus carrying D199G displayed NI (eightfold) by NAI assay and was identified as *resistant* by iART (Table 2). A(H3N2) viruses that display HRI by NAI assay were all identified as *resistant* by iART. The R-factor of the R292K virus was much greater than those harbouring either E119V or a four-amino acid deletion (del245–248). Three B/Victoria/2/87-lineage viruses – harbouring E117G, N294S or A200T – that displayed RI against oseltamivir were all identified as *resistant* by iART (Table 2). B/Yamagata/16/98-lineage viruses harbouring E117A, R150K or R374K, that displayed HRI by NAI assay were identified as *resistant*; and two viruses carrying H273Y and one carrying G407 presenting borderline NI/RI were identified as *nonresistant* by iART (Table 2). Finally, a group of viruses from both B/lineages – carrying D197N, K152N and I221V – showed borderline NI/RI by NAI assay (4–8-fold), and these viruses were identified as *resistant* by iART. These results demonstrate that iART may detect some influenza viruses harbouring NA changes in the enzyme active site (e.g. D199G in A(H1N1)pdm09 and I221V in type B) that otherwise would be classified as NI by oseltamivir using NAI assay. Notably, the criteria to separate viruses exhibiting NI from those with RI is arbitrary [9], and can be refined as more data become available. The interpretation of results obtained for viruses displaying borderline IC50 should be made cautiously.

Testing of clinical specimens

Because iART was designed to detect oseltamivir-resistant viruses in human respiratory specimens, we next tested a set of 64 well-characterised specimens collected during a clinical study conducted in 2008–2010

Table 2A

Results from neuraminidase inhibition (NAI) and iART assays for virus isolates carrying NA amino acid mutations conferring various degrees of oseltamivir resistance (n = 42) or no such mutations (controls; n= 4)

Virus	NA mutations		NAI assay			iART	
	Straight numbering	N2 numbering	IC_{50}, nM Mean ± SD[a]	Fold	Interpretation[b]	R-factor, Mean ± SD	Result[c]
A(H1N1)pdm09							
A/Washington/29/2009	H275Y	H274Y	208.76 ± 27.05	1,228	HRI	4.1 ± 0.12	*Resistant*
A/North Carolina/39/2009	H275Y	H274Y	199.43 ± 4.38	1,173	HRI	5.17 ± 0.12	*Resistant*
A/India/1027/2013	H275Y	H274Y	185.44 ± 15.95	1,091	HRI	5.30 ± 0.38	*Resistant*
A/Delaware/08/2011	H275Y	H274Y	174.53 ± 21.24	1,027	HRI	4.16 ± 0.73	*Resistant*
A/Hawaii/67/2014	H275Y	H274Y	171.48 ± 31.01	1,009	HRI	4.21 ± 0.58	*Resistant*
A/Michigan/65/2015	H275Y	H274Y	158.76 ± 28.68	934	HRI	5.77 ± 0.22	*Resistant*
A/Denmark/528/2009	H275Y	H274Y	153.26 ± 14.47	902	HRI	5.30 ± 0.26	*Resistant*
A/Georgia/31/2016	H275Y	H274Y	150.48 ± 24.48	885	HRI	5.60 ± 0.19	*Resistant*
A/Maryland/04/2011	H275Y	H274Y	145.64 ± 4.41	857	HRI	5.30 ± 0.53	*Resistant*
A/Washington/31/2016	H275Y	H274Y	141.00 ± 9.58	829	HRI	5.76 ± 0.60	*Resistant*
A/Texas/23/2012	H275Y	H274Y	145.07 ± 30.07	805	HRI	5.48 ± 0.67	*Resistant*
A/Colorado/30/2015	H275Y	H274Y	132.40 ± 32.65	779	HRI	5.84 ± 0.30	*Resistant*
A/Texas/48/2009	H275Y	H274Y	120.22 ± 18.65	707	HRI	3.85 ± 0.19	*Resistant*
A/Bolivia/1278/2014	I223R	I222R	11.68 ± 0.15	65	RI	1.99 ± 0.30	*Resistant*
A/Tennessee/24/2016	S247R	S246R	6.61 ± 0.45	37	RI	5.67 ± 0.47	*Resistant*
A/India/1819/2016	S247R	S246R	6.31 ± 0.09	35	RI	7.58 ± 0.47	*Resistant*
A/Dnipro/133/2014	S247R	S246R	5.66 ± 0.10	31	RI	3.79 ± 0.35	*Resistant*
A/Chile/1579/2009	I223K	I222K	2.84 ± 0.65	16	RI	0.42 ± 0.03	*Nonresistant*
A/Pennsylvania/05/2016	D199G	D198G	1.47 ± 0.03	8	NI	1.03 ± 0.26	*Resistant*
A/California/12/2012	Control[d]		0.18 ± 0.06	1	NI	0.24 ± 0.16	*Nonresistant*
A(H3N2)							
A/Bethesda/956/2006	R292K	R292K	>1,000	>14,285	HRI	7.22 ± 0.24	*Resistant*
A/Texas/12/2007	E119V	E119V	37.92 ± 5.56	542	HRI	1.06 ± 0.11	*Resistant*
A/Massachusetts/07/2013	E119V	E119V	37.33 ± 10.40	533	HRI	1.04 ± 0.04	*Resistant*
A/Arkansas/13/2013	E119V	E119V	34.88 ± 2.69	498	HRI	1.22 ± 0.11	*Resistant*
A/Illinois/03/2015	E119V	E119V	31.98 ± 3.70	458	HRI	1.32 ± 0.14	*Resistant*
A/Washington/33/2014	E119V	E119V	29.83 ± 6.56	426	HRI	1.19 ± 0.08	*Resistant*
A/Massachusetts/07/2013	Del245–248	Del245–248	21.70 ± 3.59	310	HRI	1.74 ± 0.06	*Resistant*
A/Washington/01/2007	Control		0.07 ± 0.02	1	NI	0.16 ± 0.07	*Nonresistant*
B/Victoria lineage							
B/Florida/103/2016	A200T	A201T	318.19 ± 37.76	23	RI	7.30 ± 0.09	*Resistant*
B/Bangladesh/3008/2013	E117G	E119G	115.54 ± 10.19	8	RI	4.34 ± 0.45	*Resistant*
B/Laos/1471/2016	N294S	N294S	108.37 ± 12.31	8	RI	2.29 ± 0.55	*Resistant*
B/Mexico/4260/2016	I221V	I222V	58.57 ± 9.38	4	NI	2.42 ± 0.03	*Resistant*
B/Laos/0425/2016	Control		13.99 ± 0.61	1	NI	0.95 ± 0.18	*Nonresistant*

Del: deletion; iART: influenza Antiviral Resistance Test; IC_{50}: inhibitory concentration 50%; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate); SD: standard deviation.

[a] Mean and standard deviation based on the results from at least three independent experiments.

[b] Criteria for reporting NAI assay results based on an IC_{50} fold increase compared with the reference IC_{50} value (control virus): for influenza A, normal (<10-fold), reduced (10–100-fold) and highly reduced (>100-fold) inhibition, and for influenza B the same criteria, but using <5-fold, 5–50-fold and >50-fold increases [9]; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition.

[c] Output result as shown on the device's display; result is based on the pre-set cutoffs for influenza A (≥0.7) and B (≥2.2) viruses.

[d] Control, a virus lacking NA changes (amino acid substitutions or deletions) associated with altered inhibition by oseltamivir, was included for each antigenic group (type/subtype/lineage) and used to determine a fold change and a degree of inhibition.

Table 2B

Results from neuraminidase inhibition (NAI) and iART assays for virus isolates carrying NA amino acid mutations conferring various degrees of oseltamivir resistance (n = 42) or no such mutations (controls; n= 4)

Virus	NA mutations		NAI assay			iART	
	Straight numbering	N2 numbering	IC_{50}, nM Mean ± SD[a]	Fold	Interpretation[b]	R-factor, Mean ± SD	Result[c]
B/Yamagata lineage							
B/Illinois/03/2008	E117A	E119A	>1,000	>112	HRI	10.44 ± 0.26	*Resistant*
B/Hong Kong/36/2005	R374K	R371K	>1,000	>112	HRI	9.11 ± 0.28	*Resistant*
B/Memphis/20/1996	R150K	R152K	591.47 ± 61.79	66	HRI	3.99 ± 0.36	*Resistant*
B/Vermont/15/2015	D197N	D198N	73.76 ± 8.17	8	RI	2.39 ± 0.18	*Resistant*
B/Santiago/75552/2015	D197N	D198N	54.81 ± 6.48	6	RI	2.59 ± 0.24	*Resistant*
B/Gorbea/75877/2015	D197N	D198N	49.51 ± 8.85	6	RI	2.49 ± 0.03	*Resistant*
B/Ontario/1110/2011	H273Y	H274Y	57.48 ± 6.98	6	RI	1.66 ± 0.16	*Nonresistant*
B/California/88/2015	H273Y	H274Y	50.18 ± 7.58	6	RI	1.78 ± 0.34	*Nonresistant*
B/Florida/05/2016	K152N	K154N	43.59 ± 4.88	5	RI	4.09 ± 0.29	*Resistant*
B/Utah/15/2016	D197N	D198N	38.72 ± 3.19	4	NI	3.06 ± 0.58	*Resistant*
B/Rochester/02/2001	D197N	D198N	37.08 ± 1.96	4	NI	2.40 ± 0.33	*Resistant*[e]
B/Wisconsin/42/2016	G407S	G402S	36.08 ± 3.52	4	NI	1.99 ± 0.10	*Nonresistant*
B/Rochester/02/2001	Control		8.93 ± 0.82	1	NI	0.97 ± 0.12	*Nonresistant*

Del: deletion; iART: influenza Antiviral Resistance Test; IC_{50}: inhibitory concentration 50%; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate); SD: standard deviation.

[a] Mean and standard deviation based on the results from at least three independent experiments.

[b] Criteria for reporting NAI assay results based on an IC_{50} fold increase compared with the reference IC_{50} value (control virus): for influenza A, normal (<10-fold), reduced (10–100-fold) and highly reduced (>100-fold) inhibition, and for influenza B the same criteria, but using <5-fold, 5–50-fold and >50-fold increases [9]; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition.

[c] Output result as shown on the device's display; result is based on the pre-set cutoffs for influenza A (≥0.7) and B (≥2.2) viruses.

[e] Two results were displayed as *resistant* and one as *nonresistant*.

[16] (Table 4). All the clinical specimens containing pre-pandemic A(H1N1) viruses harbouring H275Y (n = 32) were consistently identified as *resistant* with a mean R-factor of 6.86 ± 1.31. All other specimens were identified as *nonresistant* (Table 4). As expected, specimens negative for influenza (n = 10) displayed a signal below the level of detection (data not shown). These results serve as a proof-of-principle that iART can successfully detect oseltamivir-resistant H275Y viruses directly in clinical specimens.

Of note, the recommended volume for the iART test in its current configuration is 0.5 mL of sample, which is often unavailable at surveillance laboratories. Moreover, clinical specimens submitted to surveillance laboratories commonly undergo freeze-thaw cycles before testing, which adversely affect the integrity of virus particles. To address these concerns, we next tested a set of residual clinical specimens from the 2015/16 US national surveillance that were previously confirmed influenza virus positive; only 0.1 mL of each specimen was used for testing using iART. Of 85 tested, 17 samples (20%) had a signal below the limit of detection; 59 samples (69%) were identified as *nonresistant*; and nine samples (11%) as *resistant* (Table 5). These nine harboured H275Y, E119V or K152N. The matching isolates of these nine clinical specimens displayed RI/HRI in the NAI assay, while the other virus isolates showed NI.

Conclusion

A limitation of this study is that the effect of viral loads in relation to the performance of iART was not investigated. As the iART detects NA activity, one challenge is the difference in NA specific activities of seasonal wild-type viruses, whereby the minimal viral load needed for the iART assay may depend on the virus type/subtype and might not be generalisable. More studies are needed to establish the type/subtype specific limit of detection. Moreover, NA mutations that confer oseltamivir resistance may or may not affect the NA specific activity, so the influence of this on viral load appropriate for the assay would also have to be investigated independently for such viruses.

Taken together, however, the data presented here show that the iART assay can become a valuable tool for surveillance laboratories. iART offers a fast mean for detecting viruses displaying RI/HRI against oseltamivir in either isolates or clinical specimens. It is a simple approach where signal measurement, data analysis and interpretation are done by a compact portable device. The assay robustness is evident from its ability to test specimens under less than optimal conditions (i.e. interference from virus transport media (VTM), multiple freeze/thaw cycles, limited volume). Although iART is not a substitute for NAI assay employed by specialised laboratories, it has great potential to enable a

Table 3

Results from neuraminidase inhibition (NAI) and iART assays on mixtures of influenza A(H1N1)pdm09 viruses containing different proportions of mutants with H275Y in the neuraminidase (n = 22)

Virus	Pyrosequencing (%)[a]		NAI assay		iART	
	H275	H275Y	IC_{50}, nM (Fold)[b]	Interpretation[c]	R-factor	Result[d]
A/Louisiana/08/2013	0	100	190.84 (1,004)	HRI	5.97	*Resistant*
A/Mississippi/11/2013	3	97	177.62 (935)	HRI	6.67	*Resistant*
A/North Carolina/04/2014	3	97	199.91 (1,052)	HRI	6.17	*Resistant*
A/Michigan/73/2016	3	97	157.39 (828)	HRI	5.89	*Resistant*
A/Texas/09/2014	7	93	131.02 (690)	HRI	5.39	*Resistant*
A/Texas/100/2013	9	91	150.21 (791)	HRI	5.03	*Resistant*
A/Massachusetts/06/2016	10	90	121.85 (641)	HRI	6.03	*Resistant*
A/Pennsylvania/18/2014	11	89	127.1 (669)	HRI	6.20	*Resistant*
A/Florida/10/2014	14	86	111.35 (586)	HRI	6.46	*Resistant*
A/Colorado/07/2014	16	84	110.24 (580)	HRI	6.22	*Resistant*
A/Brazil/0257 S2/2016	25	75	97.73 (514)	HRI	4.92	*Resistant*
A/Brazil/9061/2014	32	68	39.32 (207)	HRI	3.47	*Resistant*
A/Quebec/RV1424/2016	48	52	4.14 (22)	RI	1.93	*Resistant*
Mix #1[e]	63	37	1.37 (8)	NI	1.09	*Resistant*
A/Utah/10/2013	68	32	0.98 (5)	NI	1.25	*Resistant*
A/North Carolina/21/2013	72	28	0.95 (5)	NI	1.28	*Resistant*
Mix #2	76	24	0.73 (4)	NI	0.71	*Resistant*
Mix #3	84	16	0.49 (3)	NI	0.46	*Nonresistant*
A/Michigan/36/2016	89	11	0.57 (3)	NI	0.43	*Nonresistant*
Mix #4	92	8	0.37 (2)	NI	0.28	*Nonresistant*
Mix #5	96	4	0.35 (2)	NI	0.12	*Nonresistant*
A/Maryland/08/2013	100	0	0.22 (1)	NI	0.18	*Nonresistant*

iART: influenza Antiviral Resistance Test; IC_{50}: inhibitory concentration 50%; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate).

[a] Proportion of H275 and H275Y virus subpopulations was determined by a single-nt polymorphism (SNP) pyrosequencing analysis in allele quantification mode (AQ) as described in reference [15].

[b] Fold increase calculated using the median oseltamivir IC_{50} for influenza A(H1N1)pdm09 viruses circulating during 2015/16 influenza season.

[c] Interpretation of NAI assay results based on the fold increase in IC_{50} value: normal (<10-fold), reduced (10–100-fold) and highly reduced (>100-fold) inhibition; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition.

[d] Output result as shown on the device's display; result is based on the pre-set cutoffs for influenza A (≥0.7) and influenza B (≥2.2) viruses.

[e] H275 and H275Y mixes were prepared by combining the two virus isolates A/Maryland/08/2013 and A/Louisiana/08/2013, at different ratios.

broader adoption of influenza antiviral resistance testing in various settings.

The prototype of the iART system tested in this study was configured by the developers for surveillance applications to detect viruses that could be identified by the gold standard NAI assay. Of note, samples collected by surveillance laboratories may be stored in a variety of storage media (e.g. VTM). To accommodate various types of sample media, the current iART workflow includes a buffer exchange to remove media components that interfere with the assay. If this assay is to be used at clinical care settings, this step is not needed, since a buffer optimised for the iART assay can be used for sample collection.

Larger studies are desirable to provide a better understanding of the performance and utility of the iART assay and to establish laboratory correlates (e.g. R-factor threshold) for clinically-relevant resistance. As iART was designed to test influenza A viruses, regardless of their antigenic subtype, the utility of this rapid test in detecting oseltamivir resistance in zoonotic influenza viruses (e.g. avian A(H7N9)) needs to be evaluated, as this would facilitate pandemic preparedness. Nonetheless, we are confident that the implementation of this assay, which is available for national public health agencies, e.g. the US CDC and application by its network of influenza surveillance laboratories, can facilitate timely detection of oseltamivir resistance emergence and spread.

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Table 4

Respiratory specimens from the clinical study on the efficacy of treatment with oseltamivir tested using iART[a] (n = 64)

Type and subtype	Number of specimens	Ct[b]	iART/R-factor	iART/result[c]
A(H1N1) H275Y[d]	32	24.40 ± 2.63	6.8 6 ± 1.31	*Resistant*
A(H1N1)pdm09	12	21.31 ± 3.25	0.06 ± 0.02	*Nonresistant*
A(H3N2)	10	21.75 ± 2.13	0.25 ± 0.11	*Nonresistant*
B	10	24.46 ± 1.81	0.99 ± 0.10	*Nonresistant*

Ct: cycle threshold; iART: influenza Antiviral Resistance Test; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate).

[a] Aliquots of leftover respiratory specimens (nasal or nasopharyngeal wash) from the clinical study [13] were used for testing by iART. Specimens were initially processed on ice in the laboratory within the study clinic. The study was reviewed and approved by the ethics and research review board of the International Centre for Diarrheal Diseases, Bangladesh (icddr,b) and the institutional review board of the United States Centers for Disease Control and Prevention (CDC). All index patients provided written informed consent. Clinical specimens were aliquoted and stored at -70 °C until testing using real-time reverse-transcriptase PCR assay (rRT-PCR), pyrosequencing (to detect neuraminidase amino acid substitutions previously associated with oseltamivir resistance), virus isolation and neuraminidase inhibition assay testing. All respective virus isolates, except those carrying H275Y, displayed normal inhibition in the NAI assay. For testing using iART, 0.5 mL of clinical specimen was used; all specimens tested underwent a single freeze/thaw cycle.

[b] Ct value as determined using rRT-PCR assay according to the CDC protocols.

[c] Output result as shown on the device's display; result is based on the pre-set cutoffs for influenza A (≥ 0.7) and B (≥ 2.2) viruses.

[d] Pre-pandemic A(H1N1) carrying H275Y, straight neuraminidase amino acid numbering.

Table 5

Residual clinical specimens from the 2015/16 United States national influenza surveillance tested using iART (n = 85)

Type and subtype	Number of specimens tested[a]	Number of. indeterminate[b]	Number of *nonresistant*[c]	Number of *resistant*[c]	NA mutation in resistant viruses[d]
A(H1N1)pdm09	34	9	19	6	H275Y
A(H3N2)	25	5	18	2	E119V
B	26	3	22	1	K152N
Total	85	17	59	9	Not applicable

NA: neuraminidase.

[a] Leftovers of clinical specimens submitted for United States national virological surveillance that satisfied the following criteria: (i) NA sequencing or pyrosequencing data were available; (ii) virus was recovered in cell culture and tested using NAI assay. For testing using iART, 0.1 mL of a residual clinical specimen was combined with 0.4 mL of viral transport medium (VTM, Becton Dickinson) to bring the final volume to 0.5 mL.

[b] Indeterminate: specimen displayed a low signal to noise ratio (SNR); insufficient NA activity for testing.

[c] Output result as shown on the device's display; result is based on the pre-set cutoffs for influenza A (≥ 0.7) and B (≥ 2.2) viruses.

[d] Position of amino acid residue shown using straight NA numbering (Table 1).

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Conflict of interest

EF, KW and RJ are employees of Becton Dickinson.

Authors' contributions

Designed the study: LVG, KW. Generated and analysed antiviral susceptibility data: VPM, EF and EH. Generated sequencing data: JB. Clinical data analysis and interpretation: AMF and AB. Drafted the article: LVG and KW. Revised the article: WK, LVG and KW. Provided supervisory oversight: DEW, RJ and RS. All authors further edited the manuscript and approved the final version of the paper.

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West Nile virus surveillance in Europe: moving towards an integrated animal-human-vector approach

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This article uses the experience of five European countries to review the integrated approaches (human, animal and vector) for surveillance and monitoring of West Nile virus (WNV) at national and European levels. The epidemiological situation of West Nile fever in Europe is heterogeneous. No model of surveillance and monitoring fits all, hence this article merely encourages countries to implement the integrated approach that meets their needs. Integration of surveillance and monitoring activities conducted by the public health authorities, the animal health authorities and the authorities in charge of vector surveillance and control should improve efficiency and save resources by implementing targeted measures. The creation of a formal interagency working group is identified as a crucial step towards integration. Blood safety is a key incentive for public health authorities to allocate sufficient resources for WNV surveillance, while the facts that an effective vaccine is available for horses and that most infected animals remain asymptomatic make the disease a lesser priority for animal health authorities. The examples described here can support other European countries wishing to strengthen their WNV surveillance or preparedness, and also serve as a model for surveillance and monitoring of other (vector-borne) zoonotic infections.

Introduction

West Nile fever (WNF) is a zoonotic vector-borne disease caused by a virus that is most often transmitted through mosquito bites (primarily *Culex* genus) but can also be transmitted through organ transplantation, blood transfusion, in laboratory settings and from mother to fetus during pregnancy [1]. West Nile virus (WNV) is maintained in a bird-mosquito cycle, with birds acting as amplifying hosts. Mosquitoes acquire infection by feeding on viraemic birds. Once infected, the mosquito remains infectious throughout its life, potentially transmitting the virus to every vertebrate on which it feeds. Many bird species do not develop any disease symptoms after infection. However, certain species, such as crows, jays and birds of prey, may die from the infection [2].

Humans, horses and other mammals are considered dead-end hosts. Infections in humans are generally asymptomatic. Around 20% of cases develop influenza-like symptoms, while 1% of cases, mainly elderly and immunocompromised people, develop West Nile neuroinvasive disease (WNND), which may lead to death [3]. Approximately 10% of infected horses may show neurological signs [4]. There is no specific treatment for humans or animals, and no vaccine is available for humans, although inactivated and recombinant vaccines for horses are used in Europe [5].

The epidemiological situation of WNF in Europe is heterogeneous: some European countries report outbreaks in humans and animals every year and others have never reported any autochthonous cases [6,7]. Taking five European countries (Austria, France, Greece, Italy and the United Kingdom (UK)) with very diverse WNF epidemiological situations as examples, this article describes surveillance and monitoring activities for WNV infection in humans, animals and vectors conducted at national and European levels, and suggests key actions for strengthening the intersectoral collaboration between the public health and veterinary sector.

Epidemiological situation in Austria, France, Greece, Italy and the United Kingdom

Austria

In Austria, the first autochthonous human WNF cases were diagnosed retrospectively by serology: two cases from 2009 and one case from 2010 [8] (Table 1).

In 2014, two more people were affected by WNV [9], followed by eight cases in 2015. WNV was introduced in eastern Austria in 2008 [10] causing mortality in birds. Since then, WNV has been repeatedly found in mosquito pools and birds in Vienna [9], Lower Austria [10] and in regions bordering the Czech Republic [11].

France

No evidence of WNV infections in humans or horses was identified in France from the mid-1960s until 2000 [12]. The 2000 WNV epizootic among equidae in the Camargue was the largest ever recorded in France although it did not cause massive bird die-offs [12]. In the following years, WNV circulation was reported on three occasions in the Camargue and neighbouring regions [13,14]. Seven autochthonous human cases were reported in 2003 and then none until 2015 (Table 1). Serosurveys conducted in the Camargue in the periods 2005–2007 and 2009–2010 highlighted WNV circulation in resident birds in the absence of cases in humans or horses. However, no formal proof of virus endemicity in the wild avifauna from the Camargue has yet been obtained [15,16]. In summer 2015, WNV re-emerged at the periphery of the Camargue, causing WNND in horses and one WNF human case, reminding us that the Camargue remains a potential environment for WNV circulation.

Greece

In Greece, the first outbreak of WNF in humans occurred in 2010, in the region of Central Macedonia [17]. Between 2010 and 2014, the virus spread further, with annual seasonal outbreaks recorded in humans and animals, between June and October. During that period, 624 autochthonous human WNF cases were diagnosed in Greece, in 11 of 13 regions, whereas in 2015 no human cases were diagnosed in the country (Table 1). Although neutralising antibodies against WNV had previously been detected in horses [18], the first WNF cases in equidae in Greece were detected in 2010 in Central Macedonia, after the occurrence of the first human cases. Since 2010, the number of affected horses has been decreasing (Table 1).

Italy

In Italy, WNV reappeared in the north-east of the country in the summer of 2008 after a 20-year absence; WNV was isolated in mosquitoes, birds, equidae and humans in the area surrounding the Po river delta [19,20]. From 2010 to 2015, 148 confirmed autochthonous human cases of WNND were reported in Italy from eight of the 20 regions (Table 1). Seroconversion in horses and sentinel chickens was regularly identified in the wetlands of Sicily during this period, whereas sporadic animal cases have been detected in some localities of central and southern Italy.

United Kingdom

The UK has not had any autochthonous human or animal cases of WNF, and WNV infection has never been found in vector species there. Although there have been a few limited studies in sentinel chickens and non-migratory wild birds suggesting positive WNV antibody reactions and detection of WNV RNA in avian tissues, these results have never been reproduced and validated [21].

West Nile virus surveillance and monitoring at European Union level and in the individual countries

The key characteristics of WNV infection surveillance in the European Union (EU) and the individual countries are summarised in Table 2. Notifications of human WNF cases in Europe are collected through The European Surveillance System (TESSy) [22] of the European Centre for Disease Prevention and Control (ECDC). Between June and November, the period of high vector activity, ECDC publishes weekly updated maps [7] of human cases and complementary information on animal WNV infection and vectors based on data provided by the World Organisation for Animal Health (OIE) and European countries. The yearly analyses of TESSy data are published in the ECDC annual epidemiological report [23] and jointly with the European Food Safety Authority (EFSA) [6].

EU countries report outbreaks of WNV encephalomyelitis in horses to the European Commission (EC) via the Animal Disease Notification System (ADNS) [24] and regular summaries are posted online. The data from WNV monitoring in animals is reported annually by EU countries under Directive 2003/99/EC [25] and presented in the annual EFSA/ECDC EU Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks [6]. Animal WNF outbreak data reported to the OIE are publically available on the World Animal Health Information Database (WAHIS) interface [26].

Table 1

Number of humans, horses, birds and mosquito pools tested and found to be infected with West Nile virus in Austria, France, Greece, Italy, and the United Kingdom, 2010–2015

Number of humans[a], horses, birds and mosquito pools found to be infected with West Nile virus (number of humans, horses, birds and mosquito pools tested)	2010		2011		2012		2013		2014		2015	
	Number infected	Number tested	Number infected	Number tested	Number infected	Number tested	Number infected	Number tested	Number infected	Number tested	Number infected	Number tested
Austria												
Human cases	1	NA	0	NA	0	NA	0	NA	2	NA	8	NA
Equine cases	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA
Positive birds	0	NA	0	NA	0	NA	1	NA	1	NA	2	NA
Positive mosquito pools	0	NA	1	NA	1	NA	0	NA	2	NA	3	NA
France												
Human cases	0	NA	0	NA	0	NA	0	NA	0	NA	1	NA
Equine cases	0	94	0	85	0	67	0	54	0	39	49	155
Positive birds	0	4	0	0	0	5	0	9	0	0	0	15
Positive mosquito pools	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	40
Greece												
Human cases	262	NA	100	NA	161	NA	86	NA	15	NA	0	NA
Equine cases	30	167	24	1,539	15	1,640	15	1,626	4	962	0	NA
Positive birds	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Positive mosquito pools	2	110	71	897	212	2,112	45	405	6	603	11	157
Italy												
Human cases	3	NA	14	NA	28	NA	44	NA	21	NA	38	NA
Equine cases	128	993	197	2,840	63	1,343	50	3,366	27	7,675	30	5,507
Positive birds	16	3,614	16	4,719	26	5,363	79	5,649	55	5,018	73	1,880
Positive mosquito pools	13	1,236	8	3,059	14	2,907	146	1,984	125	7,047	102	4,614
United Kingdom												
Human cases	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA
Equine cases	0	5	0	10	0	10	0	12	0	5	0	3
Positive birds	0	204	0	280	0	374	0	316	0	433	0	336
Positive mosquito pools	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA

NA: not available.

[a] Includes probable and confirmed autochthonous West Nile neuroinvasive disease (WNND) and non-WNND cases.

Through the Pan-European VectorNet project [27], presence/absence distribution maps of *Culex* species are under development. Current *Culex pipiens* maps are incomplete [28].

Austria

The Austrian Federal Ministry of Health has developed WNV guidelines for the Austrian Blood Donation System, based on the document 'West Nile Virus and Blood Safety - Introduction to a Preparedness Plan in Europe' [29]. In response to the blood donation findings in 2014, three eastern Austrian federal states switched to pooled testing of blood donations.

Veterinary surveillance of WNV in Austria covers birds and horses. Bird surveillance has been carried out since 2008. Screening is conducted in all cases of encephalitis in birds with emphasis on *Falconiformes* and *Passeriformes*; active surveillance of birds sampled under the avian influenza monitoring programme/scheme is conducted by serological testing of waterfowl (geese, ducks) sampled every year from slaughterhouses in at-risk regions. All suspected encephalomyelitis cases in horses must be notified and are tested for WNV; the first WNV-induced case of equine encephalitis was documented in September 2016. Also, a national serological screening programme for WNV antibodies in horses has been in place since 2011.

Since 2011, the Austrian Agency for Health and Food Safety (AGES) [30] has conducted active country-wide mosquito surveillance. This involves mosquito species identification and laboratory testing for various pathogens including WNV at two sampling sites per province. Any human or animal WND case leads to enhanced vector surveillance in the respective area.

A national WNV Task Force was established in 2013, with members nominated by the Ministry of Health.

This Task Force brings together representatives from all affected provinces as well as those responsible for vector surveillance/control, public and animal health. The group meets at least once a year, and more frequently when WNF cases are identified. A similar federal group already exists for investigations of outbreaks of foodborne and other zoonoses.

Austria does not have a joint reporting system across the different authorities. However, reports are published on the AGES website, including maps containing compiled information on human and animal cases as well as the results of the mosquito surveillance. Detailed reports (other than the general reports published on the AGES website) are produced exclusively for the use of the health authorities. The public is informed about WNF cases through press releases.

France

At national level, human surveillance activities include the notification of confirmed human cases by the National Reference Centre, Marseille. Enhanced surveillance of human neuroinvasive cases is implemented in the Mediterranean region between June and October. Surveillance of human non-neuroinvasive cases is conducted by the National Reference Centre and Santé Publique France.

Clinical surveillance in equidae is carried out across the entire country, with veterinary practitioners reporting suspected cases to regional veterinary services. Established in 1999, the French network Réseau d'Epidémio-Surveillance en Pathologie Equine (RESPE) supports 550 sentinel and voluntary veterinarians across France in testing symptomatic horses for the identification of causative agents for diverse conditions (e.g. neurological infections, acute respiratory infections), which includes WNV. Periodic reports on the WNV situation in horses during WNV outbreaks are made available on the online platform Epidémiosurveillance santé animale (ESA) [31].

Sentinel bird surveillance was discontinued in 2007, with the option to reactivate it should the epidemiological context change. Clinical bird surveillance, relying on WNV testing of abnormal bird fatalities from June to October in the Mediterranean area, was combined with avian influenza surveillance in 2006. The sensitivity of this bird surveillance is however very low as screening is performed on a very small subset of dead birds.

WNV screening in mosquitoes is no longer conducted outside WNV epizootics or epidemics. However, mosquito surveillance, involving the identification of mosquito species and abundance, has been systematically implemented from March to November in the Mediterranean area by the Interdepartmental Agreement for Mosquito Control on the Mediterranean coast.

WNV surveillance, prevention and control activities are described in national guidelines that were published for the first time in 2004, last updated in 2012, under the responsibility of the Ministries of Health, Agriculture and Environment. Blood safety measures described in these guidelines are in line with the EU directive [32].

Greece

Human surveillance includes awareness campaigns among physicians, support of laboratory confirmation and active laboratory-based surveillance with daily exchange of information on the diagnosed cases between the Hellenic Center for Disease Control and Prevention (HCDCP) and the laboratories testing for WNV. All probable and confirmed cases are investigated within 24 hours after diagnosis by HCDCP and there is a daily follow-up of all hospitalised cases until discharge. National and local stakeholders, including the blood safety authorities, receive daily updates on diagnosed cases and weekly surveillance reports by email, the latter also being publically available on the HCDCP website. Affected areas are defined as third administrative level areas with at least one human case.

In line with the EC directives 2004/33 [32] and 2014/110 [33] and the EC WNV and blood safety preparedness plan [29], blood safety measures have been implemented in affected areas, including screening of donor blood for WNV RNA.

Since 2010, the HCDCP's Coordinating Haemovigilance Centre has conducted active surveillance of WNV infection in the blood donor population of the affected areas during the transmission period from mid-June to mid-November. All confirmed cases of WNV infection in blood donors are notified to the Haemovigilance Centre. Specific haemovigilance procedures such as post-donation and post-transfusion information are in place: When a case of WNV infection reports a recent blood transfusion, trace back and testing of the implicated donors is initiated. Moreover, blood donors are asked to notify any suspected symptom within 15 days after donation and if they do, blood testing is performed [34].

Systematic surveillance for the most important diseases of equidae, including WNF, had previously been in place in Greece (2001–2004), carried out by the local veterinary authorities, under the coordination of the central veterinary service [18]. Since 2010, a WNF-specific surveillance programme has been implemented under the coordination of the Ministry of Rural Development and Food in cooperation with the local veterinary authorities and relevant state laboratories within the Veterinary Centres of Athens and Thessaloniki (Ministry of Rural Development and Food). The programme includes active serological surveillance of sentinel horses; active clinical surveillance of equidae around confirmed human and animal cases; passive surveillance of WNF in equidae all year round;

Table 2

Key characteristics of West Nile virus (WNV) infection surveillance in the European Union, Austria, France, Greece, Italy and the United Kingdom

Country/region	Intersectoral collaboration	Human surveillance	Animal surveillance	Vector surveillance
European Union	▪ ECDC provides WNF seasonal maps that include human cases and provides information on animal/vector WNV infection. ▪ ECDC tool for WNF risk assessment proposes a classification of risk areas based on human, animal and vector surveillance data. ▪ EFSA and ECDC publish the EU summary reports on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks every year.	▪ WNF is an EU notifiable disease; cases are reported by EU countries to TESSy according to the EU case definition. ▪ EU countries must implement a deferral of blood donations for 28 days after leaving an area with ongoing WNV transmission in humans.	▪ WNF, as a cause of equine encephalomyelitis in horses, is notifiable to the European Animal Disease Notification System. ▪ WNF in animals is a disease listed in the OIE Terrestrial Animal Health Code and must be reported to the OIE. ▪ EU countries should monitor WNV activity in animals, if warranted by the epidemiological situation, and report animal cases to the European Commission.	▪ There is no legal framework regarding mosquito surveillance at EU level. ▪ ECDC developed guidelines for surveillance of native mosquitoes to support countries to plan and implement surveillance activities.
Austria	▪A national WNV Task Force was established in 2013, with members nominated by the Ministry of Health. ▪No joint reporting system across the different authorities. However, reports are published on the AGES website.	▪ Human cases and fatalities are statutorily reportable as of September 2015. ▪ Routine examination of donated blood was introduced in eastern Austria in the summer of 2014.	▪ Neurological disease in equidae is notifiable in Austria. ▪ Surveillance covers birds and horses.	▪ Since 2011, active country-wide mosquito surveillance is conducted by AGES.
France	▪ The national guidelines for WNV surveillance, prevention and control activities are under the responsibility of the Ministries of Health, Agriculture and Environment.	▪ Suspected neuroinvasive human cases should be notified to regional health authorities in the Mediterranean region during the period of vector activity, from June to November. ▪ Measures for the safety of blood products are taken in line with the EU directive for blood safety.	▪ Notification of suspected horse cases to regional veterinary services is compulsory, whatever the time period or their location. ▪ Sentinel bird surveillance was discontinued in 2007.	▪ Mosquito surveillance is systematically implemented from March to November in the Mediterranean area.
Greece	▪ The Ministry of Rural Development and Food and the HCDCP share the results of human, animal and vector surveillance with each other and with regional and local public health authorities, local veterinary services, municipalities and local health units ▪ There are a multisectoral committee for the prevention and management of tropical diseases (including WNF) and two multisectoral working groups: for vector-borne diseases and for the designation of areas affected by such diseases.	▪ Since 2010, all laboratory probable and confirmed WNF cases should be notified. ▪ Enhanced surveillance is implemented at national level that includes awareness campaigns towards physicians, support of laboratory confirmation, active laboratory-based surveillance, cases investigation and daily dissemination of information to national and local stakeholders. ▪ Measures for the safety of blood products are taken in line with the EU directive for blood safety.	▪ WNF in animals is a notifiable disease and disease suspicions must be reported to the competent veterinary authorities. ▪ There is active serological surveillance of sentinel horses; active clinical surveillance of equidae around confirmed human and animal cases; passive surveillance of WNF in equidae all-year-round and some small scale surveillance in wild birds.	▪ Since 2010, HCDCP together with the National School of Public Health, Universities, local authorities and subcontractors, conducts active vector surveillance from June to October to detect WNV circulation in mosquitoes.
Italy	▪ A national plan for surveillance on imported and autochthonous human vector-borne disease (chikungunya, dengue and WND) integrating human and veterinary surveillance is prepared annually. ▪ Any suspected evidence of virus circulation in animals or vectors is notified to the public health authorities.	▪ Probable and confirmed human cases are notified in real time using a password-protected web-based system. ▪ In the affected area, local health authorities implement an active surveillance at risk population. ▪ Passive surveillance on human neurological cases is set up in the whole region where the affected area is located. ▪ Measures for the safety of blood products are taken in line with the EU directive for blood safety.	▪ A web-based national animal disease notification system allows the notification of animal diseases. ▪ Passive surveillance in equidae in the whole country. ▪ Random IgM screening in horses living in non-affected areas. ▪ Bird surveillance focuses on WNV detection in resident target species; immunological response among poultry of rural farms and migratory birds; and bird mortality.	▪ Entomological surveillance is systematically implemented during the period of vector activity in affected areas.
United Kingdom	▪ A national contingency plan for integrated surveillance and control of vector-borne disease has been prepared. ▪ Monthly meetings between human and animal health risk assessors to share information. ▪ Any suspect or confirmed cases would be notified to all relevant authorities.	▪ WNV in humans is a notifiable disease in Scotland, but not in the rest of the UK. It is however, a notifiable organism in England, Wales and Scotland (not Northern Ireland). ▪ Human cases of autochthonous WNV infection should be reported to National Surveillance Centres by the diagnostic laboratories. ▪ Measures for the safety of blood products are taken in line with the EU directive for blood safety.	▪ WNF, as a cause of equine encephalomyelitis, is a notifiable disease of equidae in the UK. ▪ There is no active surveillance of horses or other equidae. Monitoring relies on passive surveillance and testing of horses with neurological signs. ▪ There is no systematic WNV active surveillance of wild birds in the UK, however a passive surveillance system is in place between April and October.	▪ Some targeted surveillance for mosquitoes are carried out by PHE in areas with suitable habitat.

AGES: Austrian Agency for Health and Food Safety; ECDC: European Centre for Disease Prevention and Control; EFSA: The European Food Safety Agency; EU: European Union; HCDCP: Hellenic Center for Disease Control and Prevention; OIE: World Organization for Animal Health; PHE: Public Health England; TESSY: The European Surveillance System; UK: United Kingdom; WNF: West Nile fever; WNV: West Nile virus.

passive surveillance of wild birds by sampling dead or sick wild birds; and active surveillance of wild birds through capture and sampling in selected regions. In addition, schools of veterinary medicine perform WNV surveys in domestic and wild birds in various areas.

Since 2010, HCDCP together with the National School of Public Health, Universities, local authorities and subcontractors have conducted active vector surveillance from June to October, including WNV detection in mosquitoes [35].

The Ministry of Rural Development and Food and the HCDCP share the results of animal and vector surveillance with regional and local public health authorities, local veterinary services, municipalities and local health units for further awareness, prevention and follow-up activities.

There is a multisectoral committee for the prevention and management of tropical diseases (including WNF) of the Ministry of Health, and two multisectoral working groups: for vector-borne diseases and for the designation of areas affected by such diseases. These groups ensure communication between veterinary and human health authorities, entomologists, blood safety authorities, infectious disease specialists and other national actors.

Italy

In Italy, public and animal health surveillance is a shared responsibility between the national and regional levels.

The national plan for human surveillance defines 'affected areas' as all the provinces (NUTS-3 level) where laboratory-confirmed WNV infections in animals, vectors or humans have been notified in the previous year or during the current surveillance period (between 15 June and 30 November, the period considered to have the highest vector activity). In the affected area, local health authorities implement active surveillance in employees of farms where equine cases are identified and in individuals living or working in the surrounding area. Employees of affected farms are contacted regularly by phone and serosurveys are conducted. Moreover, measures for vector control and blood and transplant safety are implemented immediately. At the same time, passive surveillance of human WNND cases is undertaken in the whole region in which the affected area is located, requesting physicians to report all probable and confirmed WNND cases using a modified EU case definition, which includes neurological symptoms.

Probable and confirmed human cases are notified by regional authorities to the Ministry of Health and to the Istituto Superiore di Sanità using a password-protected web-based system gathering epidemiological and laboratory information about cases. The database is also accessible by the National Blood Centre and the National Transplant Network in order to implement measures on blood and transplants safety in a timely manner. Since 2013 all asymptomatic confirmed cases of WNV infection in blood donors have also been notified through the web-based system.

The surveillance activities for wild birds and vectors have been strengthened since 2013. Bird surveillance focuses on three aspects: WNV detection in resident target species such as magpies and crows; immunological response among poultry of rural farms, sentinel chickens and migratory birds; and bird mortality. Entomological surveillance includes WNV detection in mosquito pools from affected areas.

There is passive surveillance in equidae with random serological tests in non-endemic areas and monitoring of sentinel horses. However, many horses in Italy are vaccinated against WNV.

A web-based national animal disease notification system allows the notification of animal diseases. The system allows the integration of data from veterinary field services and laboratories into a national database. The database is accessible by different national stakeholders, including the Ministry of Health and the National Blood Centre, and weekly reports are published online [36,37].

United Kingdom

Surveillance activities targeting humans, animals and vector sources have been in place since 2002. Human cases of autochthonous WNV infection should be reported to National Surveillance Centres by the diagnostic laboratories as a matter of urgency. However, the causative organism of just over a third of cases of encephalitis remains undiagnosed [38].

Safeguards are in place to protect the UK blood supply from WNV. These include deferring donation for 28 days from the date of leaving a WNV-affected area, unless WNV nucleic acid test screening is in place to maintain a sufficient blood supply [39].

There is no active surveillance of horses or other equidae. Monitoring relies on passive surveillance and testing of horses with neurological signs. The Animal Health Trust has the responsibility for equine health under a contract from the Department for Environment Food and Rural Affairs (Defra). Syndromic surveillance is carried out and all suspected WNF cases must be reported to the Animal and Plant Health Agency (APHA). In addition, there is an option for private veterinary surgeons (PVS) to submit samples for testing for WNV as a differential diagnosis, which will not trigger an official investigation, as the probability of diagnosis would be considered low. The PVS must discuss the clinical and import history of the horse with APHA, and if WNV is low on the list of differential diagnoses and the owner still requests a test to rule out infection, samples may be taken at the owner's expense. If there is suspicion

of a notifiable disease, this will trigger a disease investigation. All samples must be tested by a UK reference laboratory for WNV.

APHA is responsible for testing WNV-target wild birds (e.g. small *Passeriformes*, corvids and waterside birds) found dead. There is no systematic WNV active surveillance of wild birds in the UK, but a passive surveillance system is in place between April and October. For any of the target wild bird species, birds with neurological signs or large die-offs, that are reported to the Defra helpline or via a warden patrol at specified wild bird reserves, will be collected and tested for a range of avian diseases, which include WNV. Approximately 300 to 400 birds are tested each year.

There is no formal programme for year-round country-wide vector surveillance in the UK. Instead, some targeted surveillance for mosquitoes is carried out by Public Health England (national public health agency) in areas with suitable habitat, to monitor the distribution and abundance of WNV vector species and test for WNV infection. As a result, *Culex modestus* was identified in two counties in the Thames estuary, suggesting that the vector has been endemic in this area since 2010 [40].

Discussion

Surveillance strategies adapted to the countries' epidemiological situations

Human surveillance in Austria, France, Greece and Italy targets early detection of WNV infection cases, and identification of affected areas to implement appropriate response measures: including blood safety measures, vector control and communication to relevant authorities and to the public (Table 2).

In the long term, it aims to quantify the disease burden, identifying seasonal, geographic and demographic patterns of morbidity and mortality. In the UK, where no autochthonous cases of WNV infection have ever been detected, human surveillance focuses on preparedness.

While serosurveys in horses can be used to determine absence/presence of WNV circulation in an area where no or limited data are available, the utility of serosurveys' results is limited by the background level of immunity (acquired by natural infection or by vaccination) of the population. Infection in horses may occur at the same time or even later than the identification of the first human cases [41]. The usefulness of WNV surveillance in equidae for early detection purposes is generally considered to be limited but in countries with irregular WNV outbreaks such as France, the screening of horses is considered by the French authorities to be of added value. Considering that more and more horse owners in affected countries are vaccinating their horses, it is estimated that surveillance in equidae will gradually become irrelevant.

Surveillance of birds and mosquitoes aims at early detection of WNV circulation at the beginning of a new vector season and the identification of areas of virus circulation. This surveillance is used to promptly inform public health authorities. Sentinel domestic birds are easily exposed to the mosquito fauna, handled and monitored over several months, making them suitable targets for WNV surveillance. In domestic pigeons (*Columba livia domestica*) and free-ranging chickens under 5 months old WNV circulation has been detected more than a month before the onset of an epidemic in humans [42,43]. The detection of WNV RNA in mosquitoes has resulted in detection of WNV circulation ca 2–5 weeks earlier than serological monitoring of sentinel chickens at equal spatial sampling density [44]. The downside of surveillance in captive sentinel birds and in mosquitoes are the high costs and logistical demands, which make these surveillance options cost effective only for countries that have regular large outbreaks such as Italy.

Countries free of WNV, like the UK, can achieve early warning of increased risk of WNV introduction through suitability mapping; i.e. spatial analytic studies of WNV risk predictors based on the combination of animal, human and environmental data from their own country and neighbouring countries, to identify areas at risk for WNF outbreak should the virus be introduced [45].

There is no one-fits-all surveillance strategy. Each country needs to assess its epidemiological situation and local conditions to identify the integrated approach that best meets its needs.

An integrated approach for West Nile virus surveillance

Surveillance activities are considered integrated when they are coordinated jointly by public health authorities, animal health authorities and authorities in charge of vector surveillance and control, with the aim of reaching a common understanding of threat level and disease activity. Such integration is expected to improve surveillance and monitoring efficiency and to save resources by implementing targeted measures. An integrated approach requires regular exchanges of information between all actors. In order to improve collaboration, the creation of a formal interagency working group supported by the respective ministries was found to be a crucial step (i.e. in Austria, France, Greece, Italy and the UK). Regular meetings, timely sharing of data among the working-group members and the development of a joint information exchange platform are instrumental.

An integrated collection and analysis of data from human, animal and vector surveillance, ideally in a single database, is key to obtain a comprehensive understanding of the epidemiological situation of WNV and consequently timely implement response measures. The modalities of the integrated approach are country-dependent taking into account the local context.

Challenges of the integrated approach and avenues to strengthen intersectoral collaboration

WNF is a notifiable disease in humans and in equidae at the EU level. While for public health ensuring blood safety is a key incentive to allocate sufficient resources for WNF surveillance, on the animal side, the facts that an effective vaccine is available and that most cases remain asymptomatic make the disease less of a priority. In Europe, expertise and resources in vector surveillance and control are variable but not always well integrated with the public and veterinary health sectors. Development of in-country entomology expertise and the provision of sufficient funding are key to develop adequate vector surveillance and control capacities.

The role of wild animals in the WNV transmission cycle makes the implementation of control measures challenging. In addition, as multiple stakeholders from the public health sector, the animal health sector and the environmental sector are involved in the implementation of control measures, coordination becomes complex if there are no clear guidelines and established collaboration arrangements. Setting clear common objectives can overcome some of these challenges and will allow joining resources and expertise.

Conclusions

WNV surveillance is challenging as the virus transmission cycle is complex and most human and animal cases remain asymptomatic, which poses a risk for transmission by blood products. An integrated approach, involving public health, animal health and environmental authorities offers the most efficient and effective mechanism for tackling WNV transmission. Austria, France, Greece, Italy and the UK have implemented different surveillance strategies tailored to their epidemiological situation with different degrees of integration across disciplines and authorities. The examples described here can support other European countries wishing to strengthen their WNV surveillance or preparedness plans and serve as examples for surveillance and monitoring of other (vector-borne) zoonotic infections. The example from the UK shows that even without the presence of the disease an integrated plan can support preparedness.

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Conflict of interest

None declared.

Authors' contributions

CMG coordinated the writing of the manuscript. All authors contributed to the writing of the manuscript and commented on its final version.

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Two consecutive large outbreaks of *Salmonella* Muenchen linked to pig farming in Germany, 2013 to 2014: Is something missing in our regulatory framework?

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In 2013, raw pork was the suspected vehicle of a large outbreak (n = 203 cases) of *Salmonella* Muenchen in the German federal state of Saxony. In 2014, we investigated an outbreak (n = 247 cases) caused by the same serovar affecting Saxony and three further federal states in the eastern part of Germany. Evidence from epidemiological, microbiological and trace-back investigations strongly implicated different raw pork products as outbreak vehicles. Trace-back analysis of *S.* Muenchen-contaminated raw pork sausages narrowed the possible source down to 54 pig farms, and *S.* Muenchen was detected in three of them, which traded animals with each other. One of these farms had already been the suspected source of the 2013 outbreak. *S.* Muenchen isolates from stool of patients in 2013 and 2014 as well as from food and environmental surface swabs of the three pig farms shared indistinguishable pulsed-field gel electrophoresis patterns. Our results indicate a common source of both outbreaks in the primary production of pigs. Current European regulations do not make provisions for *Salmonella* control measures on pig farms that have been involved in human disease outbreaks. In order to prevent future outbreaks, legislators should consider tightening regulations for *Salmonella* control in causative primary production settings.

Introduction

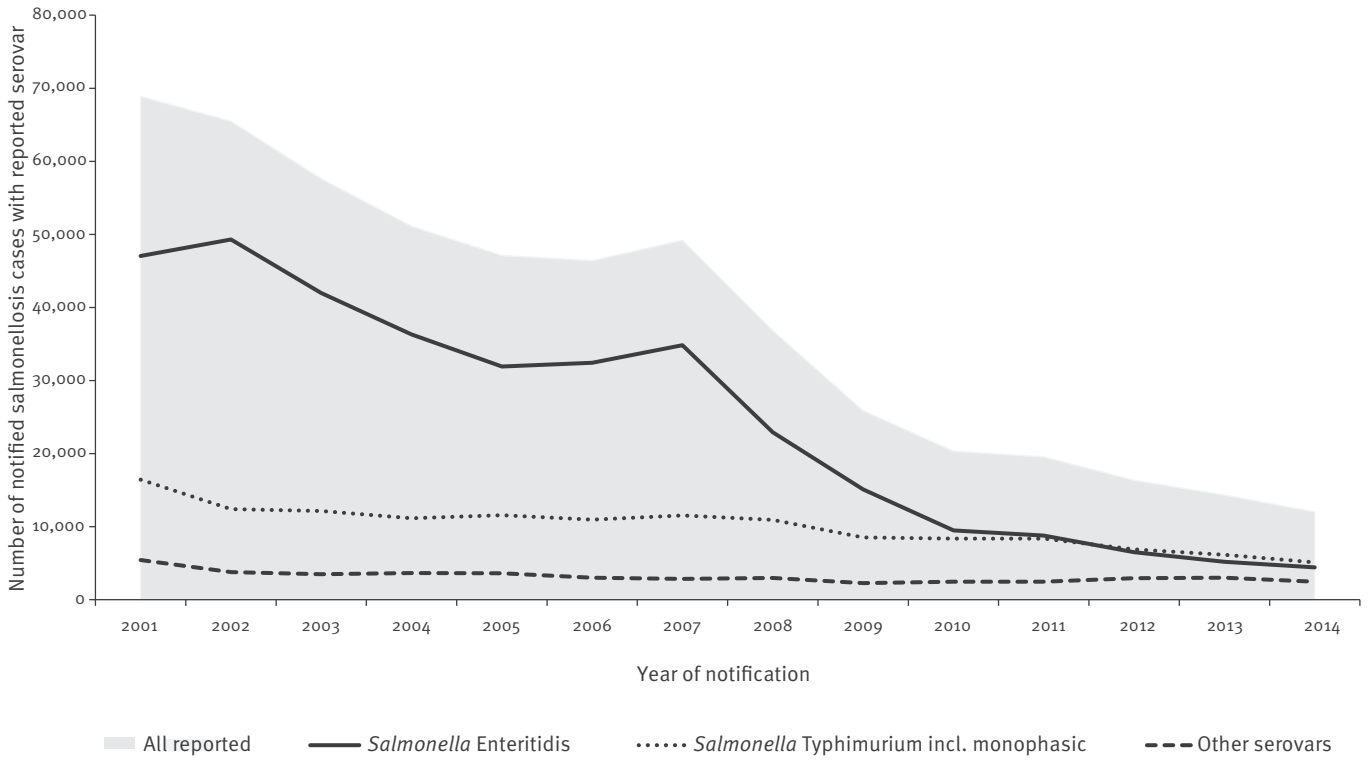
Salmonellosis is a zoonotic enteric disease caused by a multitude of non-typhoidal serological variants of *Salmonella enterica*.

The number of human cases of salmonellosis in Europe reported to the European Centre for Disease Prevention and Control (ECDC) has been declining remarkably since the first report in 1995 [1,2]. This is mainly attributable to a reduction in disease cases caused by *S. enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis), the most prevalent serovar in Europe. Human *S.* Enteritidis infections are primarily attributed to poultry and eggs [3]. Measures regarding hygiene and immunisation of chicks and young hens in broiler chicken production and in laying hens are held responsible for the decline of human cases [2,4].

With the diminishing importance of poultry as source of human salmonellosis in Europe, the relative importance of pig-related salmonellosis has increased. *S.*

Figure 1

Number of notified salmonellosis cases with reported serotype in Germany, 2001–2014



Incl. monophasic: including monophasic variant.

Typhimurium is the second most frequent serovar isolated from salmonellosis cases in Europe [2] and the most prevalent serovar identified in European pigs [4]. The number of human cases reported to the ECDC caused by this serovar has decreased only slightly since 2008. Furthermore, detection of the monophasic variant of *S.* Typhimurium remarkably increased since it was first reported to ECDC in 2010 [2]. Human cases with *S.* Typhimurium and its monophasic variants are primarily related to swine [5].

In Germany, salmonellosis was the most frequently reported bacterial disease until 2005 [6]. Very similar to the situation in Europe, there has been a decreasing trend in salmonellosis in Germany since 1992; mainly due to a reduced incidence of *S.* Enteritidis [7]. However, the number of cases caused by *S.* Typhimurium and by other *Salmonella* serovars has been relatively constant between 2001 and 2014 (Figure 1) [6]. Large salmonellosis outbreaks investigated in Germany in recent years were caused by *S.* Typhimurium or rare serovars. The majority of these outbreaks have been attributed to the consumption of raw pork and products thereof [8-10].
In June 2013, public health authorities in the federal state of Saxony, eastern Germany, investigated a salmonellosis outbreak caused by the rare serovar *S.* Muenchen, which antigenically is a group C2-C3 *Salmonella*. In that state, an annual median of three cases (range: 1–6 cases) had been reported from 2005 to 2012. During the outbreak, between 25 June and 7 August, a total of 203 cases were reported. The median age was 50 years (interquartile range (IQR): 39–62 years) and 56% of cases (113/203) were male. A convenience subset of strains (n = 21) was sent to the National Reference Centre for *Salmonella* (NRC) in Wernigerode where pulsed-field gel electrophoresis (PFGE) analysis suggested that cases were epidemiologically linked (data not shown). Raw pork was the suspected vehicle based on positive tested food specimens. Based on trace-back analysis and the detection of *S.* Muenchen on a pig breeding farm in a routine specimen in temporal relation to the outbreak, this farm was considered as the likely source of the outbreak (data not shown). Measures to stop the outbreak were mainly applied at the level of meat processing addressing severe hygienic deficits identified there.

Almost one year later in June 2014, the public health authority in Brandenburg, a federal state bordering Saxony, informed the Robert Koch Institute (RKI) of an increase in reported *S.* Muenchen infections. At that time, increased case numbers of *S.* Muenchen were also reported from Saxony, Saxony-Anhalt and Thuringia, all states located in eastern Germany. Coincident to the increase of *S.* Muenchen cases, routine or targeted testing detected *S.* Muenchen and/or *Salmonella* type C in different pork products. A possible resurgence

of the salmonellosis outbreak that had occurred in Saxony the year before was hypothesised.

We conducted a multistate inter-sectoral outbreak investigation in 2014 to strengthen or refute the evidence for raw pork or products thereof as vehicles of infection and to identify the source in order to stop the outbreak. A possible connection to the outbreak in 2013 and potential reasons for resurgence were also investigated. The existing legal basis was reviewed to identify possible gaps in the regulatory frameworks that are intended to safeguard consumers against *Salmonella* along the pork food production chain.

Methods

Outbreak case definition

For surveillance in Germany, the national case definition for salmonellosis includes patients presenting with the typical clinical picture of an acute salmonellosis (with at least one of the following symptoms: abdominal pain, diarrhoea, vomiting or fever (›38.5°C)) and either isolation of *Salmonella* spp. (laboratory confirmation) or an epidemiological link to a laboratory-confirmed case, as well as laboratory-confirmed *Salmonella* infections with an unknown or untypical clinical picture.

We defined outbreak cases as persons notified with *S.* Muenchen or *Salmonella* of group C/C2-C3 infections in affected federal states (Brandenburg, Saxony, Saxony-Anhalt, Thuringia) who fulfilled the national surveillance case definition with symptom onset between 26 May 2014 and 03 August 2014 or – if onset dates were missing – with notification weeks 22 to 31 (26 May to 03 August) of the year 2014.

Epidemiological investigations

Case information was analysed daily regarding time, place and person (age, sex, laboratory results and deaths). Information was shared by regular reports and telephone conferences, between the members of the inter-sectoral multistate outbreak team, which involved authorities for human health and food safety.

Staff of local health authorities attempted to interview all adult notified cases (18 years and older) with reported salmonellosis starting from 26 May 2014 using a specifically designed questionnaire. The questionnaire asked about consumption of pork and pork products as well as points of both purchase and consumption of the products, e.g. butcher shops and restaurants. The aim of the interviews, which took place until 14 July 2014, was to identify common points of purchase (at least 2 mentions) to provide possible starting points for trace-back investigations along the food production chain.

Cohort study among staff of a nursing home

We conducted a cohort study among staff of a nursing home with cases among staff and residents, in which raw pork sausages from unopened packages had tested positive for *S.* Muenchen. Participants were asked via an online questionnaire about symptoms, the meal they had participated in (e.g. breakfast or lunch) and the food they had consumed in the canteen of the nursing home in May and June 2014. For this study, cases were defined as staff reporting diarrhoea with symptom onset between 29 May 2014 and 09 June 2014 or detection of *S.* Muenchen or untyped *Salmonella* in a stool specimen taken in May or June 2014. Risk ratios (RR) and corresponding 95% confidence intervals (CI) were calculated.

Investigations by food safety authorities

In order to identify the food vehicle and the source of contamination, food safety authorities conducted risk-based inspections in kitchens of institutional caterers, butcher shops and supermarkets that were possibly involved in the outbreak. During these inspections, specimens of different food items with a focus on pork products and environmental swab samples were taken and analysed. Food specimens that tested positive for the outbreak strain were traced back along the food production chain to their origin (e.g. food business operator producing raw pork sausages) and further to the level of primary production. Food business operators and slaughterhouses identified through trace-back investigations were inspected and sampled. Pig farms identified by trace-back analysis and located in Brandenburg and Saxony were visited to take surface swabs and to collect animal faeces for further testing.

All information generated by the authorities of the federal states on sampling, testing, inspections, and trace-back of positive foods was summarised in situation reports by the Federal Office of Consumer Protection and Food Safety (BVL). Additionally, the collected supply chain information was provided to the central authorities for import into 'FoodChain-Lab', a relational database with integrated consistency and plausibility checks developed at the Federal Institute for Risk Assessment (BfR) [11,12]. This tool was used by the BfR for analysis and visualisation of the investigated supply chains.

Microbiological investigations

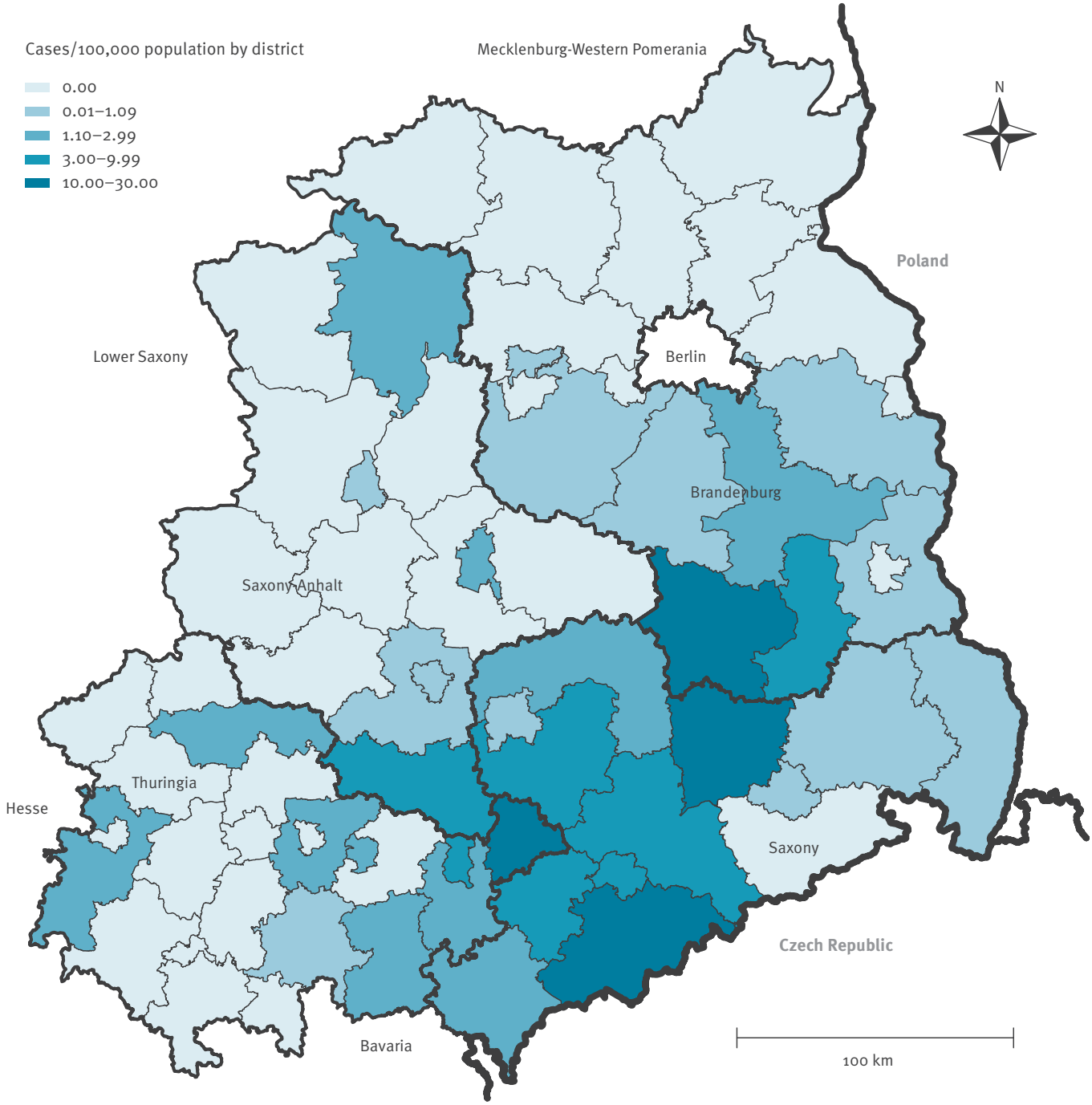
Primary diagnostic laboratories in the affected region were asked to fully serotype all detected *Salmonella* strains from patients and to forward *S.* Muenchen isolates as well as *Salmonella* isolates of group C or C2-C3 to the NRC for *Salmonella* for subtyping using PFGE according to the Pulse-Net protocol [13]. Isolates from different sources (human, food and environment) were compared with each other and to isolates from the salmonellosis outbreak in Saxony in 2013.

Review of legal framework

Finally, we reviewed the existing legal provisions targeting consumer protection against *Salmonella* when eating raw pork products to identify possible gaps in the regulatory framework.

Figure 2

Reported incidence of salmonellosis per district (number of outbreak cases/100,000 population) in four federal states affected by an outbreak, eastern Germany, May–August 2014 (n = 247 cases)



National, federal state and district borders are distinguished on the graph by line thickness, with the thickest lines for national borders, followed by federal state borders and district borders.

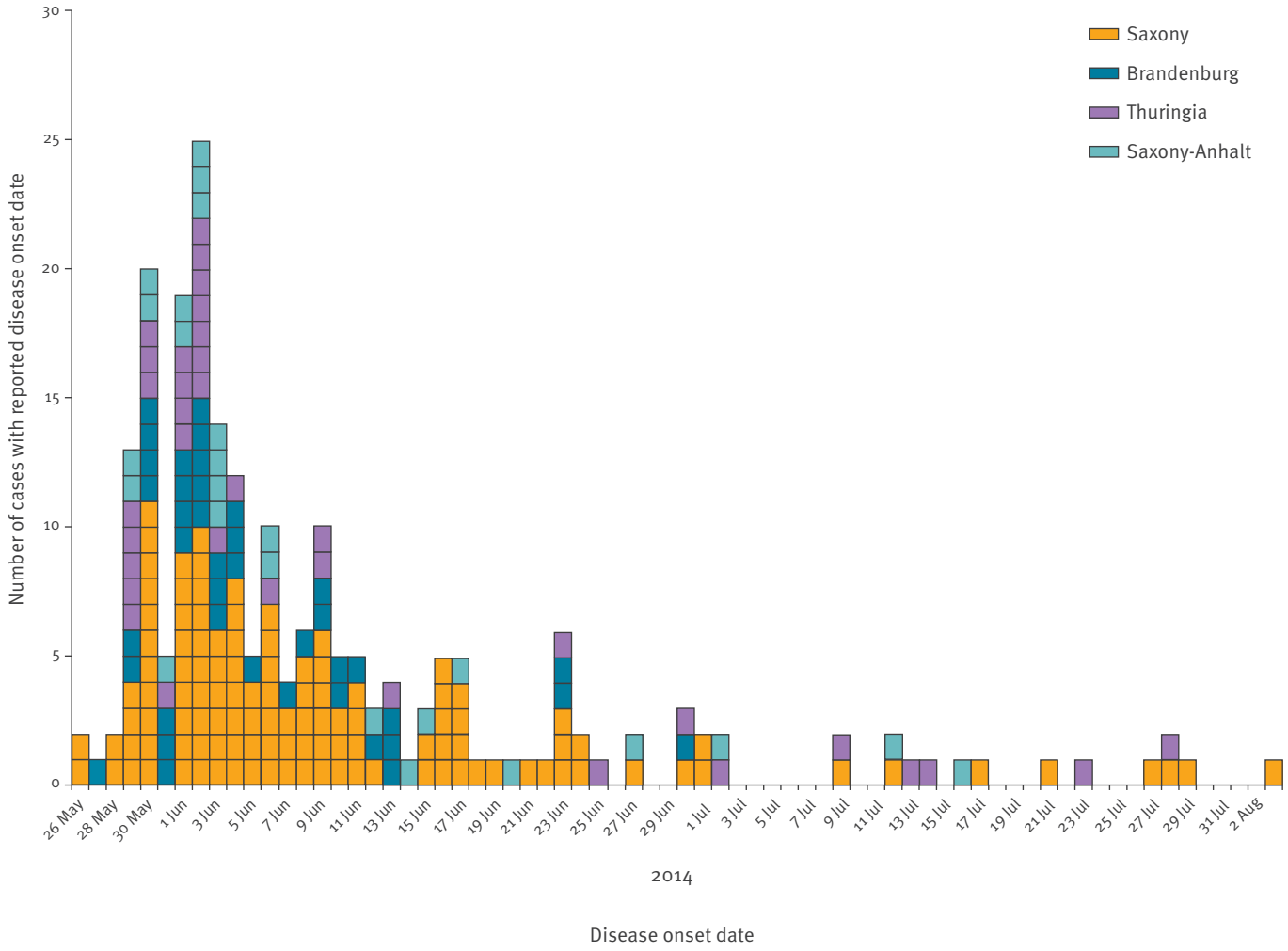
Results

Descriptive epidemiological investigations

In total, 247 notified patients with salmonellosis met the outbreak case definition. Most of them were laboratory-confirmed (n = 237) and of these, 90% (n = 213) were serotyped as *S.* Muenchen, the remaining were typed only to the group level. Most outbreak cases were reported from Saxony (n = 139; 56%). Likewise, districts in or bordering Saxony reported the highest incidence during the outbreak period between 26 May 2014 and 03 August 2014 (11–28 cases/100,000 population) (Figure 2). Median age of the outbreak cases was 56 years (IQR: 42–71 years); 54% were male (n = 133). Of all outbreak cases, 12% (n = 30) were hospitalised after their symptom onset; four patients died (all female, age range 81–93 years). For one of these patients, salmonellosis was reported as cause of

Figure 3

Reported cases with *Salmonella* Muenchen and known date of symptom onset, Germany, 26 May–3 August 2014 (n = 217 cases)



death; for the other three patients, deaths were attributed to causes other than salmonellosis but without any further information. For outbreak cases with a known symptom onset (n = 217), most contracted disease between 29 May 2014 and 23 June 2014 (n = 185; 85%) (Figure 3). At least three nursing homes were affected by the outbreak (two in Saxony and one nursing home in Brandenburg).

Cohort study among staff of a nursing home

Staff of local health authorities interviewed 160 patients with reported salmonellosis. Of these, four did not belong to the outbreak and eight did not give their consent or could not be reached, resulting in 148 questionnaires that could be analysed for consumption of pork, pork products and points of purchase or localities of consumption of the products. Of these 148 patients, 80% (n = 119) reported the consumption of pork and products thereof in the three days before symptom onset, and of these, 85% (n = 101) raw pork consumption. In total, 11 common points of purchase were identified (mainly supermarkets and butcher shops) and the information was forwarded to food safety authorities.

Analytical epidemiological investigations

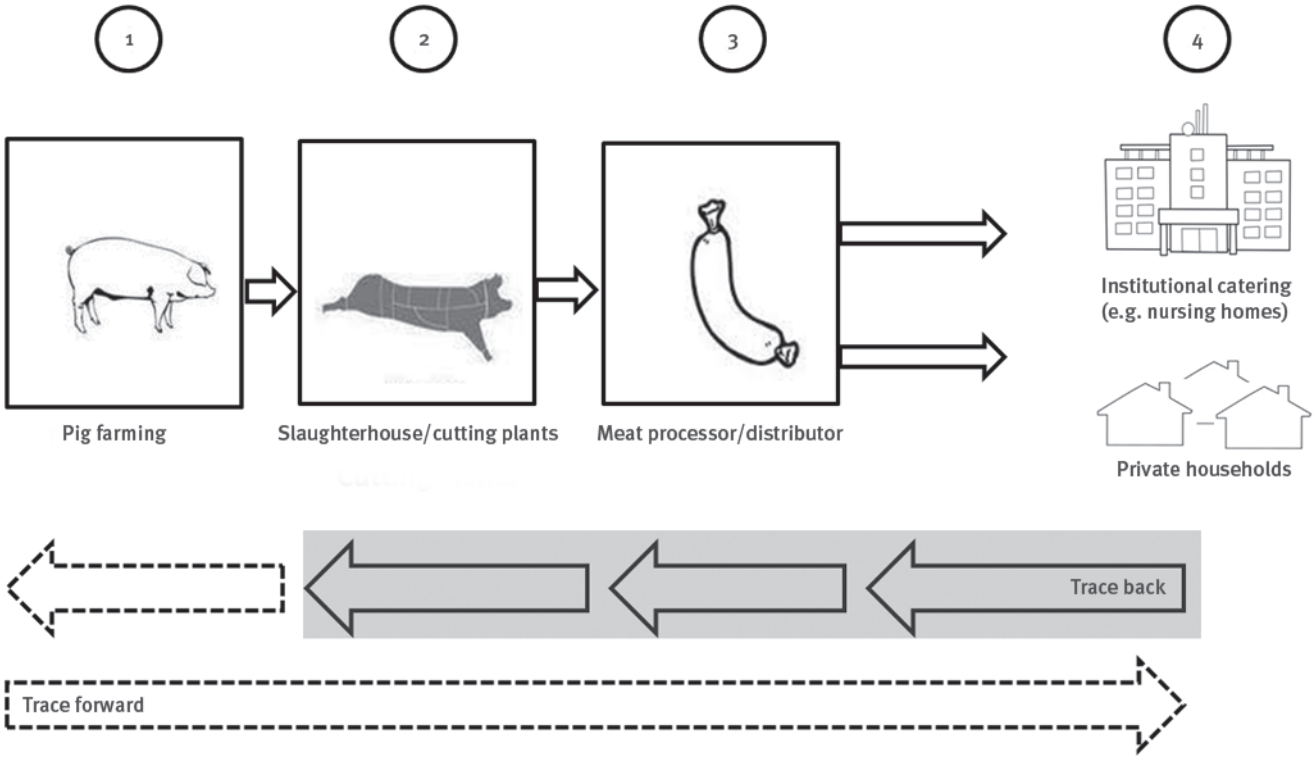
In the cohort study, 27 of 64 staff members of a nursing home affected by the outbreak completed the online questionnaire (response rate: 42%). Of these, six were defined as cases. Median age of participants was 48 years (IQR: 28–64 years) and the majority was female (n = 23 participants). Staff members eating their breakfast in the canteen at work during the time of the outbreak were 10-times more likely to become a case than staff members eating their breakfast elsewhere (RR: 10; 95% CI: 1.4–73; 5 cases among 9 exposed persons (attack rate: 56%) and one case among 18 non-exposed persons (attack rate 6%)). During breakfast, several types of raw pork sausages were served in the canteen during the respective time period.

Investigations by food safety authorities

The hypothesis that raw pork products represented the vehicle of this outbreak was generated at an early stage of the investigation, since *S.* Muenchen was

Figure 4

Schematic overview of the pork food production chain 'from farm to fork'



1 Primary production; 2 Meat productions including slaughterhouses and meat cutting plants (where the carcasses are cut into smaller pieces); 3 Meat processing; 4 Consumers.

The greyish background highlights the stages until where trace-back investigations are usually conducted.

Sources of the images: Fotolia #67699729; Rainer Zenz – own work, https://commons.wikimedia.org/w/index.php?curid=23986913; http://publicdomainvectors.org/de/kostenlose-vektorgrafiken/Vektor-Illustration-fleckig-Wurst/31268.html; http://publicdomainvectors.org/de/kostenlose-vektorgrafiken/Krankenhaus-Geb%C3%A4ude-Linie-Kunst-Vektor-Grafiken/24525.html

detected in two samples taken for routine testing of food to determine microbiological parameters. These were different pork products like minced pork intended for raw consumption, and a raw pork sausage called 'Knacker', also meant to be consumed raw. Additionally, a specimen of brine used for meat preparations tested positive.

During the outbreak investigation, food safety authorities collected further 117 food specimens. Two raw pork sausages, also intended for raw consumption, from unopened packages collected from the kitchen of the affected nursing home where we conducted the cohort study, tested positive for *S.* Muenchen as well ('Braunschweiger' and 'Schinken-Teewurst').

In total, as a result of the routine testing and outbreak investigations, four of 119 food specimens and one specimen of brine tested positive for *S.* Muenchen. In contrast, none of 227 surface swabs taken in kitchens of nursing homes, butcher shops and slaughterhouses tested positive.

Food safety authorities conducted a trace-back investigation starting with the two raw pork sausages from the nursing home that had tested positive for *S.* Muenchen and the minced pork and 'Knacker', which had been sampled during routine investigations and had tested positive for *S.* Muenchen as well. The brine was not further traced back because of probable cross-contamination. The trace-back investigation identified different meat processors and slaughterhouses, which were all visited, sampled and investigated for the presence of *Salmonella* – all tested negative. The slaughterhouses were supplied by 54 pig farms. Of these, 23 pig farms (animal faeces and surfaces in the environment of the animals, e.g. the floor of the pig houses) were tested using boot swabs. Swabs from three of the farms located in Saxony tested positive for *S.* Muenchen and isolates were sent to the NRC (n=8). One of these farms had already been the suspected source of the 2013 outbreak. The three farms belonged to the same owner and were specialised in pig breeding, rearing and fattening, respectively. Pigs were traded among the three farms. The fattening pig farm was subject to

the mandatory German *Salmonella* control programme that involves testing pigs for *Salmonella*-specific antibodies pre-slaughter and had been grouped into category I representing a low prevalence.

Microbiological investigations of human and environmental samples

S. Muenchen was confirmed at the NRC for 143 human isolates from the 2014 outbreak, 52 of which were subtyped. Of those, 47 isolates showed an identical outbreak PFGE pattern (ECDC nomenclature XbaI.1056); four isolates had slightly different band patterns and were also classified as outbreak-related. One isolate was classified as not related to the outbreak.

Furthermore, the outbreak PFGE pattern was detected in the eight isolates derived from the three pig farms, three pork-based food specimens (i.e. the Knacker found during routine testing and the two raw pork sausages found during the outbreak investigation in the nursing home) and the brine. One of the food specimens (minced pork, which was found with *S.* Muenchen during routine testing) showed the same pattern variation as the outbreak-related human strains mentioned above. Remarkably, the outbreak PFGE pattern had already been identified for human and minced pork isolates during the 2013 outbreak. The NRC-PFGE-database of *S.* Muenchen contains 59 PFGE patterns derived from altogether 218 isolates from humans, food, and animals between 2000 and 2014. The outbreak pattern had been detected before 2013 but only in single isolates from sporadic cases.

Review of legal framework

We identified European Union (EU) regulations, as well as German national laws and recommendations aiming to form a multi-barrier to protect consumers from *Salmonell*a with provisions at the different stages of the pork production chain, from farm to fork (Figure 4).

Stage 1 (primary production)

Regulation (EC) No 2160/2003 provides the general framework for the control of food-borne zoonotic agents in the EU demanding the establishment of targets for the reduction of prevalence of specified zoonoses and zoonotic agents (e.g. for all *Salmonella* serotypes with public health significance in breeding herds of pigs) [14]. In Germany two regulations are implemented regarding *Salmonella* in pigs at primary production: the pig production hygiene regulation ('Schweinehaltungshygieneverordnung', SchHaltHygV) [15] and the pig *Salmonella* ordinance ('Schweine-Salmonellen-Verordnung', SchwSalmoV) [16]. The former generally describes hygienic requirements for pig farms, specifying that perished or certain sick animals have to be tested for the causative agent and epidemiological investigations have to be conducted to identify the cause. The pig *Salmonella* ordinance mandates the routine testing of fattening pigs for the presence of *Salmonella*-specific antibodies. Pig farms are categorised based on the resulting seroprevalence into three categories with category I denoting pig farms with the lowest seroprevalence (0–20%). Pig farms of category II show seroprevalences between 21% and 40%. Measures to reduce seroprevalence on the farm are only obligatory for pig farms belonging to category III with a seroprevalence of more than 40%.

Regulation (EC) No 178/2002 [17] lays down general requirements for food safety in Europe, but basically does not apply to pig farms because it does not define food producing animals as food. The German national law concerning The Foods, Consumer Goods and Feedstuffs Code ('Lebensmittel-Bedarfsgegenstände- und Futtermittelgesetzbuch', LFGB) [18] aligns with that definition.

Stage 2 (slaughterhouse)

At the stage of the slaughterhouse, process hygiene criteria stated in Regulation (EC) No 2073/2005 [19] apply. This regulation specifies that when more than 6% of a minimum of 50 tested swine carcasses (3/50) are found positive for *Salmonella* using cultural techniques, improvements in slaughter hygiene have to be taken, and process controls, the origin of animals, and biosecurity measures on the farms of origin have to be reviewed.

Stage 3 (meat processor/distributer)

Minced meat, meat preparations and meat products intended to be eaten raw (food safety criteria, also Regulation (EC) No 2073/2005), are not allowed to contain *Salmonella* [19]. Five pooled specimens of at least 25g each taken on one day per week have to test negative using cultural techniques.

Stage 4 (consumer)

To protect especially vulnerable population groups, the BfR recommends that raw pork and products thereof should not be served in institutional catering (e.g. nursing homes). This recommendation was published in 2011 [20]. Next to that, the German Institute for Standardisation (DIN) published the DIN standard 10506:2012–3 on 'Food hygiene – Mass catering', which gives detailed advice to caterers providing food for vulnerable persons not to serve raw foods of animal origin such as raw minced meat and raw pork sausages [21].

Discussion

Our epidemiological, microbiological and product tracing investigations suggest that (i) raw pork products were the vehicles in the outbreak of *S.* Muenchen infections in 2014, (ii) the outbreaks in 2013 and 2014 were connected and (iii) the common source was at the farm-level. The negative tested surface swabs taken at slaughterhouses, meat processors and butcher shops in 2014 are compatible with good hygiene practices at the stages following primary production.

Raw pork and products thereof are a traditional and popular food in Germany, particularly in the northern

and eastern parts [22]. Consumption of raw pork is also the cardinal risk factor for yersiniosis [22] and likely also important for toxoplasmosis in Germany [23]. Dependent on the region in Germany, different raw pork products are consumed, including, for example, spreadable sausages. These may sometimes not even be recognised by the consumer as containing raw pork, and hence risk awareness regarding consumption of these products is likely low.

Despite national recommendations in Germany to the contrary [20,21], raw pork products were served in at least three affected nursing homes. In one of these homes, the reported reason was that many elderly people strongly requested this traditional food, e.g. spreadable sausages containing raw pork. We also suspected that even when raw pork products were not served in a nursing home, visitors would provide residents with these products. For the other identified institutions, it was unclear whether failure to follow this recommendation was due to deliberate non-compliance or lacking knowledge.

The resource-intensive trace-back investigations, involving food safety authorities from all levels, were pivotal in identifying the three pig farms as likely source of the outbreak in 2014. Despite this and other successes in the recent past [12], product tracing (back and especially forward) investigations are seldom conducted in Germany and Europe. This may be one reason why the source of many food-borne outbreaks is not identified. We advocate conducting product tracing investigations more frequently, especially in the investigation of geographically diffuse food-borne outbreaks. As this outbreak exemplifies, without identifying the source, the risk of further illnesses or outbreaks including deaths may remain. *Salmonella*, often introduced by feed or animal trade [24], may persist on a farm, either in the environment, for example, on surfaces where bacteria may be protected against disinfection in a biofilm matrix, or in the pigs themselves. Intermittent shedding induced by external factors (e.g. change of feed, stress of the animals) or unintentional sloughing of biofilms may then lead to a re-contamination on the farm [25].

To our knowledge, all relevant regulations and laws were followed in the aftermath of the outbreak in 2013. Yet, only one year later the same strain caused another even larger outbreak, including at least one death, showing that the multi-barrier approach to prevent human *Salmonella* infections failed.

When reviewing current provisions to control *Salmonella* in pigs, we identified weaknesses from an infection control point of view. Most notably, competent authorities, such as veterinary control services, have no legal mandate to initiate measures at farms even if they can be linked to human cases. Yet, they should be able to demand mandatory preventive measures at primary production after a food-borne outbreak.

In poultry, measures need to be taken when presence of certain *Salmonella* serovars is suspected. In contrast, presence of *Salmonella*, either antibodies or bacteria, in (asymptomatic) pigs does not require action, at least up to a farm seroprevalence of 40%. The fattening pig farm identified as possible source of the outbreaks had a seroprevalence of less than 20% and was thus grouped into the lowest *Salmonella* prevalence category for pig farms. This case in point adds to the view that antibody prevalence alone inadequately reflects the infection risk posed by pigs [26,27]. Furthermore, the proportion of farms in Germany with low *Salmonella* seroprevalence remained relatively constant or even slightly decreased over time (79% in 2006; 74% in 2016) (personal communication: Sabrina Heß, Qualität und Sicherheit GmbH, Bonn, April 2016) reflecting that the German *Salmonella* control programme failed to notably reduce *Salmonella* prevalence at the farm level. By testing sick or dead animals, as requested by the national legal provisions on pig farm hygiene, *Salmonella* positive pigs may not be detected because human pathogenic *Salmonella* strains mainly cause mild or even asymptomatic infections in pigs [28,29]. Admittedly, control of *Salmonella* in pigs is intricate and eradication of *Salmonella* on pig farms might not be achievable. However, this underscores the need not only for quality control programmes but also for mandatory regulations when farms are linked or suspected to be linked to human salmonellosis cases.

Limitations

Suspicion of raw pork(-products) as vehicles existed early on in this outbreak due to detection of *S.* Muenchen in several pork specimens. Epidemiological investigations served mainly to support the evidence and did not explore other potential vehicles with the same rigour. The validity of the cohort study is somewhat compromised by the rather low participation and identified only a plausible surrogate (breakfast) as a risk factor – not raw pork products themselves. Yet, evidence from different lines of investigations is consistent and points to raw pork products as outbreak vehicles.

Our review of the regulatory framework focused on Germany. Some requirements anchored in EU regulations remain general, allowing for interpretation. Thus, their implementation into national regulations may not be uniform across Europe.

Lessons learnt and recommendations for the future

Raw pork remains a risky, yet frequently consumed food product in Germany that may cause salmonellosis and other infectious diseases, e.g. yersiniosis. Recommendations, e.g. not to serve raw pork(-products) to vulnerable populations, are necessary building blocks of food-borne illness prevention strategies, but are apparently not sufficient to prevent salmonellosis outbreaks caused by pork in Germany. We recommend a survey regarding knowledge, attitude and practices

or an anthropological approach to understand underlying reasons for non-compliance with guidance documents, which might also improve formulation of future recommendations. Only if missing knowledge about existence of the recommendation was the main reason for non-compliance, intensifying tailored communication would be a sensible strategy. Furthermore, legislators should review the existing regulatory framework regarding protection of the consumer against *Salmonella* in raw pork products with a focus on primary production to critically assess where regulations can be tightened to better prevent future outbreaks and to protect the consumer from infectious diseases. Most notably, we recommend that competent authorities, such as veterinary control services, should have a legal mandate to initiate measures at farms if they can be linked to human cases.

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Conflict of interest

None declared.

Authors' contributions

AS, DW, BR, CF and KS drafted the manuscript. AS, DW, CF, BR, KS and AG were the principle investigators at RKI. All of these substantially contributed to collection, analysis and interpretation of descriptive data. AS and DW conducted the cohort study. CF designed the questionnaire about points of both purchase and consumption of pork products; AS and BR analysed and forwarded the data to food safety authorities. WR, RP, AF and SS performed the laboratory investigations. On state level, CS and SW were the principle investigators in Brandenburg, SSc and BA in Thuringia, SSM, RH and MS in Saxony and HO and SP in Saxony-Anhalt. AAW was the main developer of 'FoodChain-Lab' and contributed to analysis and visualisation of the food chain. PL, MS, RH, SW and SP substantially contributed to the review and interpretation of the legal framework regarding Salmonella in pigs and pork. All authors critically revised the manuscript and approved of the final version.

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Passive enhanced safety surveillance for Vaxigrip and Intanza 15 µg in the United Kingdom and Finland during the northern hemisphere influenza season 2015/16

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Enhanced safety surveillance (ESS) was conducted in the United Kingdom and Finland for Vaxigrip and Intanza 15 µg to comply with the European Medicines Agency interim guidance aimed to detect any potential increase in reactogenicity in near real time following the annual update of the influenza vaccine strain composition. This pilot passive ESS was established to strengthen safety monitoring by facilitating spontaneous vaccinee reports and estimating near real-time vaccinee exposure. The primary objective was to estimate the reporting rates of suspected adverse reactions (ARs) occurring within 7 days post vaccination during the northern hemisphere 2015/16 influenza season. Among the Vaxigrip vaccinees (n = 1,012), 32 (3.2%) reported a total of 122 suspected ARs, including 110 suspected ARs that occurred within 7 days post vaccination. Among the Intanza 15 µg vaccinees (n = 1,017), 31 (3.0%) reported a total of 114 suspected ARs, including 99 that occurred within 7 days post-vaccination. These results were consistent with the known safety profile of the two vaccines and did not show any change in reactogenicity or safety concerns. This passive ESS showed improved data reporting and demonstrated its suitability to health authorities' requirements; further fine tuning of the methodology is under discussion between all stakeholders.

Introduction

Influenza is an acute viral respiratory infection that affects 5% to 20% of the global population annually [1]. This rate amounts to ca 25 to 100 million persons each influenza season in Europe. The epidemiology of seasonal influenza has been well characterised, particularly in the northern hemisphere (NH), where the influenza season typically falls between November and April [2].

Vaccination is the only preventive measure for seasonal influenza. As recommended by the World Health Organization (WHO), the current trivalent or quadrivalent marketed influenza vaccines are composed of antigens from two influenza A strains and one or two influenza B virus strain [1]. The recommendation is based on extensive surveillance of influenza strains through the WHO Global Influenza Surveillance network as the influenza strains continue to evolve, causing an antigenic mismatch between the virus strains in the vaccine and the circulating viruses in the subsequent influenza season [3,4]. Consequently, the strain composition of the influenza vaccine is adapted to the epidemiological situation to provide optimal protection for the population.

The European Medicines Agency (EMA) requests annual enhanced safety surveillance (ESS) for all seasonal influenza vaccines. The purpose of this requirement is to rapidly detect a clinically significant change (beyond what was known or expected with the previous vaccine composition) in the frequency and/or severity of reactogenicity (local, systemic or allergic reactions) that may indicate the potential for more serious risks as exposure to the vaccine increases. To avoid false attribution of such a signal to the general intrinsic safety profile of a product, it is recommended that ESS should involve subanalysis of more than one batch [5].

Interim guidance was issued by the Pharmacovigilance Risk Assessment Committee (PRAC) in April 2014 to outline the principles to be followed for improved continuous routine surveillance of influenza vaccines [5]. Experiences and limitations faced during the NH 2014/15 pilot influenza season were discussed between the vaccine marketing authorisation holders (MAHs)

Table 1

Safety report cards distributed for Vaxigrip and Intanza 15 µg vaccinees, by age group, United Kingdom and Finland, 2015/16 (n = 2,029)

Age group	Safety report cards distributed	
	Number	Percentage
Vaxigrip		
6 months to < 6 years	496	49.0
≥ 6 to < 13 years	111	11.0
≥ 13 to < 18 years	19	1.9
≥ 18 to ≤ 65 years	149	14.7
> 65 years	237	23.4
Total Vaxigrip	1,012	100.0
Intanza[a]		
Total Intanza	1,017	100.0

[a] All Intanza 15 µg vaccinees were ≥ 60 years-old.

through a dedicated safety task force within Vaccines Europe (European Vaccines Manufacturers Association within the European Federation of Pharmaceutical Industries and Associations (EFPIA)) and were presented to the EMA/PRAC/Vaccine Working Party in November 2014. By December 2014, the PRAC recommended establishing a passive ESS for the NH 2015/16 influenza season to the MAHs. Thus, a new design was developed to monitor Vaxigrip (intramuscular trivalent split-virion inactivated influenza vaccine) and Intanza 15 µg (intradermal trivalent split-virion inactivated influenza vaccine) reactogenicity that relied on enhanced routine pharmacovigilance early in the influenza season.

In the United Kingdom (UK), Vaxigrip is recommended for adults older than 65 years, risk groups between 18 and 64 years and children between 6 months and 2 years of age. In Finland, Vaxigrip is recommended to be used in children 6 months to 2 years of age and in at-risk groups from 3 to 18 years of age. Children aged 2 to 3 years in Finland and 2 years and older in the UK preferentially receive another influenza vaccine (live attenuated influenza vaccine) per respective national recommendations [6,7]. Intanza 15 µg is only used in the UK and recommended for individuals 60 years and older. Notably, the Vaxigrip trade name in the UK is Inactivated Influenza (split virion) BP vaccine, but it will be referred to as Vaxigrip in this manuscript.

The principle of this passive ESS was to rapidly estimate vaccine usage or coverage (number of vaccinees or doses administered) and to facilitate passive reporting of suspected adverse reactions (ARs) from vaccinees in order to derive AR reporting rates from the same source of population. For these spontaneous reports, causality assessment was not requested from the vaccinee or healthcare professionals (HCPs) and was not performed by the MAH.

The primary objective was to estimate the reporting rates of suspected ARs occurring within 7 days following routine vaccination with Vaxigrip or Intanza 15 µg during the NH 2015/16 influenza season. The secondary objectives were to estimate the reporting rates of suspected ARs occurring within 7 days following routine vaccination with Vaxigrip or Intanza 15 µg according to age group and of serious suspected ARs post vaccination not limited to 7 days. This ESS also aimed to provide reference reporting rates for comparison in the next influenza season (2016/17). As an exploratory objective, a batch analysis would be performed if a signal was detected, whenever possible, to avoid false attribution of the signal to the general intrinsic safety profile of the product.

Methods

Design

This was a multicentre, non-interventional, observational, passive ESS conducted in the UK and Finland to ensure the representativeness of all age groups indicated for each vaccine and the use of at least two different batches. The passive ESS relied on enhanced (facilitated) reporting of suspected ARs by increasing the awareness of vaccinees, through trained HCPs, regarding the importance of reporting suspected ARs post vaccination (especially those occurring within 7 days post vaccination) and by distributing safety report cards (SRCs) that allowed vaccinees to report suspected ARs through a dedicated toll-free telephone number. Near real-time, age-specific, brand-specific influenza vaccination coverage was achieved in addition to near real-time analysis estimating suspected AR reporting rates within 7 days post vaccination during the NH 2015/16 influenza season.

Setting

The passive ESS started on 13 October 2015 for Vaxigrip and 17 October 2015 for Intanza 15 µg and ended when 1,000 SRCs each had been distributed (on 2 December 2015 for Vaxigrip and on 8 December 2015 for Intanza 15 µg). Any reports received outside the ESS period were handled as routine spontaneous reports but were not included in the analysis.

Participants

Vaccinees who received Vaxigrip or Intanza 15 µg in routine practice during the NH 2015/16 influenza season and who accepted the SRC (or their parents, in cases of child vaccinees) were eligible for participation in this ESS. There were no exclusion criteria.

Procedures and data collection method

A paper SRC specific to Vaxigrip or Intanza 15 µg provided the following information to the vaccinee: details regarding the ESS, instructions on how to report suspected ARs, the dedicated local toll-free telephone number, the site identifier, a unique SRC identification number, vaccine brand and batch, vaccination date and name of the treating physician.

Table 2

Summary of suspected adverse reactions by age group, time of onset and brand, United Kingdom and Finland, 2015/16 (n = 2,029)

	Time of onset after vaccination					
	≤7 days		>7 days		Total[a]	
	n	%	n	%	n	%
Vaxigrip (n=1,012)						
Total number of suspected AR	110	10.9	12	1.2	122	12.1
Total number of PRAC AEI	42	4.1	4	0.4	46	4.5
Total number of vaccinees with at least 1 suspected AR	31	3.1	3	0.3	32	3.2
Total number of vaccinees with PRAC AEI	22	2.2	3	0.3	25	2.5
6 months to<6 years (n=496)						
Number of suspected AR	40	8.1	2	0.4	42	8.5
Number of PRAC AEI	20	4.0	1	0.2	21	4.2
Number of vaccinees with at least 1 suspected AR	14	2.8	1	0.2	14	2.8
Number of vaccinees with PRAC AEI	11	2.2	1	0.2	12	2.4
≥6 to<13 years (n=111)						
Number of suspected AR	8	7.2	0	0	8	7.2
Number of PRAC AEI	7	6.3	0	0	7	6.3
Number of vaccinees with at least 1 suspected AR	2	1.8	0	0	2	1.8
Number of vaccinees with PRAC AEI	2	1.8	0	0	2	1.8
≥13 to<18 years (n=19)						
	No data reported for this age group					
≥18 to≤65 years (n=149)						
Number of suspected AR	12	8.0	0	0	12	8.0
Number of PRAC AEI	4	2.7	0	0	4	2.7
Number of vaccinees with at least 1 suspected AR	4	2.7	0	0	4	2.7
Number of vaccinees with PRAC AEI	2	1.3	0	0	2	1.3
>65 years (n=237)						
Number of suspected AR	50	21.1	10	4.2	60	25.3
Number of PRAC AEI	11	4.6	3	1.3	14	5.9
Number of vaccinees with at least 1 suspected AR	11	4.6	2	0.8	12	5.1
Number of vaccinees with PRAC AEI	7	3.0	2	0.8	9	3.8
Intanza[b] (n=1,017)						
Total number of suspected AR	99	9.7	15	1.5	114	11.2
Total number of PRAC AEI	53	5.2	3	0.3	56	5.5
Total number of vaccinees with at least 1 suspected AR	29	2.9	3	0.3	31	3.0
Total number of vaccinees with PRAC AEI	26	2.6	3	0.3	28	2.8

AEI: adverse event of interest; AR: adverse reaction; PRAC: pharmacovigilance risk assessment committee.

[a] Not all numbers add up as vaccinees could report suspected AR in both time intervals.

[b] All Intanza 15 µg vaccinees were ≥ 60 years-old.

Vaccine coverage data were collected at practice level by the HCP/vaccinator(s) on a real-time basis (at least once a day) using an electronic data capture system. Vaccinees were encouraged to report any suspected post-vaccination ARs, especially those occurring within 7 days (although reports of ARs after 7 days were also considered for the analysis). A structured telephone interview was developed to ensure the appropriateness and completeness of data collection when vaccinees called to report suspected ARs.

All events reported spontaneously by vaccinees were considered suspected ARs and were recorded and reported according to Good Pharmacovigilance Practice module VI [8]. All suspected ARs were described. PRAC Adverse Events of Interest (AEIs), as listed in the guidance, were also specifically described [5]. Per protocol, side effects reported by a vaccinee or HCP were considered suspected ARs (unless the reporters specifically stated the events to be unrelated or excluded a causal relationship).

Safety signals were defined per Good Pharmacovigilance Practice Annex I revision 3 [9].

Table 3

Most frequently reported suspected adverse reactions (with reporting rates ≥ 1%) by age group and time of onset, United Kingdom and Finland, 2015/16 (n = 2,029)

Preferred term	Time of onset								
	≤7 days			>7 days			Total		
	n	%	CI	n	%	CI	n	%	CI
Vaxigrip (n=1,012)									
6 months to<6 years (n=496)									
Cough	5	1.0	0.1–1.9	0			5	1.0	0.1–1.9
Pyrexia	7	1.4	0.4–2.4	1	0.2	0.0–1.1	8	1.6	0.5–2.7
Rhinorrhoea	5	1.0	0.1–1.9	1	0.2	0.0–1.1	6	1.2	0.2–2.2
≥6 to<13 years (n=111)									
Vaccination site erythema	2	1.8	0.2–6.4	0			2	1.8	0.2–6.4
≥13 to<18 years (n=19)									
	No data reported for this group								
≥18 to≤65 years (n=149)									
	No suspected AR≥1% of total reported for this group								
>65 years (n=237)									
Cough	3	1.3	0.3–3.7	1	0.4	0.0–2.3	4	1.7	0.5–4.3
Fatigue	2	0.8	0.1–3.0	1	0.4	0.0–2.3	3	1.3	0.3–3.7
Headache	3	1.3	0.3–3.7	1	0.4	0.0–2.3	4	1.7	0.5–4.3
Influenza-like illness	5	2.1	0.3–3.9	0			5	2.1	0.3–3.9
Malaise	3	1.3	0.3–3.7	2	0.8	0.1–3.0	5	2.1	0.3–3.9
Nasopharyngitis	3	1.3	0.3–3.7	0			3	1.3	0.3–3.7
Oropharyngeal pain	2	0.8	0.1–3.0	1	0.4	0.0–2.3	3	1.3	0.3-3.7
Intanza [b] 15 µg (n=1,017)									
Vaccination site pain	10	1.0	0.4–1.6	0			10	1.0	0.4-1.6

AR: adverse reaction; CI: confidence interval.

[a] Not all numbers add up as vaccinees could report suspected AR in both time intervals.

[b] All Intanza 15 µg vaccinees were ≥ 60 years-old.

Population size

The number of SRCs needed to be distributed per brand (n=1,000) was estimated based on the expected AR reporting rate and the ability to detect common or very common ARs. The number of sites (six in Finland and 14 in the UK) was based on the expected volume of vaccinations with Vaxigrip and Intanza 15 µg and their ability to distribute SRCs within a short time period. Age representativeness of the population was ensured through country/site selection (in Finland, only paediatric vaccination centres were selected); nevertheless, the SRC distribution at site level followed routine vaccination practices. The number of vaccinees who would potentially report suspected ARs could only be stimulated but not controlled.

Statistical analysis

The ESS population included all vaccinees who were vaccinated in routine practice with either Vaxigrip or Intanza 15 µg and who received the SRC. No confirmatory hypothesis testing was conducted for the analyses. All analyses were descriptive and were produced using SAS version 9.2. Verbatim ARs were coded with Medical Dictionary for Regulatory Activities terminology (version 18.0) and processed according to routine pharmacovigilance processes.

ESS reporting rates were calculated per brand using the following formula:

ESS reporting rate=(Number of vaccinees reporting ARs within 7 days x 100) / total number of SRCs distributed

Suspected AR reporting rates were estimated per brand using the following method:

Suspected AR reporting rate=(Number of ARs within 7 days x 100) / total number of SRCs distributed

Confidence intervals (CIs) for ESS reporting rates were computed using the Wald method if the AR count was≥5 and using exact method if the AR count was<5.

All suspected ARs (including PRAC AEIs, serious suspected ARs and other suspected ARs) and corresponding AR reporting rates were reported and summarised by vaccine, age groups (Vaxigrip: 6 months to < 6 years;

Table 4

Most frequently reported PRAC adverse events of interest (events reported at least twice) with onset within 7 days, by severity, United Kingdom and Finland, 2015/16 (n = 2,029)

Preferred term	Mild			Moderate			Severe			Unknown			Total		
	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
Vaxigrip (n=1,012)															
Number of vaccinees with PRAC AEI	9	0.9	0.3–1.5	3	0.3	0.1–0.9	5	0.5	0.1–0.9	9	0.9	0.3–1.5	22	2.2	1.3–3.1
Headache	0	0	0	0	0	0	1	0.1	0.0–0.5	4	0.4	0.1–1.0	5	0.5	0.1–0.9
Pyrexia	3	0.3	0.1–0.9	1	0.1	0.0–0.5	2	0.2	0.0–0.7	3	0.3	0.1–0.9	9	0.9	0.3–1.5
Vaccination site erythema	1	0.1	0.0–0.5	1	0.1	0.0–0.5	2	0.2	0.0–0.7	1	0.1	0.0–0.5	5	0.5	0.1–0.9
Intanza 15 µg (n=1,017)															
Number of vaccinees with PRAC AEI	18	1.8	1.0–2.6	2	0.2	0.0–0.7	3	0.3	0.1–0.9	7	0.7	0.2–1.2	26	2.6	1.6–3.5
Malaise	3	0.3	0.1–0.9	0	0	0	1	0.1	0.0–0.5	2	0.2	0.0–0.7	6	0.6	0.1–1.1
Vaccination site erythema	6	0.6	0.1–1.1	1	0.1	0.0–0.5	0	0	0	2	0.2	0.0–0.7	9	0.9	0.3–1.5
Vaccination site pain	9	0.9	0.3–1.5	0	0	0	0	0	0	1	0.1	0.0–0.5	10	1.0	0.4–1.6
Vaccination site pruritus	4	0.4	0.1–1.0	0	0	0	1	0.1	0.0–0.5	0	0	0	5	0.5	0.1–0.9
Vaccination site swelling	5	0.5	0.1–0.9	0	0	0	0	0	0	0	0	0	5	0.5	0.1–0.9

AEI: adverse event of interest; PRAC: pharmacovigilance risk assessment committee.

Note: PRAC AEIs as listed in the guidance were specifically described as follows: Injection site reactions (pain, erythema, pruritus, swelling, induration and ecchymosis) and systemic reactions (fever › 38 °C, headache, malaise, myalgia, shivering, rash, vomiting, nausea, arthralgia, decreased appetite, irritability (for vaccinees younger than 5 years), crying (for vaccinees younger than 5 years), and events indicative of allergic and hypersensitivity reactions including ocular symptoms).

≥ 6 years to ‹ 13 years; ≥ 13 years to ‹ 18 years, ≥ 18 years to ≤ 65 years, and › 65 years; Intanza 15 µg: ≥ 60 years), seriousness (Yes/No), severity (Grade 1 (mild), Grade 2 (moderate), Grade 3 (severe), and unknown per protocol severity definition), and day of onset since vaccination (≤7 and›7 days). A similar analysis was also performed on serious suspected ARs.

The mean number of ARs per vaccinee who reported at least one suspected AR was also calculated. For each brand, weekly reports for signal detection were generated and analysed. A 1-month interim report (1 month after the first SRCs were distributed) and a final report were compiled and submitted to the relevant health authorities. AR reporting rates were calculated and compared with the frequency of the AEIs reported during the NH 2014/15 influenza season clinical trials and with the expected rates based on current product-specific data from the Summary of Product Characteristics (SmPC) [10]. No statistical tests were performed [11].

Ethics

The ESS was conducted in accordance with Good Epidemiological Practice, the European Network of Centres for Pharmacoepidemiology and Pharmacovigilance Guide on Methodological Standards in Pharmacoepidemiology [12,13] and Good Pharmacovigilance Practices [8]. The ESS was submitted to national authorities as required by the local regulations and was approved by national ethics committees.

Results

Exposure data

A total of 1,012 SRCs for Vaxigrip and 1,017 SRCs for Intanza 15 µg were distributed to different age groups in the UK and Finland during the 8-week period from 13 October to 8 December 2015 (Table 1). We also considered in the analysis additional SRCs distributed on the same day the 1,000th SRC was reached.

The ESS covered 21 different batches of Vaxigrip and three different batches of Intanza 15 µg. Approximately half (51%) of the Vaxigrip vaccinees received the same batch; the other half (49%) received Vaxigrip from 20 different batches. Almost all of the Intanza vaccinees (except three vaccinees) received the same batch. Because no safety signal was detected for either Vaxigrip or Intanza 15 µg, no specific batch analysis was conducted.

Vaxigrip safety data

Among the Vaxigrip vaccinees, 32 (3.2%) reported a total of 122 suspected ARs (mean of 3.8 ARs/vaccinee who reported at least one AR), including 110 suspected ARs that occurred within 7 days post-vaccination (Table 2).

The highest reporting rate of suspected ARs occurring within 7 days post vaccination was observed in vaccinees older than 65 years; 11 of these vaccinees reported 50 suspected ARs (4.5 ARs within 7 days/vaccinee who reported at least one AR; Table 2).

Table 5

Comparison of other reactions (not solicited in the northern hemisphere 2014/15 clinical trial) with the Vaxigrip Summary of Product Characteristics, United Kingdom and Finland, 2015/16 (n = 2,029)

Adverse reaction[a]	ESS 2015/16 (≤7 days)		Vaxigrip SmPC (≤7 days)	Frequency[b]		Comparison result
	Age group	Observed frequency per age group	Age group	SmPC	ESS 2015/16	Higher or equal or lower than SmPC
Diarrhoea	6 months to < 6 years	0.2%	6 to 35 months	Very common	Uncommon	Lower
Diarrhoea	≥ 18 years[c]	0.3%	≥ 18 years	Uncommon	Uncommon	Equal
Dizziness	≥ 18 years[c]	0.5%	≥ 18 years	Uncommon	Uncommon	Equal
Influenza- like illness	≥ 18 years[c]	1.3%	≥ 18 years	Uncommon	Common	Higher
Asthenia	≥ 18 years[c]	0.3%	≥ 18 years	Very common	Uncommon	Lower
Sweating increased	≥ 18 years[c]	0.3%	≥ 18 years	Common	Uncommon	Lower

ESS: enhanced safety surveillance; SmPC: summary of product characteristics.

Note: A vaccinee with multiple occurrences of an adverse reaction is counted only once under the applicable system organ class/preferred term.

[a] Only not solicited adverse reactions in the northern hemisphere 2014/15 clinical trial and reported in this ESS are compared with the SmPC and included in this table.

[b] Very common (≥1/10 or≥10%); common (≥1/100 to<1/10 or≥1% to<10%); uncommon (≥1/1,000 to<1/100 or≥0.1% to<1%); rare (≥1/10,000 to<1/1,000 or≥0.01% to<0.1%); very rare (<1/10,000 or<0.01%).

[c] Combined age groups of adult and elderly vaccinees.

There was no obvious distribution pattern in the type of suspected ARs across age groups, with the majority of individual ARs occurring at a frequency of less than 1%. The total number of suspected ARs that occurred at a frequency of 1% or higher are presented by age group and time of onset in Table 3.

One serious suspected AR was reported following Vaxigrip vaccination. A person in their late 70s experienced a chest infection (lower respiratory tract infection, which was considered to be an important medical event) 18 days after vaccination, which started with sore throat, headache, coughing and feeling 'unpleasant' and hot. The vaccinee's medical history included a previous chest infection 2 weeks before the influenza vaccination. The vaccinee was later reported to be recovering from the second chest infection following vaccination.

Overall, 46 suspected PRAC AEIs were reported by 25 vaccinees (1.8 suspected AEIs/vaccinee who reported at least one AR). Of these AEIs, 42 suspected AEIs occurred within 7 days post vaccination (Table 2). The most frequent (n≥2) PRAC AEIs with an onset within 7 days post vaccination are presented by severity in Table 4.

There was no obvious distribution pattern in the type of AEIs, their severity or their frequency observed across age groups for Vaxigrip. All AEIs were considered not serious.

Intanza safety data

Among the Intanza 15 µg vaccinees, 31 (3.0%) reported 114 suspected ARs (3.7 ARs/vaccinee who reported at least one AR), including 99 suspected ARs that occurred within 7 days post vaccination (Table 2).

All of the suspected ARs were non-serious. One vaccinee could not be included in the analysis because of insufficient information to identify the SRC number. This vaccinee had reported the non-serious suspected ARs of cough and pain. The most frequently reported suspected ARs within 7 days post vaccination (those reported by ≥1% of vaccinees) are listed in Table 3.

Overall, 56 suspected PRAC AEIs were reported by 28 vaccinees (2 AEIs/vaccinee who reported at least one AR). Of these AEIs, 53 AEIs occurred within 7 days post vaccination (Table 2). All AEIs were considered non-serious. The most frequent (n≥2) PRAC AEIs with an onset within 7 days post-vaccination are presented by severity in Table 4.

Comparison of the reported frequencies with the reference data from the northern hemisphere 2014/15 enhanced safety surveillance

No increase was noted in the observed AEI frequencies for Vaxigrip or Intanza 15 µg during the NH 2015/16 ESS when compared with the frequencies observed during the NH 2014/15 ESS (data not shown).

Comparison of the reported frequencies with the Summary of Product Characteristics

Vaxigrip

Influenza-like illness (ILI) was found to have a higher reporting frequency in this ESS compared with the Vaxigrip SmPC. ILI was reported by five vaccinees

Table 6

Comparison of other reactions (not solicited in the northern hemisphere 2014/15 clinical trial) with Intanza 15 µg summary of product characteristics, United Kingdom and Finland, 2015/16 (n = 2,029)

Adverse reaction	ESS 2015/16 (≤7 days + >7 days)		Intanza 15 µg SmPC (≤7 days + >7 days)	Frequency[b]		Comparison result
	Age group[c]	Reported frequency per age group	Age group	SmPC	ESS 2015/16	Higher or equal or lower than SmPC
Fatigue	≥ 18 years	0.2%	>60 years	Uncommon	Uncommon	Equal
Sweating	≥ 18 years	0.2%	>60 years	Uncommon	Uncommon	Equal

ESS: enhanced safety surveillance; SmPC: summary of product characteristics.

Note: A vaccinee with multiple occurrences of an adverse reaction is counted only once under the applicable system organ class/preferred term.

[a] Only not solicited adverse reactions in the northern hemisphere 2014/15 clinical trial and reported in this ESS are compared with the SmPC and included in this table.

[b] Very common (≥1/10 or≥10%); common (≥1/100 to<1/10 or≥1% to<10%); uncommon (≥1/1,000 to<1/100 or≥0.1% to<1%); rare (≥1/10,000 to<1/1,000 or≥0.01% to<0.1%); very rare (<1/10,000 or<0.01%).

[c] Combined age groups of adult and elderly vaccinees.

(2.1%; 95% CI: 0.3–3.9%) older than 65 years but was not reported by vaccinees aged 18–65 years, which led to a combined ILI reporting rate of 1.3%. This observed frequency was slightly higher than the 'uncommon' (≥0.1% to<1%) frequency for ILI in the groups of adults and elderly people in the SmPC. However, the slightly higher reporting rate observed was not considered clinically relevant upon medical review. The other suspected ARs had frequencies lower than or equal to the SmPC frequencies (Table 5).

Intanza 15 µg

Fatigue and sweating (hyperhydrosis) were reported following Intanza vaccination, and the reported frequencies in the ESS were similar to those referenced in the SmPC (Table 6).

Discussion

In this ESS, vaccinees were encouraged to report any suspected ARs that they experienced, with an emphasis on those occurring within 7 days post vaccination. Hence, the reporting of suspected ARs was stimulated but remained spontaneous in nature (i.e. not solicited). We observed higher reporting rates when spontaneous notification was stimulated (3.2% for Vaxigrip and 3.0% for Intanza 15 µg) compared with reporting rates in routine pharmacovigilance (passive spontaneous non-stimulated system). Spontaneous reporting rates after seasonal influenza vaccination range from 20 to 90 reports per 1,000,000 people vaccinated [14-19]. Passive ESS has been shown to increase reporting rates two- to fivefold when switched from routine pharmacovigilance [20,21].

This study was executed in a time-efficient manner. Approximately 1.5–2 hours per HCP were dedicated to protocol training, processes to be used, management of vaccinees, site management and the end of the ESS process, depending on staff involved. The contact centre needed ca 15 min per vaccinee to record the suspected AR. However, information on the time spent per vaccinee by the HCP to explain the ESS, distribute the SRCs, and explain how ARs were to be reported was not collected as part of this ESS.

The strengths of this ESS were that the number of SRCs distributed was consistent with the estimated sample size and that weekly analyses were performed, which allowed for near real-time investigation of the reactogenicity of Vaxigrip and Intanza 15 µg. The safety reports received were well documented in terms of exposure data (brand, batch and date of vaccination), which is not always the case with routine pharmacovigilance. The overall reporting rates for the two products were of the same order of magnitude. By considering two countries and using a thorough site selection process, we were able to gather data across all age groups as recommended by the guidance, including data in paediatric age groups, over-represented compared with paediatric routine coverage rate.

Overall, the mean numbers of suspected ARs per vaccinee who reported at least one AR within 7 days post-vaccination were 3.8 for Vaxigrip and 3.7 for Intanza 15 µg, ranging between 0 (for vaccinees in the 13–18 years age group owing to the small number of SRCs distributed) and 13 (for vaccinees older than 65 years). The higher average number of suspected ARs in the group of elderly people could be due to the well-known correlation between increasing age and AR reporting rate. Frailty, medical history and concomitant use of medication are common causes of this phenomenon [22]. No obvious distribution pattern in the type and frequency of suspected ARs was observed across age groups for either vaccine.

All of the reported ARs were non-serious, except for one serious AR reported after Vaxigrip vaccination. The passive ESS results do not raise any concerns about the safety of Vaxigrip and Intanza 15 µg. None of the observed frequencies of AEIs in the current ESS were above the frequencies observed during the NH 2014/15

clinical study [11]. No safety issues were observed, and the safety profile of the two vaccines was consistent with what is known for both products. Per EMA interim guidance, data was to be generated from at least two batches of the vaccines. This requirement was fulfilled for Vaxigrip but was not feasible for Intanza 15 µg owing to the fragmented market share.

The passive ESS had the following potential limitations: firstly, there was no control over the actual reporting (under-reporting was still possible) or the timing of a suspected AR report relative to the time since vaccination (suspected ARs that occurred within 7 days could still be reported outside the ESS period). Secondly, the age groups in which the vaccine was used could not be controlled and depended on national recommendations for influenza vaccination, as well as the vaccine coverage rates per age group observed in routine practice. In addition, the choice to conduct the ESS in two countries, with Finland dedicated to the distribution of paediatric SRCs, affected the age group distribution for the SRC. During the ESS, all age groups were represented. However, for Vaxigrip, most of the SRCs were distributed in the age groups 6 months to < 6 years (n = 496) and in the age group older than 65 years (n = 237). Only 19 SRCs were distributed in the age group 13 to 18 years. Therefore, data from this specific paediatric age group were difficult to capture owing to low influenza vaccine coverage. Thirdly, some degree of selection bias may have occurred because vaccinees who accepted the SRC might have reported more (or fewer) ARs than those who refused the SRC. Moreover, HCPs could have preselected the vaccinees to whom the SRC was proposed, even if the instructions were to distribute the SRC on an ongoing basis to all eligible vaccinees. In addition, the vaccinees who received the vaccine early in the season might have been different from those who received the vaccine later in the season. However, this bias is most probably limited, as some sites distributed the SRCs very quickly to large vaccinee groups on days of massive organised influenza vaccinations.

Finally, some operational constraints were faced before initiating this ESS at the site level. In the UK, there was no official start date and influenza vaccination started by the middle of October in the context of ESS at the selected sites. In Finland, the national official start date for the influenza vaccination was 9 November 2015. The start date at the site level for the ESS depended on HCP availability for initiation, contract signature, local practice organisation for seasonal influenza vaccination and different local approval dates. The first site in Finland started SRC distribution on 10 November 2015, immediately after the official national start date for influenza vaccination. The ESS started as closely as possible to the time of the first influenza vaccinations in the selected sites in both countries; however, these start dates may have been some weeks after the first administration of Vaxigrip doses elsewhere in Europe and may have affected the speed at which potential safety issues could have been detected.

The estimated AR reporting rates will provide baseline AR reporting rates to improve comparison during the next NH influenza season (2016/17) using a similar passive methodology. A limitation in the current comparison is that the reference data from ESS NH 2014/15 were obtained from active safety surveillance (clinical trial) and not from spontaneous reporting. The finalisation of the guidance related to ESS is currently under discussion at EMA, and current passive ESS pilot experiences will provide data to further support recommendations. Despite the historic success of immunisation in reducing the morbidity and mortality of several diseases, some public concerns about the safety of vaccines remain. These concerns occasionally erode public confidence in immunisation and sometimes lead to vaccine hesitancy and disease outbreaks. Therefore, enhanced influenza vaccine safety monitoring can contribute to increase public confidence in vaccine safety.

In the absence of a more systemic, centralised, pan-European safety surveillance system, we believe that the passive ESS experience presents a suitable model for enhanced passive surveillance of seasonal influenza vaccines.

Conclusions

The current pilot ESS used a passive approach and showed higher AR reporting rates than previously shown for routine spontaneous reporting. There was no obvious distribution pattern in the type and frequency of suspected ARs for Vaxigrip or Intanza 15 µg. We did not observe any clinically significant changes compared with what is known or expected for either vaccine, nor any safety concerns during the current ESS period. The ESS results have improved data reporting and demonstrated its suitability to health authorities' requirements; further fine tuning of the methodology is under discussion between all stakeholders. A continuous dialogue between the MAHs (through Vaccines Europe) and the European health authorities will help optimise and scale up the ESS system for future seasons.

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Conflict of interest

The study was sponsored and funded by Sanofi Pasteur MSD. HB, ALC, and CS are employees at Sanofi Pasteur MSD.

Authors' contributions

HB and ALC contributed to the design, setup, data analysis, interpretation of results, and outline and manuscript writing.

CS participated in the design and the outline and manuscript review. AS contributed to the statistical analysis plan writing, data analysis, interpretation of results, and outline and manuscript review. TC and TV reviewed the design, participated in the setup and study conduct, and reviewed the content of the manuscript.

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