DOI:10.1111/j.1750-2659.2010.00149.x
www.influenzajournal.com

Original Article

Real time reverse transcription (RRT)-polymerase chain reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A virus infections in pigs

Marek J. Slomka,[a] Anstice L. E. Densham,[a] Vivien J. Coward,[a] Steve Essen,[a] Sharon M. Brookes,[a] Richard M. Irvine,[a] Erica Spackman,[b] Jonathan Ridgeon,[a] Rebecca Gardner,[a] Amanda Hanna,[a] David L. Suarez,[b] Ian H. Brown[a]

[a]Avian Virology Workgroup, Virology Department, Veterinary Laboratories Agency (VLA Weybridge), Surrey, UK. [b]Southeast Poultry Research Laboratory, Athens, GA, USA.
Correspondence: Dr Marek J Slomka, Avian Virology Workgroup, Virology Department, Veterinary Laboratories Agency (VLA-Weybridge), Woodham Lane, Addlestone, Surrey KT15 3NB, UK. E-mail: m.slomka@vla.defra.gsi.gov.uk

Accepted 30 May 2010. Published Online 18 August 2010.

Background There is a requirement to detect and differentiate pandemic (H1N1) 2009 (H1N1v) and established swine influenza A viruses (SIVs) by real time reverse transcription (RRT) PCR methods.

Objectives First, modify an existing matrix (M) gene RRT PCR for sensitive generic detection of H1N1v and other European SIVs. Second, design an H1 RRT PCR to specifically detect H1N1v infections.

Methods RRT PCR assays were used to test laboratory isolates of SIV (n = 51; 37 European and 14 North American), H1N1v (n = 5) and avian influenza virus (AIV; n = 43). Diagnostic sensitivity and specificity were calculated for swabs (n = 133) and tissues (n = 116) collected from field cases and pigs infected experimentally with SIVs and H1N1v.

Results The ''perfect match'' M gene RRT PCR was the most sensitive variant of this test for detection of established European SIVs and H1N1v. H1 RRT PCR specifically detected H1N1v but not European SIVs. Validation with clinical specimens included comparison with virus isolation (VI) as a ''gold standard'', while field infection with H1N1v in swine was independently confirmed by sequencing H1N1v amplified by conventional RT PCR. ''Perfect match'' M gene RRT PCR had 100% sensitivity and 95·2% specificity for swabs, 93·6% and 98·6% for tissues. H1 RRT PCR demonstrated sensitivity and specificity of 100% and 99·1%, respectively, for the swabs, and 100% and 100% for the tissues.

Conclusions Two RRT PCRs for the purposes of (i) generic detection of SIV and H1N1v infection in European pigs, and for (ii) specific detection of H1N1v (pandemic influenza) infection were validated.

Keywords H1N1v, Pandemic H1N1 2009 virus, RRT PCR, swine influenza virus (SIV), validation.

Please cite this paper as: Slomka *et al.* (2010) Real time reverse transcription (RRT)-polymerase chain reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A virus infections in pigs. Influenza and Other Respiratory Viruses 4(5), 277–293.

Introduction

The pandemic (H1N1) 2009 influenza A virus (H1N1v) emerged in April 2009 and was first detected in a cluster of human respiratory cases in Mexico and the USA; initially, the virus could not be subtyped using available molecular tests.[1–3] Genetic and antigenic characterisation of the H1N1v virus showed it to be distinct from current seasonal H1N1 influenza A viruses circulating in humans.[4,5] Rapid spread of H1N1v to other continents and frequent escalating human transmission resulted in an official declaration of a new human influenza A pandemic by the World Health Organization (WHO) on 11 June 2009.[6,7]

Regardless of their host of origin, influenza A viruses usually have the potential to infect and, during dual infections, re-assort in other host species. Pigs are particularly susceptible to infections from other hosts because receptors for influenza A viruses of both human and avian origin are present in their upper respiratory tract.[8,9] Swine influenza viruses (SIVs) circulating in pig herds include H1N1, H3N2 and H1N2 subtypes and these have been observed globally.[10–13] H1N1 SIVs were first isolated in the 1930s,

and phylogenetic studies have shown that these ''classical swine'' isolates were originally closely related to the human H1N1 ''Spanish 'flu''' pandemic influenza virus of 1918, but subsequently these classical H1N1 SIVs evolved into a unique genetic lineage.[11] H3N2 SIVs have also evolved distinctly in pigs following the human ''Asian 'flu''' pandemic of 1968. During the early 1990s, ''avian-like'' H1N1 SIVs became common in European farmed pigs and have replaced classical swine H1N1.[10,11] However, classical swine H1N1 remained present in the USA and many parts of Asia.[10–13] Evolution of SIVs in North America has included emergence of a triple re-assortment H3N2 in 1998 that contained genes of human, avian, and classical H1N1 swine influenza origin genes.[12,13] These triple reassortant viruses have become endemic in North America, and, through additional reassortment events, triple reassortant variants of the H1N1 and H1N2 subtypes have been detected.[5,12,13] In the case of H1N2 SIVs isolated in Europe, the H1 gene is of human H1 seasonal influenza origin.[11,14]

The first instance of infection of pigs with H1N1v was reported in May 2009 in Canada where it was suspected that pigs were infected through contact with infected humans.[15,16] SIVs are known to infect humans[17,18] and poultry,[19–22] while human influenza A viruses are also known to transmit to pigs.[23] Continuing human cases of H1N1v during 2009 sustained veterinary concerns that this virus may become established in pigs, with a degree of concern that this host may then serve as a source for further influenza A reassortment and future zoonotic transmission.[24]

Although swine influenza is not listed as a notifiable disease by the World Organisation for Animal Health (OIE),[25,26] SIV surveillance programmes have been carried out in Europe and North America and have provided valuable epidemiological information.[10–13] These surveillance programmes have been based on conventional testing as recommended by the OIE,[27] using attempted virus isolation (VI) in cell culture and embryonated fowls' eggs (EFEs), followed by typing with defined antisera to identify the haemagglutinin (H) and neuraminidase (N) subtypes using well-established haemagglutination and neuraminidase inhibition tests (HI and NI), respectively.[27]

In recent years, highly sensitive and specific real time reverse transcription polymerase chain reaction (RRT PCR) technology has been exploited by veterinary institutes and reference laboratories to develop and validate appropriate tests for avian influenza viruses (AIVs).[28] These include validated RRT PCR tests for notifiable AI caused by H5 and H7 subtype viruses, following concerns resulting from the spread of H5N1 highly pathogenic (HP) AI in poultry in the Eastern Hemisphere, together with a number of accompanying zoonotic cases. Many veterinary laboratories have already embraced generic AI RRT PCRs that detect all sixteen H subtypes of influenza A, typically through amplifying within the highly conserved M gene, and also as specific assays for AIVs of H5 and H7 subtypes.[28] While public health institutions have recently described RRT PCRs for the detection of H1N1v in the context of the current human pandemic,[29–32] in this study, we describe and validate similar approaches for the detection of H1N1v in pigs. Full genome sequence analysis of the current H1N1v reveals the virus to be putatively of swine origin.[4,5] Segment 7, which includes the matrix (M) gene that encodes the matrix M1 and M2 proteins, appears to be of Eurasian SIV origin, while segment 4, which encodes the H1 haemagglutinin (HA), is related to American classical SIV H1 genes.[4,5] This study outlines the adaptation of an existing M gene RRT PCR assay for the generic detection of SIVs and H1N1v, plus a novel RRT PCR that amplifies within the H1 gene for the specific detection of H1N1v in European pigs.

Materials and methods

Viruses

Ninety-nine laboratory isolates of influenza A viruses were obtained from the influenza virus repository at the Veterinary Laboratories Agency (VLA-Weybridge), grown in 9- to 10-day-old specific-pathogen-free embryonated fowls' eggs (EFEs) and typed using standard protocols.[27,33] These included 51 swine influenza virus (SIV) isolates, 43 avian influenza virus (AIV) isolates plus five H1N1v isolates, which included two from humans and three from pigs (Table 1). These EFE-grown influenza A viruses were diluted at least 100- to 1000-fold prior to RNA extraction to give levels of virus that approximate to those present in clinical specimens.[34] Biological infectivity of EFE-grown influenza A viruses was determined as the median egg infectious dose (EID_{50}) per ml.[35] Fourteen laboratory isolates of other non-influenza A viruses known to infect swine were kindly provided by the Mammalian Virology group at VLA and included: Aujeszky's disease virus, border disease virus (isolate 137/4), bovine viral diarrhoea virus (C24V isolate), four isolates of classical swine fever virus (Alfort, Elsenburg 2006-07, Rilmser C and UK 2000), encephalomyocarditis virus (V12050), haemagglutinating encephalomyelitis virus, porcine circovirus type 2 (isolate 1010), porcine parvovirus (RC 6/06), porcine reproductive and respiratory syndrome virus (Euro strain), porcine respiratory coronavirus (isolate 135328) and Talfan virus.

Field clinical specimens from pigs

Ninety-seven frozen (−70°C) archived respiratory tissues were originally obtained during 1991–2009 from the routine swine influenza surveillance programme in United Kingdom, in which acute respiratory disease of a suspected

Table 1. List of influenza A virus laboratory isolates (n = 99) used to assess (i) M gene RRT PCRs (original avian protocol and two variants) and (ii) H1-118 RRT PCR

Influenza A category	Influenza A subtype	Isolate name	Ct values			
			M gene RRT PCR			"H1-118" RRT PCR
			Original "avian"	"Perfect match"	"Combo"	
Pandemic (H1N1) 2009 influenza, human and swine isolates	H1N1v	A/California/07/09	*30·86*	20·96	*21·03*	20·98
	H1N1v	A/England/195/09	*32·77*	20·78	*23·48*	24·14
	H1N1v	A/swine/Singapore-Q/929/09	*31·02*	23·45	*24·20*	24·90
	H1N1v	A/swine/N Ireland/1012/09	*No Ct*	35·68	*36·59*	35·99
	H1N1v	A/swine/England/P0433/09	*30·09*	22·92	*23·66*	24·40
UK swine (classical) H1N1 isolates	H1N1	A/swine/England/117316/86	23·19	*32·28*	*23·73*	No ct
	H1N1	A/swine/England/604718/96	*26·58*	*24·81*	24·07	No ct
European swine (avian-like) H1N1 isolates	H1N1	A/swine/Finistere (France)/2899/82	*23·94*	22·69	*23·56*	No ct
	H1N1	A/swine/Eire/89/96	24·90	*30·41*	*25·84*	No ct
	H1N1	A/swine/England/195852/92	*27·17*	25·04	*25·32*	No ct
	H1N1	A/swine/England/452670/94	*35·02*	31·61	*34·07*	No ct
	H1N1	A/swine/England/600475/96	*27·81*	25·18	*27·33*	No ct
	H1N1	A/swine/England/604718/96	*30·29*	27·37	*29·66*	No ct
	H1N1	A/swine/England/95953/97	*31·11*	28·08	*30·48*	No ct
	H1N1	A/swine/England/101692/97	*26·30*	23·41	*25·23*	No ct
	H1N1	A/swine/England/108640/97	*29·58*	26·43	*28·31*	No ct
	H1N1	A/swine/England/147452/97	*30·47*	27·53	*29·63*	No ct
	H1N1	A/swine/England/159981/97	*29·01*	26·55	*28·55*	No ct
	H1N1	A/swine/England/167655/97	*30·40*	25·56	*27·00*	No ct
	H1N1	A/swine/England/706565/97	*32·59*	28·76	*30·95*	No ct
	H1N1	A/swine/Belgium/1/98	*23·00*	20·44	*20·84*	No ct
	H1N1	A/swine/Italy/1513-1/98	*29·57*	22·12	*22·59*	No ct
	H1N1	A/swine/Isles de Valles (France)/1455/99	*24·54*	21·33	*22·01*	No ct
	H1N1	A/swine/Brno (Czech Republic)/1/02	*23·09*	22·75	*23·27*	No ct
	H1N1	A/swine/England/1195/07	*27·67*	24·95	*25·87*	No ct
	H1N1	A/swine/England/589/2/07	*28·23*	26·03	*27·02*	No ct
	H1N1	A/swine/England/663/08	*23·27*	21·21	*21·93*	No ct
European swine H1N2 isolates	H1N2	A/swine/England/410440/94	*27·30*	24·69	*26·55*	No ct
	H1N2	A/swine/England/448813/94	*29·33*	26·77	*28·72*	No ct
	H1N2	A/swine/England/17394/96	*26·35*	23·91	*26·03*	No ct
	H1N2	A/swine/Cotes d'Armor (France)/790/97	*24·36*	23·12	*23·88*	No ct
	H1N2	A/swine/Italy/1521/98	*23·83*	22·55	*23·25*	No ct
	H1N2	A/swine/England/053307/00	*27·07*	24·17	*25·98*	No ct
	H1N2	A/swine/England/997/08	*29·63*	27·16	*28·07*	No ct
	H1N2	A/swine/England/3/09	*23·62*	21·26	*21·87*	No ct
European swine (human-like) H3N2 isolates	H3N2	A/swine/Cotes d'Armor (France)/3633/84	*26·28*	25·22	*25·98*	No ct
	H3N2	A/swine/England/285044/93	*25·24*	23·44	*24·15*	No ct
	H3N2	A/swine/England/502321/94	*25·96*	24·04	*25·02*	No ct
	H3N2	A/swine/England/742104/95	*24·94*	20·76	*22·02*	No ct
	H3N2	A/swine/Flanders (Belgium)/1/98	*23·07*	20·21	*20·89*	No ct
	H3N2	A/swine/Italy/1477/96	*22·99*	21·42	*22·09*	No ct
	H3N2	A/swine/Italy/1523/98	*24·83*	23·49	*24·19*	No ct
North American swine flu isolates	H1N1	A/swine/Indiana/1726/88	*20·18*	*21·22*	20·09	No ct*
	H1N1	A/swine/Wisconsin/H04YS2/04	*23·09*	*31·80*	22·50	28·18*
	H1N1	A/swine/Ontario/11112/04	*23·80*	*34·88*	22·97	No ct*
	H1N1	A/swine/Minnesota/02011/08	*21·99*	*22·98*	21·13	No ct*
	H1N1	A/swine/North Carolina/02084/08	*23·58*	*24·25*	22·81	No ct*
	H1N1	A/swine/Nebraska/02013/08	23·21	*24·64*	*23·87*	28·36*
	H1N1	A/swine/Minnesota/02093/08	*22·57*	*23·70*	21·77	No ct*
	H1N2	A/swine/Indiana/9K035/99	*25·42*	*32·88*	24·35	21·93*
	H1N2	A/swine/Ontario/48235/04	*24·60*	*33·51*	24·49	No ct*
	H3N2	A/swine/Iowa/8548-1/98	*22·29*	*22·07*	20·54	No ct
	H3N2	A/swine/Minnesota/593/99	*28·22*	*35·98*	27·00	No ct
	H3N2	A/swine/Wisconsin/H02AS8/02	*26·17*	*34·09*	25·09	No ct
	H3N2	A/swine/North Carolina/307408/04	*22·78*	*23·62*	21·69	No ct
	H3N2	A/swine/Ontario/33853/05	*21·23*	*29·12*	20·07	No ct

Table 1. (Continued)

Influenza A category	Influenza A subtype	Isolate name	Ct values			
			M gene RRT PCR			"H1-118" RRT PCR
			Original "avian"	"Perfect match"	"Combo"	
Avian influenza viruses, with highly pathogenic (HP) H5 and H7 isolates indicated	H1N1	A/duck/Alberta/35/76	26·71	*30·65*	*28·61*	No ct
	H1N1	A/turkey/Netherlands/07014290/07	22·93	*29·81*	*24·51*	No ct
	H1N1	A/turkey/Netherlands/07016245/07	22·68	*29·62*	*24·45*	No ct
	H1N1	A/mallard/Germany/R355/07	23·79	*27·88*	*25·85*	No ct
	H1N1	A/mallard/Italy/357-24/07/08	23·34	*26·74*	*25·29*	No ct
	H1N1	A/Egyptian goose/Germany/R1419/06/07	24·82	*28·76*	*26·69*	No ct
	H1N1	A/wild duck/Germany/R30/06/07	22·85	*27·13*	*24·39*	No ct
	H1N1	A/teal/N Ireland/784/07	24·43	*29·04*	*26·34*	No ct
	H1N2	A/wigeon/England/4/06	26·01	*30·90*	*28·25*	No ct
	H2N3	A/duck/Germany/1215/73	29·21	*32·77*	*31·23*	No ct
	H2N3	A/mallard/England/7277/06	32·27	*36·06*	*34·30*	No ct
	H3N2	A/turkey/England/69	27·35	*30·77*	*29·23*	No ct
	H3N2	A/duck/Malaysia/F11107/02	30·66	*33·66*	*33·05*	No ct
	H4N6	A/duck/Czechoslovakia/56	29·46	*32·90*	*31·20*	No ct
	H4N6	A/duck/Italy/473/07	22·52	*22·65*	*22·72*	No ct
	H5N2	A/wild birds/Denmark/04	33·77	*38·09*	*35·44*	No ct
	H5N1	A/turkey/England/614/07 HP	34·85	*37·23*	*35·39*	No ct
	H5N3	A/teal/England/06	36·52	*36·94*	*39·03*	No ct
	H5N1	A/turkey/England/50/92 HP	35·17	*39·10*	*35·93*	No ct
	H6N2	A/teal/England/7440/06	29·24	*33·30*	*30·86*	No ct
	H6N8	A/duck//Denmark/883/02	28·55	*32·73*	*30·56*	No ct
	H7N1	A/African starling/Q-England/983/79	27·31	*31·25*	*29·23*	No ct
	H7N2	A/psittacine/Italy/1384//91	25·10	*28·60*	*26·86*	No ct
	H7N7	A/chicken/England/08 HP	34·98	*No ct*	*36·70*	No ct
	H7N7	A/mallard/Sweden/08	36·86	*No ct*	*No ct*	No ct
	H7N1	A/chicken/Italy/99 HP	29·27	*32·18*	*30·50*	No ct
	H8N4	A/turkey/Ontario/6118/68	28·84	*32·60*	*30·71*	No ct
	H8N4	A/teal/England/06	33·74	*37·04*	*36·03*	No ct
	H9N2	A/chicken/Pakistan/99	29·13	*No ct*	*31·15*	No ct
	H9N1	A/teal/N Ireland/07	35·34	*No ct*	*36·34*	No ct
	H9N2	A/goose/England/07	20·58	*23·03*	*21·43*	No ct
	H10N7	A/chicken/England/279/01	30·67	*34·58*	*32·89*	No ct
	H10N7	A/mallard/England/England/7495/06	29·72	*34·03*	*31·95*	No ct
	H11N6	A/duck/England/56	28·87	*33·23*	*30·80*	No ct
	H11N3	A/duck broiler/Singapore/F107/05/02	29·09	*33·00*	*30·81*	No ct
	H12N5	A/duck/Alberta/60/76	26·84	*30·83*	*28·79*	No ct
	H12N2	A/duck/Belgium/10157/07	*19·76*	19·46	*19·83*	No ct
	H13N6	A/gull/Maryland/704/77	30·04	*39·67*	*31·85*	No ct
	H13N6	A/herring gull/Finland/Li9875/05	28·34	*36·33*	*29·83*	No ct
	H14N6	A/Mallard/Gurjev/244/82	33·54	*36·92*	*35·66*	No ct
	H15N6	A/shearwater/Western Australia/79	28·44	*37·09*	*29·91*	No ct
	H16N3	A/gull/Denmark/68110/02	29·53	*No ct*	*31·30*	No ct
	H16N3	A/gull/Sweden/03	32·40	*No ct*	*34·38*	No ct

For (i) the Ct values for the most sensitive M gene RRT PCR for a given isolate is shown in normal type, while Ct values for the two less sensitive M gene RRT PCR variants are shown in italic type.
*These samples produced bands when the amplified products were run on a 3% w/v agarose gel.

viral aetiology forms the primary submission selection criterion. All were tissue pools that included varying proportions of trachea and lung specimens. Virus isolation (VI) in EFEs at the time of submission divided these into 31 and 66 tissue specimens from pigs that were infected with SIV (Table 2) and uninfected, respectively. In addition, 104 nasal swabs from pigs in United Kingdom were collected by the VLA Regional Laboratories at Thirsk and Bury St Edmunds during the summer of 2009. These were obtained from swine that had been submitted for endemic disease investigations.

Thirty-nine clinical specimens were received between September and November 2009 from nine pig herds at nine different locations in six countries and included 24 respiratory swabs and 15 respiratory tissues (Table 3). Fifteen of these specimens (six swabs and nine tissues) were shown to be

Table 2. List of archived tissue specimens collected in UK (n = 31) from pigs that were VI positive at the time of submission

Influenza A category	Influenza A subtype	Isolate name	Ct values				VI result (+/−) during current validation
			M gene RRT PCR			H1-118 RRT PCR	
			Original ''avian''	''Perfect match''	''Combo''		
UK pigs positive for avian-like swine H1N1	H1N1	A/swine/England/205200/92	*34·63*	34·21	*35·72*	No ct	–
	H1N1	A/swine/England/0204212/92	*30·95*	28·80	*30·61*	No ct	–
	H1N1	A/swine/England/0377301/93	*29·12*	27·96	*28·98*	No ct	–
	H1N1	A/swine/England/0281321/93	*38·04*	36·78	*No* ct	No ct	–
	H1N1	A/swine/England/0280326/93	No ct	No ct	No ct	No ct	–
	H1N1	A/swine/England/0378916/93	*25·60*	24·27	*25·27*	No ct	–
	H1N1	A/swine/England/020883/93	*27·42*	24·25	*25·96*	No ct	–
	H1N1	A/swine/England/0359774/93	*38·36*	34·65	*35·50*	No ct	–
	H1N1	A/swine/England/0281319/93	*36·13*	35·09	*35·90*	No ct	–
	H1N1	A/swine/England/079270/96	*32·42*	31·27	*32·57*	No ct	–
	H1N1	A/swine/England/076235/96	*22·20*	21·10	*21·76*	No ct	+
	H1N1	A/swine/England/108640/97	*32·82*	31·65	*33·01*	No ct	–
	H1N1	A/swine/England/095953/97	*28·33*	27·21	*28·29*	No ct	–
	H1N1	A/swine/England/101692/97	*19·39*	18·45	*19·56*	No ct	–
	H1N1	A/swine/England/113833/97	*27·97*	26·42	*27·63*	No ct	–
	H1N1	A/swine/England/379/08	*31·96*	29·63	*30·81*	No Ct	–
	H1N1	A/swine/England/471/08	*35·39*	32·43	*33·60*	No Ct	–
	H1N1	A/swine/England/57455/08	*No Ct*	35·43	*37·11*	No Ct	–
UK pigs positive for swine H1N2	H1N2	A/swine/England/410439/94	*24·42*	23·10	*24·09*	No Ct	–
	H1N2	A/swine/England/645913/96	*25·97*	24·18	*25·33*	No Ct	–
	H1N2	A/swine/England/661264/97	No Ct	No Ct	No Ct	No Ct	–
	H1N2	A/swine/England/997/08	*22·50*	20·12	*20·90*	No Ct	+
	H1N2	A/swine/England/3/09 (lung)	*38·11*	36·23	*37·47*	No Ct	–
	H1N2	A/swine/England/3/09 (trachea)	*No Ct*	37·39	*37·98*	No Ct	–
UK pigs positive for human-like swine H3N2	H3N2	A/swine/England/119404/91	33·65	*34·30*	*35·12*	No Ct	–
	H3N2	A/swine/England/241009/92	*30·11*	28·76	*29·69*	No Ct	–
	H3N2	A/swine/England/266546/93	No Ct	No Ct	No Ct	No Ct	–
	H3N2	A/swine/England/285044/93	*32·97*	30·64	*31·65*	No Ct	–
	H3N2	A/swine/England/399890/93	*29·22*	28·00	*28·65*	No Ct	+
	H3N2	A/swine/England/399892/93	*No Ct*	35·89	*36·03*	No Ct	–
	H3N2	A/swine/England/502321/94	*25·47*	22·44	*23·18*	No Ct	+

Archived tissues were pools that contained varying proportions of respiratory tissues, including trachea and/or lung, except for A/swine/England/3/09 where two distinct tissues were tested from the same pig. These samples were used to assess (i) M gene RRT PCRs (original avian protocol and two variants) and (ii) H1-118 RRT PCR. Ct values for the most sensitive M gene RRT PCR for a given tissue specimen is shown in normal type, while Ct values for the two less sensitive M gene RRT PCR variants are shown in italic type.

positive for H1N1v by non-RRT PCR approaches (Table 3), i.e. amplification of RNA extracted from the clinical specimen by conventional RT PCR using primers that had been designed specifically for the HA gene of current H1N1v isolates, available at: http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf

Amplicons were electrophoresed in 2% agarose and stained with RedSafe™ (iNtRON Biotechnology, Kyungki-Do, Korea) for visualisation, excised and purified from agarose using the QIAquick® Gel Extraction Kit (Qiagen, Crawley, UK). Nucleotide sequencing was performed using the Big Dye® Terminator v3·1 cycle sequencing kit (Applied Biosystems, Warrington, UK), and checked by BLAST analysis at: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome=blasthome, which confirmed these 15 specimens as H1N1v positive. In the case of four of these 15 H1N1v specimens, virus was grown in EFEs (Table 3) and RNA extracted from infective allantoic fluid, then similarly PCR amplified and sequenced to provide confirmation of H1N1v infection.

Table 3. Examination of clinical specimens derived from field cases of H1N1v-infected pigs using RRT PCR assays

Country of origin	Clinical Specimen (numbers)	Ct values of RRT PCR		Confirmatory criterion for H1N1v infection in herd
		''Perfect match'' M gene RRT PCR	H1-118 RRT PCR	
Singapore (Quarantine)	Nasal swab (1)	25·84	25·50	Sequencing and BLAST confirmation as H1N1v for both clinical specimens and egg-isolated virus
	Tracheal swab (1)	26·05	25·99	
N Ireland (a)	Lung (4)	21·48	22·34	Sequencing and BLAST confirmation as H1N1v for all four clinical specimens
		20·84	20·89	
		28·29	29·05	
		23·99	24·33	
N Ireland (b)	Lung (1)	30·26	30·20	Sequencing and BLAST confirmation as H1N1v for clinical specimen and egg-isolated virus
Norway	Nasal swabs (12)	23·04	23·14	Sequencing and BLAST confirmation as H1N1v for two swabs
		20·64	20·74	
		26·67	26·40	
		26·08	26·02	
		No Ct	No Ct	
		34·14	34·66	
		27·97	28·15	
		32·23	32·48	
		28·84	28·45	
		28·71	29·00	
		24·15	23·80	
		29·83	29·55	
Ireland	Lung (4)	23·71	24·24	Sequencing and BLAST confirmation as H1N1v for two lung specimens
		22·21	22·69	
		26·77	27·22	
		24·98	25·31	
N Ireland (c)	Lung (2)	37·50	37·93	Epidemiologically linked to other cases of H1N1v in swine in N Ireland & Ireland
		33·17	32·84	
Iceland	Nasal swabs (10)	30·64	29·92	Sequencing and BLAST confirmation as H1N1v for swab
		27·62	28·02	
		27·60	27·87	
		29·00	29·04	
		25·98	26·18	
		24·09	24·20	
		26·41	26·40	Sequencing and BLAST confirmation as H1N1v for swab
		28·17	28·25	
		23·87	24·45	
		23·23	23·38	
England (a)	Lung tonsil & trachea pools (3)	28·02	27·77	Sequencing and BLAST confirmation as H1N1v for this pooled tissue
		23·95	23·86	
		30·41	29·86	
England (b)	Lung tonsil & trachea pool (1)	21·95	22·30	Sequencing and BLAST confirmation as H1N1v for clinical specimen and egg-isolated virus

Ct values are shown for both generic ''Perfect match'' M gene RRT PCR and the specific ''H1-118'' RRT PCR assays.

Clinical specimens from pigs infected experimentally

Six Landrace hybrid pigs (age 4–5 weeks) were each infected experimentally by inoculation with 2 ml H1N1v (A/California/07/09), which contained $10^{5·8}$ EID_{50} per animal via the intranasal route.[36] Twenty-three nasal swabs were obtained by swabbing the infected pigs daily from 1 to 7 days post-infection (dpi), and 10 pig tissues (lung $n = 5$, thoracic trachea $n = 5$) were obtained from animals killed humanely for post-mortem examination on 2,4 and 7 dpi (Table 4).

Table 4. Results of testing clinical specimens from pigs infected experimentally with H1N1v (A/California/07/09)

Pig identifier	Days post-infection	Sample	Ct values		VI
			Perfect match M gene RRT PCR	H1-118 RRT PCR	
1	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	28·57	29·67	+
	3	Nasal swab	26·68	28·86	+
	4	Nasal swab	29·53	32·36	+
	4	Lung	24·30	24·67	+
	4	Thoracic trachea	21·34	22·02	+
2	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	30·51	31·69	+
	2	Nasal swab	23·02	25·59	+
	3	Nasal swab	30·01	32·24	+
	4	Nasal swab	31·29	34·47	+
	5	Nasal swab	25·19	27·44	+
	6	Nasal swab	27·95	29·77	+
	7	Nasal swab	34·48	34·08	–
	7	Lung	29·72	30·25	+
	7	Thoracic trachea	No Ct	No Ct	–
3	1	Nasal swab	34·43	36·84	+
	2	Nasal swab	30·67	33·20	+
4	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	27·66	29·36	+
	2	Nasal swab	22·20	24·23	+
	2	Lung	No Ct	No Ct	–
	2	Thoracic trachea	No Ct	No Ct	–
5	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	27·51	29·35	+
	2	Nasal swab	22·24	24·25	+
	2	Lung	26·61	26·43	+
	2	Thoracic trachea	34·28	34·42	+
6	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	27·03	29·20	+
	2	Nasal swab	26·07	28·28	+
	3	Nasal swab	31·07	32·68	+
	4	Nasal swab	29·96	33·01	+
	5	Nasal swab	28·15	30·18	+
	6	Nasal swab	27·68	29·79	+
	7	Nasal swab	34·29	36·52	+
	7	Lung	20·33	21·17	+
	7	Thoracic trachea	30·82	31·51	+

Ct values are shown for ''perfect match'' M gene and ''H1-118'' RRT PCRs. ND indicates ''not done''.

Processing of clinical specimens, VI and RNA extraction

Swabs

One hundred and ten swabs from the field (104 from VLA Regional Laboratories during summer 2009; six from proven field cases of H1N1v infection, Table 3) and 23 swabs from pigs infected experimentally (Table 4) were stored at −70°C in 1 ml brain heart infusion broth containing antibiotics (BHIB). Swabs from the VLA Regional Laboratories and H1N1v experimentally infected pigs ($n = 127$) were tested by VI in 9- to 10-day-old EFEs by means of double inoculations via the allantoic and amniotic cavities, followed by a second EFE passage in the event of the first passage being VI negative. Influenza A virus growth was detected by a positive haemagglutination assay (HA) result

obtained by testing harvested amnio/allantoic fluid with chicken red blood cells.[27] In the case of six swabs from H1N1v-field-infected pigs, infection with this pandemic virus was independently proven by conventional PCR amplification, sequencing and BLAST searching as described earlier (Table 3).

Tissues

Organ homogenates (approximately 10% w/v in BHIB) were prepared from 97 archived UK tissue specimens (31 from SIV-positive pigs (Table 2) and 66 SIV-negative pigs by VI) and 15 tissues from H1N1v-field-infected pigs (September–November 2009; Table 3) by grinding with sterile sharp sand. For nine of 15 tissues from H1N1v-field-infected pigs, infection with this pandemic virus was independently proven by conventional PCR amplification, sequencing and BLAST searching as described earlier (Table 3). Ten tissue specimens from the experimentally infected pigs (Table 4) were disrupted in BHIB using a General Laboratory Homogenizer (Omni International, Marietta, GA, USA) to provide a similar 10% w/v suspension. All tissue homogenates were clarified by centrifugation for 1 minute prior to VI and RNA extraction. VI was carried out by inoculating clarified tissue homogenates into 9- to 10-day-old EFEs as described earlier for pig swabs.

The Mini Viral RNA kit (Qiagen) was used to extract RNA from allantoic fluids, BHIB swabs fluids and clarified tissue homogenates. This was performed either manually by the ''spun column'' method in accordance with the manufacturer's protocol, or by robotic RNA extraction by the same kit chemistry adapted to a Universal Biorobot (Qiagen).[34]

M gene RRT PCR assays

Comparison of the M gene primer and probe sequence were performed on the Influenza Research Database website http://www.fludb.org. Several programmes available on the site were used for the comparison of available swine influenza sequence information including the SNP Analysis, Blast and Alignment Viewer.[37] The primers and hydrolysis probe used initially were those from the M gene RRT PCR originally described by Spackman *et al.*[38] (Table 5) for global and generic detection of AIVs at final concentrations of 0·4 and 0·3 μm, respectively.[34] The reverse primer was modified in two variations of the M gene RRT PCR assay to investigate the detection of SIVs and H1N1v isolates. The first variant (''perfect match'' M gene RRT PCR) included modification of the AIV reverse primer used by Spackman *et al.*[38] to provide a perfect match primer (reverse modified, i.e. ''Rev-mod'') with the corresponding region in the M gene of H1N1v isolates (Table 5),[39] i.e. accession number FJ966975 for the M gene sequence of A/California/07/09. This was Rev-mod: 5′-tgc aaa Gac aCT ttc Cag tct ctg-3′ [76–99] in which the four altered nucleotides in the reverse primer are indicated in upper case, while square brackets indicate nucleotide positions within FJ966975.

The second variant (''combo'' M gene RRT PCR) included an equimolar mix of the original AIV reverse primer plus the above Rev-mod primer, with each included at 0·2 μm final concentration. Otherwise cycling conditions, temperatures and chemistry details for both variants were as outlined for the M gene RRT PCR,[34] in which the original M gene RRT PCR and the two variants produced the same 101- bp product. Two microlitres of extracted RNA were tested in each M gene RRT PCR in a final 25 μl volume using Mx 3000 RealTime PCR instruments (Stratagene, Amsterdam, The Netherlands).[34] Fluorescence thresholds for all RRT PCR experiments were determined using default settings in the supplied Stratagene MxPro software, and these were inspected visually after every experiment to ensure consistency.

H1-118 RRT PCR

Sequences of the H1 gene were acquired from a database listing pandemic H1N1v isolates at: http://www.ncbi.nlm.nih.gov/genomes/FLU/SwineFlu.html.[39] primerselect software from the Lasergene package (DNAstar, Madison, WI, USA) was used to guide RealTime PCR primer and probe design. Primers and a hydrolysis probe were designed to amplify a 118- bp product within the HA2 region (''H1-118'' RRT PCR) of H1N1v isolates, these were

F118: 5′- AAT GCC GAA CTG TTG GTT CT -3′ [1315–1334] and

R118: 5′- CAA TTT CCT TGG CAT TGT TTT 3′ [1412–1432]

with nucleotide positions in square brackets corresponding to the H1 gene sequence in A/California/07/09 (accession number FJ981613).

The antisense hydrolysis probe (31 nucleotides long) was

118 pro-rev: FAM-5′- CTG GCT TCT TAC CTT TT*C ATA TAA GTT CTT C-3′ [1377-1407],

which was labelled with the FAM fluorophore at the 5′ end and with black hole quencher (BHQ1) at an internal T nucleotide (indicated as T*), while the 3′ terminal C nucleotide was modified to a dideoxy C to prevent probe extension by polymerase activity (Eurogentec, Liège (Luik), Belgium).

Primers and probe were included at final concentrations of 0·4 and 0·3 μm, respectively, and 2 μl of extracted RNA were tested in each H1-118 RRT PCR in a final 25 μl volume. Other chemistry details and cycling conditions were as outlined for the H7 HA2 RRT PCR[34] where Stratagene Mx 3000 RealTime PCR instruments were used. Occasionally, ''H1-118'' RRT PCR products were visually checked by electrophoresis in 3% (w/v) agarose gels according to standard procedures.[40]

Table 5. Identification of nucleotide substitutions at the primer and probe binding sites for the matrix RRT-PCRs with 16 representative SIV and H1N1v isolates

Examples of influenza A isolates (subtype)	Accession number	M+24 F primer* AGA TGA GTC TTC TAA CCG AGG TCG	M+64 probe* TCA GGC CCC CTC AAA GCC GA	M−124 R primer* TGC AAA AAC ATC TTC AAG TCT CTG
A/California/07/09 (H1N1v)	FJ966975	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -CT --- C-- --- ---
A/England/195/09 (H1N1v)	GQ166660	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -CT --- C-- --- ---
A/swine/Italy/29021/09 (H1N1v)	CY053622	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -CT --- C-- --- ---
A/swine/England/WVL16/98 (H1N1 avian-like)	CY037955	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- --- --- C-- --- ---
A/swine/Geldern (Germany)/IDT2888/04 (H1N1 avian-like)	EU478821	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -C- --- C-- --- ---
A/swine/Stadtlohn (Germany)/IDT3853/05 (H1N2)	EU478843	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -C- --- C-- --- ---
A/swine/Sweden/1021/09 (H1N2)	GQ495135	--- --- --- --- --- --- --- ---	--C --- --- --- --- --- --	--- --- --- -C- --- C-- --- ---
A/swine/England/742104/95 (H3N2)	Unpublished	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- --- --- C-- --- ---
A/swine/Hungary/13509/07 (H3N2)	FJ798769	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -C- --- C-- --- ---
A/wildboar/Germany/WS169/06 (H3N2)	AM746618	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- --- --- C-- --- ---
A/swine/Quebec/5393/91 (H1N1)	AF188004	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- --- --- --- --- ---
A/swine/Kansas/77778/07 (H1N1)	GQ484361	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---
A/swine/Ontario/48235/04 (H1N2)	DQ280234	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- G-- --- --- --- --- --- ---
A/swine/Minnesota/SG-00239/07 (H1N2)	CY041906	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---
A/swine/North Carolina/R08-001877-D08-013371/08 (H3N2)	CY041850	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---
A/swine/Oklahoma/001142/09 (H3N2)	CY045554	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---

*Primer and probe sequences at head of this table are as originally named and described by Spackman *et al* for the ''avian'' M gene RRT PCR,[38] written 5′ to 3′ from left to right. M gene sequences from H1N1v isolates guided modification of the original M–124 R primer to the ''Rev-mod'' primer used in the ''perfect match'' M gene RRT PCR described in this study.

Production of T7 *in vitro* RNA transcripts

In vitro RNA transcripts were made corresponding to M gene and ''H1-118'' amplified sequences of A/California/07/09 (H1N1v) and to the M gene amplified sequence of A/swine/England/742104/95 (H3N2). First, both H1N1v and H3N2 were amplified using the M gene and ''H1-118'' RRT PCR primer pairs as outlined earlier, but with the M gene reverse primer modified to identically match the target sequence for the two viruses (Table 5), and where the 5′ end of all reverse primers included the T7 promoter.[41] Conventional RT PCR conditions were as for the corresponding M gene and ''H1-118 RRT'' PCRs described in this study, except that the final magnesium chloride concentration was 2·5 mm, with ROX and probes excluded.[34] Amplicons were purified after gel electrophoresis in 3% agarose, and 10–20 ng/ul of each was added to a 100 μl volume T7 *in vitro* RNA transcription reaction (T7 RiboMAX Large Scale RNA Production System; Promega, Southampton, UK) that included 40 units of RNAsin (Promega). The reaction was incubated for four hours at 37°C. The volume was then adjusted to 120 μl by the addition of buffer and nine Kunitz units of DNAse I (RNase-Free DNase Set; Qiagen) where digestion proceeded for 30 minutes at 37°C, followed by T7 RNA transcript purification by spun columns using the outlined protocol for small transcript products <200 nucleotides (RNeasy Mini Kit; Qiagen).[34] The recovered 20 μl was again digested with fresh DNase I (7·5 Kunitz units) in an adjusted volume of 100 μl for 30 minutes at 37°C, followed by a second purification by spun columns. The concentration of the T7 *in vitro* transcripts in the 20 μl final eluate was determined using the RiboGreen RNA Quantitation kit (Invitrogen, Paisley, Scotland). The supplied ribosomal RNA standards were used to construct a calibration curve for RiboGreen fluorescence using the Mx3000 RealTime PCR instrument (Stratagene) in the quantitative plate read function and was used to calculate the concentration of the purified T7 *in vitro* transcribed RNA preparations. Successful DNase I digestion of template was established as previously.[34]

Conventional RT PCRs to identify H1N2 SIV infection

These were designed to investigate any UK clinical specimens that may be positive by M gene RRT PCR but negative by ''H1-118'' RRT PCR and negative by VI. Three HA gene alignments that included European SIV isolates of the H1N1 (avian-like swine), H1N2 and human-like swine H3N2 subtypes were constructed using the Megalign software from the Lasergene package. The Primerselect programme was then used to design appropriate primers to generate small (i.e. <230 bp) amplicons that were of sufficient size for sequencing and identification by BLAST searching. Five clinical specimens (one UK archived swine tissue submitted in January 2009 and four nasal swabs from UK Regional Labs, summer 2009) were positive by ''perfect match'' M gene RRT PCR but ''H1-118'' RRT PCR and VI negative. These were identified as being H1N2 SIV positive using the following five HA primer pairs for conventional RT PCR with forward (F) and reverse (R) primers indicated by suffixes: 218: 218F: AAA CGG GTG TTT TGA ATT CTA CCA CAA G, 218R: TGA TTG CCC CCA GGG AGA CTA AA; 226: 226F: GTT CAC CCC AGA AAT TGC AAA AAG AC, 226R: CCC CCG GGG TGT TTG ACA CT; 210: 210Fa: CAT GGC CCA AAC ACA GCG TAA AC, 210Fb: CAT GGC CCA AAC ACA ACG TAA CC (use as an equimolar mixture of two forward (F suffix) primers in 210 RT PCR), 210R: AGA TGG CCC TTT GAT CCT CTA TGT TAG A; 234: 234F: CAC TAC AAT TGG GGA AAT GCA GCA T, 234R: TGT GTT TGG GCC ATG AAC TTT CYT TAG; 187: 187F: ATG CCC AAA GTA CGT CAG GAG TAM AAA, 187R: TCC GCA GCA TAG CCA GAT CCC. These conventional RT PCR products were electrophoresed in 2% w/v agarose, purified and sequenced. Conventional RT PCR primers were similarly designed to amplify portions of the HA gene of European H1N1 (avian-like swine) and human-like H3N2 SIVs, and these sequences are available from the corresponding author on request.

Results

Bioinformatics evaluation of primer/probe-binding sequences for M gene RRT PCR

Analysis of the M gene primers and probe to available swine influenza sequences shows several different patterns based on geographical and temporal origins of the viruses (Table 5). M gene sequences are available from over 500 SIV isolates from North America, Europe and Asia, with different sequence patterns in each region (data not shown). The sequence for the forward M gene RRT PCR primer and the probe remains highly conserved for most isolates (Table 5). However, the reverse primer has extensive variability at primarily five different positions for H1N1v and SIV isolates compared to the ''avian'' M gene RRT PCR primers and probes originally designed by Spackman *et al.*[38] Current SIVs and H1N1v isolates have from zero to four nucleotide mismatches in the reverse primer (Table 5) that could potentially contribute to a decrease in sensitivity for some viruses. This influenced the design of the ''perfect match'' M gene RRT PCR ''Rev-mod'' primer for H1N1v detection, which is located at the same corresponding position as the ''M−124 R'' primer for the ''avian'' M gene RRT PCR (Table 5).

Analytical sensitivity and efficiency of M gene and H1-118 RRT PCRs

H1N1v isolate A/California/07/09 and SIV isolate A/swine/England/742104/95 (H3N2) were grown in EFEs

and EID_{50} titres determined. RNA was extracted from these influenza A preparations and used to construct 10-fold dilution series, and both were tested by the three M gene RRT PCR assays, namely the ''avian'',[38] ''combo'' and ''perfect match''. The 10-fold dilution series from A/California/07/09 (H1N1v) was also tested by the ''H1-118'' RRT PCR. The detection limit corresponded to a viral titre (pre-RNA extraction) of 1×10^1 EID_{50}/ml for the ''perfect match'' M gene and ''H1-118'' RRT PCRs, which typically registered at Ct values 34–36. When a dilution series of quantified T7 *in vitro* RNA transcripts were tested, the detection limit of the ''perfect match'' M gene RRT PCR at these Ct values was 200 RNA copies and 2000 RNA copies for the H1N1v and H3N2 targets, respectively. This discrepancy in sensitivity may relate to differences in the M gene reverse primer binding sequence for the two influenza A viruses (Table 5), where A/swine/England/742104/95 (H3N2) has two mismatches with the ''perfect match'' M gene RRT PCR ''Rev-mod'' primer. The Stratagene Mx 3000 instrumentation software plotted a standard curve of Ct values against the logarithmic dilutions. An R^2 value of >0·98 indicated a straight line, in which the slope corresponded to an efficiency in the range 90–110% for the ''combo'' and ''perfect match'' M gene RRT PCRs for both the influenza A viruses, as well as for testing A/California/07/09 by the ''H1-118'' RRT PCR. These observations indicated optimised RRT PCR protocols.[42] However, the efficiency of the ''avian'' M gene RRT PCR fell outside these limits when testing both these viruses. This was unsurprising in view of M gene sequence mismatches for both A/California/07/09 and A/swine/England/742104/95 (H3N2) in the reverse primer (Table 5).

Influenza A virus laboratory isolates: M gene RRT PCRs

RNA extracted from 99 influenza A viruses were tested by the ''avian'',[38] ''combo'' and ''perfect match'' variations of the M gene RRT PCR assays. These RNA samples included those from influenza A virus laboratory isolates of veterinary significance, i.e. 51 SIV and 43 AIV isolates plus five H1N1v isolates (Table 1). For the five H1N1v isolates, the ''perfect match'' M gene RRT PCR was most sensitive as this test yielded the lowest threshold cycle (Ct) value, followed by ''combo'' RRT PCR, but the ''avian'' M gene RRT PCR was clearly the least sensitive with the highest Ct values (Table 1). In the case of A/swine/N Ireland/1012/09, the ''avian'' M gene RRT PCR failed to detect this RNA sample, presumably owing to it being of low titre (Table 1). It would appear that the modification of the reverse M gene RRT PCR primer in the ''perfect match'' variant assay contributed to this being the most sensitive approach for detecting the RNA of H1N1v isolates.

Fifty-one laboratory isolates of established SIVs were divided into five categories based on combinations of subtype and temporal and geographical origin (Table 1). For the two classical H1N1 SIVs isolated in United Kingdom in 1986 and 1996, the ''avian'' and ''combo'' M gene variant RRT PCR assays appeared to be the most sensitive. However, when 35 contemporary UK and European SIVs were tested, the ''perfect match'' M gene RRT PCR was the most sensitive for detecting 34 of the isolates in the avian-like swine H1N1, H1N2 and human-like swine H3N2 categories (Table 1). These three categories are representative of the SIVs that have been circulating in Europe for up to 30 years.[11] It should be noted that the ''combo'' M gene RRT PCR assay was slightly less sensitive, detecting the 35 contemporary European SIVs at Ct values that were higher, but by no more than 2 for the majority of these isolates (Table 1). The one exception was A/swine/Eire/89/96 (H1N1 avian-like), which was most sensitively detected by the original ''avian'' M gene RRT PCR (Table 1).

In contrast, the 14 North American SIVs were most sensitively detected by the ''combo'' M gene RRT PCR assay in which the lowest Ct values were obtained, followed by the ''avian'' M gene RRT PCR. The ''perfect match'' M gene RRT PCR assay was the least sensitive for detection of the 14 North American SIVs as the Ct values obtained were consistently higher than in the other two assays (Table 1). It can be inferred from these findings that the ''perfect match'' M gene RRT PCR assay would be the most sensitive to use for generic testing of contemporary European SIVs as well as any pig infections caused by H1N1v. However, for North American pigs, the ''combo'' M gene RRT PCR provided the most sensitive approach to generic SIV testing for 13 of 14 North American isolates, the one exception being A/swine/Nebraska/02013/08 (H1N1) where the ''avian'' M gene RRT PCR registered a slightly lower Ct value compared to the ''combo'' M gene RRT PCR (Table 1).

Representative isolates of all sixteen H-types were included in the 43 AIVs tested (Table 1). Forty-two of 43 were detected most sensitively by the ''avian'' M gene RRT PCR as this test consistently gave the lowest Ct values, the one exception being A/duck/Belgium/10157/07 (H12N5) (Table 1). This was followed by the ''combo'' M gene RRT PCR, while the ''perfect match'' M gene RRT PCR assay proved to be the least sensitive for the detection of the AIV isolates. The low sensitivity of the ''perfect match'' M gene RRT PCR assay resulted in ''No Ct'' being recorded for six AIV isolates, possibly because their RNA had been extracted from highly diluted virus preparations. One AIV RNA (A/mallard/Sweden/08, H7N7) was so highly diluted that a high Ct value (36·86) was obtained with the ''avian'' M gene RRT PCR, but ''No Ct'' registered by both the ''perfect match'' and ''combo'' M gene RRT PCR assays (Table 1).

Influenza A virus laboratory isolates: "H1-118" RRT PCR

The "H1-118" RRT PCR assay was used to test 94 SIV and AIV isolates plus five H1N1v isolates (Table 1). In the tests, comparable Ct values were obtained for the five H1N1v isolates to those observed in the "perfect match" M gene RRT PCR assay (Table 1). None of the 37 European SIV laboratory isolates, including the 22 H1N1 (20 avian-like swine and two classical) and eight H1N2 SIVs, were detected by the "H1-118" RRT PCR assay. The "H1-118" RRT PCR assay was designed specifically for differential detection of H1N1v influenza A viruses. Non-detection of contemporary, established European H1 SIVs by the "H1-118" RRT PCR as well as H1 AIVs indicated that it fulfils this condition (Table 1).

However, positive fluorescence signals were obtained in "H1-118" RRT PCR tests with two of the North American H1N1 SIVs, namely A/swine/Wisconsin/H04YS2/04 and A/swine/Nebraska/02013/08 (Table 1). Tests with the five other North American H1N1 SIVs produced negative fluorescence signals, but gel electrophoresis in 3% w/v agarose showed that all seven of the North American H1N1 isolates tested produced a band of the predicted 118 -bp size (Table 1). H1 gene sequences are available in the public databases for two of these North American H1N1 isolates, namely A/swine/Ontario/11112/04 (accession no. DQ280250) and A/swine/Indiana/1726/88 (accession no. M81707 or CY039925). A degree of conservation was observed in the primer binding sequences for the "H1-118" RRT PCR, while mismatches in the probe-binding region appeared to account for the failure to generate fluorescence signals (data not shown).

"H1-118" RRT PCR testing of both North American H1N2 isolates also produced the 118- bp band of predicted size. A/swine/Indiana/9K035/99 (H1N2) (AF250124) produced fluorescence in this test, but A/swine/Ontario/48235/04 (H1N2) (DQ280236) was fluorescence negative (Table 1). The A/swine/Indiana/9K035/99 H1 gene sequence has a perfect match with the primer and probe-binding regions for the "H1-118" RRT PCR, while the corresponding H1 gene sequence in A/swine/Ontario/48235/04 included significant mismatches in its probe-binding region (data not shown).

Non-influenza A viruses

The 14 non-influenza A viruses were tested by the three M gene RRT PCR assays and by the "H1-118" RRT PCR assay and only negative results were obtained.

Archived clinical specimens from pigs in UK

Ninety-seven frozen archived respiratory tissues that had been obtained from pigs in the United Kingdom since 1991 were tested. These were divided into two groups, those that had been obtained from pigs that were (i) SIV positive (n = 31) and (ii) SIV negative (n = 66) based on VI at the time of sample submission. All archived tissue specimens from positive pigs were retested by VI in the course of this study, as well as by the three M gene RRT PCR approaches and "H1-118" RRT PCR (Table 2). The 31 tissues from SIV-positive pigs included 18 tissues from pigs infected with avian-like swine H1N1 viruses between 1992 and 2008, six from pigs with H1N2 virus infections from 1994 to 2009 and seven from pigs infected with human-like swine H3N2 virus during 1991–1994 (Table 2). Three of these archived tissue samples from VI-positive pigs gave no Ct value with any of the M gene RRT PCR assays. No Ct value was obtained by the "avian" M gene RRT PCR with three further samples, although these were positive by the other two M gene assays (Table 2). One tissue specimen was negative by the "combo" M gene RRT PCR but gave high Ct's by the other two M gene RRT PCRs (Table 2). As in the case of the more contemporary SIV laboratory isolates from United Kingdom and European origin (Table 1), the "perfect match" M gene RRT PCR was shown to be the most sensitive of the M gene RRT PCR variants for the majority of the archived SIV-positive tissue specimens, as for 27 of 28 tissues the M gene "perfect match" RRT PCR registered the lowest Ct compared to the two other M gene RRT PCRs (Table 2). The one exception was the oldest tissue specimen, from which A/swine/England/119404/91 (H3N2) had been isolated; in this case, the original avian M gene RRT PCR was most sensitive (Table 2). These test results with archived tissue specimens obtained from the field reinforced the observation that the "perfect match" M gene RRT PCR is the most sensitive for detecting contemporary European and UK SIVs. All 31 archived UK tissue samples from SIV-positive pigs were negative by the "H1-118" RRT PCR assay (Table 2). Only four of these archived tissues were VI positive during the current validation study, this may reflect the age and/or storage history of the tissues, plus the use of only two egg passages for VI.

Sixty-six archived tissue specimens from SIV-negative pigs from United Kingdom (organ pools of lung, trachea and tonsil, or lung only) were tested by the "perfect match" M gene RRT PCR assay to assess its specificity. All gave "No Ct" results except one, which was submitted in January 2009 and produced a Ct value of 25·45 by the "perfect match" M gene RRT PCR. Repeat VI testing during the current validation study failed to recover viable influenza A virus from this specimen. However, investigation of this specimen by conventional RT PCR and amplicon sequencing identified an H1 gene that was highly suggestive of an H1N2 SIV. All 66 of these archived tissues from SIV-negative pigs were "No Ct" by the "H1-118" RRT PCR.

Nasal swabs collected in the field by VLA Regional Laboratories

One hundred and four nasal swabs obtained from pigs in United Kingdom during the summer of 2009 were tested by VI in EFEs, the ''perfect match'' M gene RRT PCR assay and the ''H1-118'' RRT PCR assay. One hundred of these swabs were negative by all three tests. However, four swabs that originated from two UK pig herds gave negative results by VI and ''H1-118 RRT'' PCR but resulted in positive amplifications by ''perfect match'' M gene RRT PCR, with Ct values of 27·56 and 30·77 for one herd and 34·54 and 38·01 for the second herd. Investigation by conventional RT PCR and sequencing revealed an H1 gene that was suggestive of H1N2 SIV.

Respiratory swabs and tissues from H1N1v-field-infected pigs

Of the 39 specimens (24 respiratory swabs and 15 respiratory tissues) received between September and November 2009 from nine geographically diverse pig herds (Table 3), fifteen (six swabs and nine tissues) originating from eight pig herds had been shown to be H1N1v positive by non-RRT PCR approaches (Table 3). These and a further 24 specimens (18 swabs and six tissues) that included two lung specimens from an epidemiologically related ninth herd in Northern Ireland (NI ''c''; Table 3) were tested by both the ''perfect match'' M gene and ''H1-118'' RRT PCRs. Twenty-two of the 24 swabs were positive by both RRT PCRs, with Ct value ranges of 20·64–34·14 and 20·74–34·66 for the ''perfect match'' M gene and ''H1-118'' RRT PCR assays, respectively (Table 3). The submission derived from herd ''c'' in NI that included two lung tissues gave positive results by both RRT PCRs for one specimen, but the second gave high Ct values (37·50 and 37·93 by ''perfect match'' M gene and ''H1-118'' RRT PCRs, respectively) that were just above the positive cut-off for the two tests (Table 3). However, the clear positive result for the first lung specimen had identified H1N1v infection in this pig herd. One swab from the Norwegian herd gave ''No Ct'' by both RRT PCR assays, but the other 11 nasal swabs from the same herd gave similar positive Ct values by both methods. The 15 tissues were all positive by both tests, with Ct value ranges of 20·84–33·17 and 20·89–32·84 for the ''perfect match'' M gene and ''H1-118 RRT'' PCR assays, respectively (Table 3).

Nasal swabs and tissues from pigs infected experimentally with H1N1v

Thirty-three clinical specimens collected from pigs infected experimentally with H1N1v [36] were tested by VI, the ''perfect match'' M gene RRT PCR assay and the ''H1-118'' RRT PCR assay (Table 4). These samples included 23 nasal swabs taken between 1 and 7 dpi, and post-mortem tissues (lung, $n = 5$; thoracic trachea, $n = 5$). Twenty-two of the 23 swabs gave positive results by VI and both RRT PCR assays, while one swab was weakly positive by the two RRT PCRs and VI negative (7 dpi, pig 2; Table 4). The Ct ranges for these swabs were 22·20–34·48 and 24·23–36·52 for ''perfect match'' M gene and ''H1-118'' RRT PCR assays, respectively. The 10 post-mortem tissues from the experimentally infected pigs gave concordant results by all three tests, i.e. seven were positive and three were negative (Table 4). The Ct ranges for these positive tissues were 20·33–34·28 and 21·17–34·42 for the ''perfect match'' M gene and ''H1-118'' RRT PCR assays, respectively.

Diagnostic sensitivity and specificity

The sensitivity and specificity were calculated for the ''perfect match'' M gene and ''H1-118'' RRT PCR assays from the clinical specimens (respiratory swabs and tissues) used in this validation study. For 249 clinical specimens, confirmatory results were available either as VI, or in the case of 15 H1N1v field specimens, through independent confirmation by amplicon sequencing (Table 3).

For 133 swabs, a diagnostic sensitivity of 100% and a specificity 95·2% were calculated for the ''perfect match'' M gene RRT PCR. With this assay, there were five results that were VI negative, but ''perfect match'' M gene RRT PCR positive (Table 6a). Evidence presented elsewhere in the Results strongly suggests that these five were genuine positives. When the swabs were tested by ''H1-118 RRT'' PCR, a diagnostic sensitivity of 100% and specificity of 99·1% was determined (Table 6b). There was only one swab, obtained from an experimentally infected pig at 7 dpi, that was VI negative but positive by ''H1-118 RRT'' PCR (Table 6b), this swab from pig 2 was also positive by ''perfect match'' M gene RRT PCR (Table 4).

In the case of the 97 archived pig tissues collected in United Kingdom between 1991 and 2009, the specimens were divided into those from SIV-positive ($n = 31$; Table 2) and SIV-negative ($n = 66$) pigs that had been identified by VI at the time of the original submission. By inclusion of 19 additional tissues from H1N1v-field-infected (Table 3) and experimentally infected (Table 4) pigs, a diagnostic sensitivity of 93·6% and a specificity of 98·6% were determined for the ''perfect match'' M gene RRT PCR (Table 7a). Three archived tissues from pigs that were VI positive gave negative results by ''perfect match'' M gene RRT PCR (Table 2), and one archived tissue was positive by ''perfect match'' M gene RRT PCR but VI negative (Table 7a). When the same tissues were tested by ''H1-118'' RRT PCR assay, the diagnostic sensitivity and specificity were both 100%, respectively (Table 7b).

Table 6: Diagnostic sensitivity and specificity for nasal swab samples (n=133) tested by (a) ''perfect match'' M gene RRT PCR assay and (b) ''H1-118'' RRT PCR assay.

n=133		VI or independent H1N1v identification +	–	Total
(a) * ''Perfect match'' M gene RRT PCR	+	28	5	33
	–	0	100	100
Total		28	105	133
(b) † ''H1-118'' RRT PCR	+	28	1	29
	–	0	104	104
Total		28	105	133

Sensitivity for both assays: 28/28+0 = 100%
* Specificity: 100/5+100 = 95·2%
† Specificity: 104/1+104 = 99·1%

Table 7: Diagnostic sensitivity and specificity for tissue samples (n=116) tested by (a) ''perfect match'' M gene RRT PCR assay and (b) ''H1-118'' RRT PCR assay.

n=116		VI or independent H1N1v identification +	–	Total
(a) * ''Perfect match'' M gene RRT PCR	+	44	1	45
	–	3	68	71
Total		47	69	116
(b) † ''H1-118'' RRT PCR	+	16	0	16
	–	0	100	100
Total		16	100	116

* Sensitivity: 44/44+3 = 93.6%; Specificity: 68/1+68 = 98.6%
† Sensitivity: 16/16+0 = 100%; Specificity: 100/0+100 = 100%

Discussion

In recent years, the M gene RRT PCR assay [38] has gained widespread acceptance as a validated method for the global detection of all AIV subtypes, and this method is referred to as a suitable screening assay in the OIE and EU Diagnostic Manuals for AI.[33,43] Although no international recommendations have been made concerning RRT PCR testing for SIVs, the highly conserved nature of the M gene in all influenza A viruses led to the M gene RRT PCR being considered as a candidate for an initial screening assay for detecting pigs infected with all SIVs, including any infections because of H1N1v. In this study, we have investigated the application of the ''avian'' M gene RRT PCR,[38] but sequence differences observed in the M gene of H1N1v isolates also prompted investigation of two new variations of the original M gene RRT PCR. The original ''avian'' M gene RRT PCR reverse primer (M−124 R),[38] with four nucleotide differences to H1N1v viruses (Table 5), clearly resulted in a significantly lower sensitivity when testing H1N1v laboratory isolates (Table 1). Consequently, the original avian M gene RRT PCR[38] should not be used for detection of H1N1v, and this deficiency of the diagnostic test needed to be addressed. Among the three variants of the M gene RRT PCR that were evaluated, the ''perfect match'' with the revised reverse primer (''Rev-mod'') provided the most sensitive results for many of the European SIVs (Tables 1 and 2). M gene sequence analysis showed that the ''Rev-mod'' primer compensated for two or three nucleotide mismatches in the corresponding region of the M gene from contemporary European SIVs (Table 5). However, experiments with T7 *in vitro* RNA transcripts corresponding to the amplified M gene region of A/swine/England/742104/95 (H3N2) suggested that the ''perfect match'' M gene RRT PCR may not be maximally sensitive for detection of all contemporary European SIVs, which again reflected sequence mismatches with the ''Rev-mod'' primer. Nevertheless, the ''perfect match'' M gene RRT PCR demonstrated successful detection of contemporary European SIVs in archived tissues from infected pigs (Table 2), plus five specimens (four nasal swabs from UK herds and one archived tissue, collected in 2009, highly likely to be H1N2), that were negative by VI (Tables 6 and 7). Importantly, the ''perfect match'' RRT PCR successfully detected both H1N1v laboratory isolates (Table 1) and clinical specimens from pigs that had been infected with H1N1v in the field (Table 3) and experimentally (Table 4).[36] The ''combo'' M gene RRT PCR assay was slightly less sensitive for detection of contemporary European SIVs and H1N1v (Tables 1 and 2).

However, for the testing of most North American SIVs that were included in this study, the ''perfect match'' M gene RRT PCR suffered from reduced or even poor sensitivity (Table 1). For these SIVs, improved sensitivity was restored by employing the ''combo'' M gene RRT PCR, where inclusion of the original ''avian'' M−124 R reverse primer reduced the number of nucleotide mismatches within the corresponding sequence of the North American SIVs' M gene (Tables 1 and 5). It must also be emphasised that the ''avian'' M gene RRT PCR remains the most sensitive of the three M gene RRT PCRs for the detection of AIVs (Table 1).

The diagnostic sensitivity and specificity of the ''perfect match'' M gene RRT PCR assay were 100% and 95·2%, respectively, from the testing of 133 swabs (Table 6a). The

five discrepant results included a VI-negative nasal swab obtained from a H1N1v-experimentally infected pig at 7 dpi when viral shedding appeared to be waning (Ct 34·48), and this low titre was mirrored by the corresponding H1-118 RRT PCR (Ct 34·08; Table 4). The other four discrepant results were VI-negative swabs collected by UK Regional Laboratories during summer 2009 that were shown to be likely H1N2 SIV positives, so affirming these ''perfect match'' M gene RRT PCR results. This inferred a greater sensitivity for the RRT PCRs compared to VI. As regards ''perfect match'' M gene RRT PCR testing of the 116 swine tissues, however, there were four discrepant results, where a diagnostic sensitivity and specificity of 93·6% and 98·6%, respectively, were observed (Table 7a). The 96 UK archived swine tissues were classified as SIV positive or negative at the time of original submission based on VI. Three of the 31 tissues from SIV-positive pigs were negative (No Ct) by the ''perfect match'' M gene RRT PCR (Table 2). It was speculated that at the time of the original submission these pigs may have been classified as SIV positive based on a VI result from another localised tissue portion or clinical specimen that was no longer available for the current validation. It was also observed that only 4 of 31 tissues obtained from SIV-positive pigs were positive when VI was repeated through two egg passages during the current validation (Table 2). This may be a consequence of the long-term storage history of these archived tissues, where infectivity may have been compromised by repeated freeze-thawing, or even because of limiting VI to two egg passages. One archived tissue from an SIV-negative pig collected in 2009 was positive by ''perfect match'' M gene RRT PCR, negative by ''H1-118'' RRT PCR and VI (Table 7), but additional investigations suggested that this was a non-viable H1N2 specimen.

The H1-118 RRT PCR assay was designed for the differential detection of H1N1v infections of pigs. This method successfully detected five H1N1v laboratory isolates (Table 1) and the presence of H1N1v in 48 clinical specimens (29 swabs and 19 post-mortem tissues) in field-infected and experimentally infected pigs (Tables 3 and 4). It was also shown that the H1 genes from nine H1 AIVs, 30 European H1 SIVs (i.e. classical H1N1, contemporary avian-like swine H1N1 and H1N2) were not detected (Table 1) by this assay. In addition, no positive signal was obtained when 24 archived tissue samples obtained from pigs infected with European H1N1 and H1N2 SIVs were tested with the H1-118 RRT PCR assay (Table 2). This affirmed the ability of the H1-118 RRT PCR assay to differentiate H1N1v infections from those caused by the SIVs endemic in Europe. However, this test did show some cross-reactivity with North American H1 SIVs (Table 1). This was not unexpected in view of the evolutionary relationship between the H1 gene of North American triple reassortment SIVs and H1N1v.[5] Consequently, it would not be an appropriate test for differentiating H1N1v in North American pig infections.

The diagnostic sensitivity and specificity of the ''H1-118'' RRT PCR assay for 133 swabs were 100% and 99·1%, respectively (Table 6b); the one discrepant result was the swab obtained at 7 dpi from pig 2 infected experimentally with H1N1v when the titre of viral shedding was declining (Table 5). For the ''H1-118'' RRT PCR using tissue samples, both the diagnostic sensitivity and specificity of this assay were 100% (Table 7b).

Fifteen clinical specimens from H1N1v-field-infected pigs (Table 3) were independently proven as H1N1v positive by conventional RT PCR and amplicon sequencing because VI was not consistently successful for the identification of H1N1v. This is because H1N1v does not consistently haemagglutinate chicken red blood cells, which may not be observed until the third passage in EFEs (data not shown). A further 24 clinical specimens (18 swabs and six tissues) from H1N1v-field-infected herds were not included in the diagnostic sensitivity and specificity calculations, but testing by both RRT PCRs gave highly concordant results as judged by a comparison of Ct values (Table 3).

Public health laboratories have already described RRT PCR protocols for the detection of H1N1v in humans,[29–32] and in this study, we have validated RRT PCR testing for detection of H1N1v for veterinary purposes, specifically for detecting H1N1v in pigs. This included evaluation of a generic approach to detect known SIVs as well as H1N1v. This was achieved for European SIVs by the ''perfect mach'' M gene RRT PCR assay, which sensitively detected all known types of European SIVs in addition to H1N1v. However, it was noted that contemporary Irish SIVs may be the exception to this observation in Europe (Table 1). Testing of recent Irish SIVs by the various M gene RRT PCR assays suggests that the original ''avian'' M gene RRT PCR may be the more sensitive approach (Raleigh and Flynn, Personal Communication). In the case of North American SIVs, the ''combo'' M gene RRT PCR assay was most sensitive for generic detection, and this method was slightly less sensitive for the detection of H1N1v than the ''perfect match'' M gene RRT PCR assay. The ''H1-118'' RRT PCR assay was shown to be able to differentiate H1N1v from SIV infections in clinical samples derived from field-infected and experimentally infected pigs relevant to a European setting. Validation of the generic and differential RRT PCR assays involved not only testing of a range of laboratory-grown influenza A virus isolates, but also crucially included testing of the same set of clinical specimens from pigs infected with SIVs or H1N1v. Primers and probe for the differential ''H1-118'' RRT PCR were deliberately designed within the relatively conserved HA2 region of the HA gene of H1N1v. However, continuing molecular evolution the HA gene may

yet result in nucleotide changes within these primer and probe-binding sequences. Vigilance for relevant nucleotide changes by sequencing the HA genes of new H1N1v swine isolates may identify potential future modifications to the ''H1-118'' RRT PCR.

Validation of the RRT PCR assays was conducted from the perspective of veterinary contingency planning for the eventuality of H1N1v outbreaks in the European pig population. To date, H1N1v infections have been already documented in pigs in the Americas, Eurasia and Australasia [15,16,44] and examples of field cases have been included in this study (Table 3). As the human pandemic caused by H1N1v continues to spread globally, it is anticipated that further pig infections with H1N1v will be reported, and precedent suggests that these will be followed by sustained transmission within pig populations.[36] Analogous contingency planning was conducted in 2005, when the westward spread of H5N1 highly pathogenic AIV prompted the validation of a H5 RRT PCR for the detection of both this H5 virus and other Eurasian H5 AIVs.[45] In conclusion, it is possible to present these approaches as sensitive and specific methods for use in surveillance for SIV and current H1N1v isolates in European pig populations.

Acknowledgements

The authors thank all laboratories that supplied specimens of swine influenza and pandemic (H1N1) 2009 influenza used in this study, in particular the VLA Regional Laboratories at Bury St Edmunds and Thirsk for their long-standing contribution to swine influenza surveillance in United Kingdom. The contribution of Dr Dennis Alexander to the checking of this manuscript is also gratefully acknowledged, as is the funding that was provided from the UK Department of the Environment, Food and Rural Affairs (Defra).

References

1 Perez-Padilla R, Rosa-Zamboni DD, Leon SP *et al.* Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. N Engl J Med 2009; 361:680–689.
2 Centers for Disease Control and Prevention (CDC). Swine influenza A (H1N1) infection in two children – Southern California, March-April 2009. MMWR Morb Mortal Wkly Rep 2009; 58:400–402.
3 Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009; 360:2605–2615.
4 Garten RJ, Davis CT, Russell CA *et al.* Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science 2009; 325:197–201.
5 Smith GJ, Vijaykrishna D, Bahl J *et al.* Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 2009; 459:1122–1125.
6 Fraser C, Donnelly CA, Cauchemez S *et al.* Pandemic potential of a strain of influenza A (H1N1): early findings. Science 2009; 324:1557–1561.
7 Chan M. World Now at the Start of 2009 Influenza Pandemic. WHO Statements 2009. Available at: http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html 2009 (accessed 1 March 2010).
8 Nicholls JM, Chan RW, Russell RJ, Air GM, Peiris JS. Evolving complexities of influenza virus and its receptors. Trends Microbiol 2008; 16:149–157.
9 Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR, Richt JA. The role of swine in the generation of novel influenza viruses. Zoonoses and Public Health 2009; 56:326–337.
10 Brown IH. The epidemiology and evolution of influenza viruses in pigs. Vet Microbiol 2000; 74:29–46.
11 Brown IH. The role of pigs in interspecies transmission; in Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza. Monogr Virol 2008; 27:88–100. Basel: Karger.
12 Olsen C. The emergence of novel swine influenza viruses in North America. Virus Res 2002; 85:199–210.
13 Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. Swine influenza viruses: a North American perspective. Adv Virus Res 2008; 72:127–154.
14 Brown IH, Harris PA, McCauley JW, Alexander DJ. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J Gen Virol 1998; 79:2947–2955.
15 OIE disease notification in pigs Canada. (early May). Available at: http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8065 2009 (accessed 1 March 2010).
16 Anonymous. Pigs destroyed following flu outbreak. Vet Rec 2009; 165:3.
17 Rota PA, Rocha EP, Harmon MW *et al.* Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza. J Clin Microbiol 1989; 27:1413–1416.
18 Gregory V, Bennett M, Thomas Y *et al.* Human infection by a swine influenza A (H1N1) virus in Switzerland. Arch Virol 2003; 148:793–802.
19 Lipkind M, Weisman Y, Shihmanter E. Isolation of viruses antigenically related to the swine influenza virus from an outbreak of respiratory disease in turkey farms in Israel. Vet Rec 1984; 28:426–428.
20 Wright SM, Kawaoka Y, Sharp GB, Senne DA, Webster RG. Interspecies transmission and reassortment of influenza A viruses in pigs and turkeys in the United States. Am J Epidemiol 1992; 136:488–497.
21 Wood GW, Banks J, Brown IH, Strong I, Alexander DJ. The nucleotide sequence of the HA1 of the haemagglutinin of an H1 avian influenza virus isolate from turkeys in Germany provides additional evidence suggesting recent transmission from pigs. Avian Pathol 1997; 26:347–355.
22 Suarez DL, Woolcock PR, Bermudez AJ, Senne DA. Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis 2002; 46:111–121.
23 Yu H, Zhou Y-J, Li G-X *et al.* Further evidence for infection of pigs with human-like H1N1 influenza viruses in China. Virus Res 2009; 140:85–90.
24 Van Reeth K. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet Res 2007; 38:243–260.
25 Olsen CW, Brown IH, Easterday BC, Van Reeth K. Swine Influenza; in: Straw BE, Zimmerman JJ, Taylor DJ, D'Allaire S (eds): Diseases of Swine. Ames: Iowa State University Press, 2006; 469–482.
26 OIE (World Organisation for Animal Health). Swine Influenza Technical Disease Card. Paris: OIE, 2009. Available at: http://www.oie.int/eng/maladies/Swine%20Influenza_OIE%20TDC_FNL.pdf 2009 (accessed 1 March 2010).

27 OIE (World Organisation for Animal Health). Swine Influenza: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter 2.8.8. Paris: OIE, 2008. Available at: http://www.oie.int/Eng/Normes/Mmanual/2008/pdf/2.08.08_SWINE_INFLUENZA.pdf 2008 (accessed 1 March 2010).

28 Hoffmann B, Beer M, Reid SM *et al.* A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Vet Microbiol 2009; 139:1–23.

29 CDC protocol of realtime RT PCR for influenza (H1N1). Available at: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf 2009 (accessed 1 March 2010).

30 Ellis J, Iturriza M, Allen R *et al.* Evaluation of four real-time PCR assays for detection of influenza A(H1N1)v viruses. Euro Surveill 2009; 14: pii 19230. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19230 2009 (accessed 1 March 2010).

31 Poon LLM, Chan KH, Smith GJ *et al.* Molecular detection of a novel human influenza (H1N1) of pandemic potential by conventional and Real-Time quantitative RT-PCR assays. Clin Chem 2009; 55:1555–1558.

32 Wang R, Sheng ZM, Taubenberger JK. Detection of novel (swine origin) H1N1 influenza A virus by quantitative real-time RT-PCR. J Clin Microbiol 2009; 47:2675–2677.

33 OIE (World Organisation for Animal Health). Avian Influenza: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter 2.3.4. Paris: OIE, 2009. Available at: http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf 2009 (accessed 1 March 2010).

34 Slomka MJ, Pavlidis T, Coward VJ *et al.* Validated RealTime reverse transcriptase PCR methods for the diagnosis and pathotyping of Eurasian H7 avian influenza viruses. Influenza Other Respi Viruses 2009; 3:151–164.

35 Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938; 27:493–497.

36 Brookes SM, Núñez A, Choudhury B *et al.* Replication, pathogenesis and transmission of pandemic (H1N1) 2009 virus in non-immune pigs. PLoS ONE 2010; 5(2): e9068. doi:10.1371/journal.pone.0009068.

37 Squires B, Macken C, Garcia-Sastre A *et al.* BioHealthBase: informatics support in the elucidation of influenza virus host pathogen interactions and virulence. Nucleic Acids Res 2008; 36:D497–D503.

38 Spackman E, Senne DA, Myers TJ *et al.* Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 haemagglutinin subtypes. J Clin Microbiol 2002; 40:3256–3260.

39 Bao Y, Bolotov P, Dernovoy D *et al.* The influenza virus resource at the National Center for Biotechnology Information. J Virol 2008; 82: 596–601. Available at: http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/select.cgi 2008 (accessed 1 March 2010).

40 Sambrook J, Russell D The Condensed Protocols from ''Molecular Cloning: A Laboratory Manual''. Cold Spring Harbor Laboratory Press, 2006.

41 Fronhoffs S, Totzke G, Stier S *et al.* A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction. Mol Cell Probes 2002; 16:99–110.

42 McPherson MJ, Møller SG. Real-time RT-PCR, chapter 9; in PCR, 2nd edn. New York and Oxford: Taylor and Francis, 2006; 209–231.

43 EU. Diagnostic Manual for Avian Influenza. Brussels: European Union, 2006. Available at: http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_237/l_23720060831en00010027.pdf 2006 (accessed 1 March 2010).

44 OIE (World Organisation for Animal Health). Immediate notification report. Report reference: NSWpigs1_2009, Ref OIE: 8332, Report Date: 31/07/2009, Country: Australia. Available at: http://www.oie.int/wahis/reports/en_imm_0000008332_20090731_174520.pdf 2009 (accessed 1 March 2010).

45 Slomka MJ, Pavlidis T, Banks J *et al.* Validated H5 Eurasian real-time reverse transcriptase–polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. Avian Dis 2007; 51:373–377.