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Short Communication

Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany

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A natural reassortant influenza A virus consisting of seven genome segments from pandemic (H1N1) 2009 virus and a neuraminidase segment from a Eurasian porcine H1N1 influenza A virus was detected in a pig herd in Germany. The obvious reassortment compatibility between the pandemic (H1N1) 2009 and H1N1 viruses of porcine origin raises concern as to whether swine may become a reservoir for further reassortants of pandemic (H1N1) 2009 viruses with unknown implications for human health and swine production.

In April 2009, an H1N1 influenza virus with a unique genome constellation emerged as a human pandemic virus and subsequently spread to more than 200 countries. The pandemic spread was only associated with a minor clinical impact on the human population. Until the end of 2010 pandemic (H1N1) 2009 virus appeared to have replaced the previously circulating seasonal strains of H1N1 in most places (World Health Organization, 2010a). Phylogenetic analyses of pandemic (H1N1) 2009 virus revealed a mixed ancestry of this multi-reassortant virus with a strong participation of porcine influenza viruses, although the exact parentage of this virus and its geographical origins still remain to be elucidated (Smith *et al.*, 2009). Nevertheless, pandemic (H1N1) 2009 started out as an infection entirely of humans, but incursions into domestic porcine populations have been reported repeatedly (Howden *et al.*, 2009; Pasma & Joseph, 2010; Pereda *et al.*, 2010). Pigs proved to be highly susceptible to experimental infection with pandemic (H1N1) 2009, and the virus was efficiently transmitted among pigs (Lange *et al.*, 2009; Brookes *et al.*, 2010). This raised concerns as to whether the virus will become established in pig populations and whether its future evolution with respect to possible reassortment events with other porcine influenza A viruses will possibly lead to altered virulence and/or transmissibility (Vijaykrishna *et al.*, 2010). This situation prompted us to investigate swine influenza viruses (SIV) detected in Germany in 2009–2010 in greater detail.

Swabs (n=230) and lung tissues (n=411) obtained from pigs with respiratory symptoms in Germany were examined for the presence of influenza A virus genome by a modified generic real-time RT-PCR detecting part of the M gene (Hoffmann *et al.*, 2010) and positive samples were further characterized by conventional RT-PCR assays (Lee *et al.*, 2001; Gall *et al.*, 2008; Fereidouni *et al.*, 2009a), and finally subjected to sequence analysis of full-length genome segments (Haase *et al.*, 2010). In addition, RT-PCR assays which amplify the H1 gene in three overlapping parts were developed and used to enhance the recovery of H1 sequences in case full-length amplification was unsuccessful (Starick *et al.*, 2011). Influenza RNA-positive samples were also processed for virus isolation in Madin–Darby canine kidney cells, and isolates were antigenically characterized by haemagglutination inhibition assays (Kaden *et al.*, 2008). Twenty viruses of the subtype H1, all detected in pig populations in Germany during 2009–2010, were characterized by sequencing the haemagglutinin (HA) and neuraminidase (NA) encoding genes (n=17) or the whole genome (n=3). The viruses originated from seven Federal States with a focus in Lower Saxony (n=9), the region with the highest density of pig populations in Germany. The other samples came from Saxony (n=3), Schleswig-Holstein (n=3), North-Rhine Westphalia (n=2), Rhineland-Palatinate (n=1), Thuringia (n=1) and Brandenburg (n=1). Viral sequences were characterized as subtype H1N1 (n=13,

A supplementary table is available with the online version of this paper.

see Table 1 for accession nos), pandemic (H1N1) 2009 (n=3, all detected in nasal swabs, accession no. see Table 1), and as H1N2 (n=4, accession no. EPI278733–EPI278740, EPI301666–EPI301671). One of the pandemic (H1N1) 2009 viruses was detected in Rhineland-Palatinate in 2009, while the other originated from herds in Schleswig-Holstein and North Rhine-Westphalia in 2010. The HA and NA sequences of the pandemic (H1N1) 2009 viruses detected in 2009 (R3314/09) and one of the 2010 viruses (R397/10) showed highest identity scores (about 99.5 % on the nucleotide level) to pandemic (H1N1) 2009 influenza viruses isolated from humans. Isolate R708/10, in contrast, carried the HA and six further genome segments of the human pandemic virus (H1N1) 2009, but the NA sequence was characterized, by phylogenetic analysis, as a typical representative of the European porcine N1 lineage: this NA sequence, as determined concordantly by neighbour-joining, maximum-likelihood and Bayesian inference methods, clustered most closely with porcine (avian-like) H1N1 viruses detected in Germany in 2009 (Fig. 1). In contrast, another NA sequence derived from an avian-like H1N1 porcine virus (R705/10) circulating in the same region and time as the reassortant virus was more distantly related. Thus it cannot be discerned whether the pandemic (H1N1) 2009 reassortant virus R708/10 originated in this pig herd or had already been in circulation and was introduced from other sources. This again demonstrates considerable gaps in the knowledge on the molecular epidemiology of porcine influenza viruses. A nearly full genome sequence of the reassorted virus R708/10 comprising all eight segments (with the exception of the NP gene of which only a partial sequence could be determined) was created using RNA prepared from the cell culture-isolated virus and was verified by sequencing cDNA preparations of the corresponding original swab sample of that animal (R707/10) and an additional one from another pig of the same herd (R710/10). HA (720 nt) and NA (500 nt) sequences of both preparations were identical to those of virus R708/10. When analysing the nucleotide composition of the genes HA, M, NP, NS and PB2 which carry signatures for the differentiation of human pandemic (H1N1) 2009 into two different clusters (Fereidouni *et al.*, 2009b), the R708/10 sequences belonged to cluster 2. Antigenic characterization by haemagglutination inhibition assays using specific antisera revealed a clear grouping of most isolates into either cluster H1N1 Eurasian swine, H1N2 Eurasian swine or pandemic (H1N1) 2009 (Supplementary Table S1, available in JGV Online). This is corroborated by the phylogenetic analysis which yielded essentially the same clustering (Fig. 1). Only isolate R708/10 reacted with antisera against both Eurasian swine H1N1 (A/swine/Belzig/2/01) and the H1N1 pandemic virus (A/Regensburg/D26/2009) (Supplementary Table S1) possibly caused by the presence of the Eurasian N1 and the pandemic H1 antigens.

The samples yielding the reassortant virus originated from a two-site farrow-to-finish herd with 170 sows at the end of May 2009. Clinically, the weaning pigs revealed retarded growth and lameness, while the sows appeared to be healthy. Two piglets were subjected to pathological investigations. Non-purulent interstitial pneumonia and

Table 1. Overview of sequence data generated from porcine influenza A viruses (subtype H1) from Germany 2009–2010 and their nucleotide identity compared to the NA gene segment of reassortant virus H1N1 R708/10

NA, Not applicable.

Influenza virus	Subtype	Identity* (%)	Segments†	Accession no.
A/swine/Germany-BB/siv-Leipz11308/09	H1N1	93, 62	8	EPI248486–EPI248493‡
A/swine/Germany-SN/siv-Leipz243/09	H1N1	94, 18	HA, NA	EPI248502–EPI248503
A/swine/Germany-SN/siv-Leipz6340/09	H1N1	94, 11	HA, NA	EPI248504–EPI24505
A/swine/Germany-SN/siv-Leipz8826/09	H1N1	94, 11	HA, NA	EPI248506–EPI248507
A/swine/Germany-NI/R211/09	H1N1	96, 67	HA, NA	EPI278637–EPI278638
A/swine/Germany-NI/R248/09	H1N1	96, 67	HA, NA	EPI278639–EPI278640
A/swine/Germany-NI/R255/09	H1N1	NA	HA part. (nt 1073–1701)	EPI278641
A/swine/Germany-NI/R258/09	H1N1	96, 45	HA, NA	EPI278642–EPI278643
A/swine/Germany-SH/R1737/09	H1N1	93, 97	8	EPI248478–EPI248485
A/swine/Germany-TH/R2241/09	H1N1	93, 97	HA, NA	EPI278644–EPI278645
A/swine/Germany-NI/R3394/09	H1N1	95, 96	HA, NA	EPI1278646–EPI1278647
A/swine/Germany-SH/R402/10	H1N1	93, 83	HA, NA	EPI1278648–EPI1278649
A/swine/Germany-NRW/R705/10	H1N1	93, 69	HA part., NA (nt 214–1701)	EPI278650–EPI278651
A/swine/Germany-RP/R3314/09	Pandemic (H1N1) 2009	90, 21	HA part., NA (nt 524–1745)	EPI244101–EPI244102
A/swine/Germany-SH/R397/10	Pandemic (H1N1) 2009	90, 28	HA, NA	EPI301656–EPI301657
A/swine/Germany-NRW/R708/10	H1N1 reassortant	100	8 (NP part.: nt 1–764)	EPI301658–EPI301665

*Percentage nucleotide identity of the NA genome segment sequence of the isolate indicated and R708/10.
†Number or type of genome segment sequenced; part: partial sequence.
‡The numbers represent the eight accession numbers for the segments NP, NS, M, PA, PB2, PB1, NA and HA.

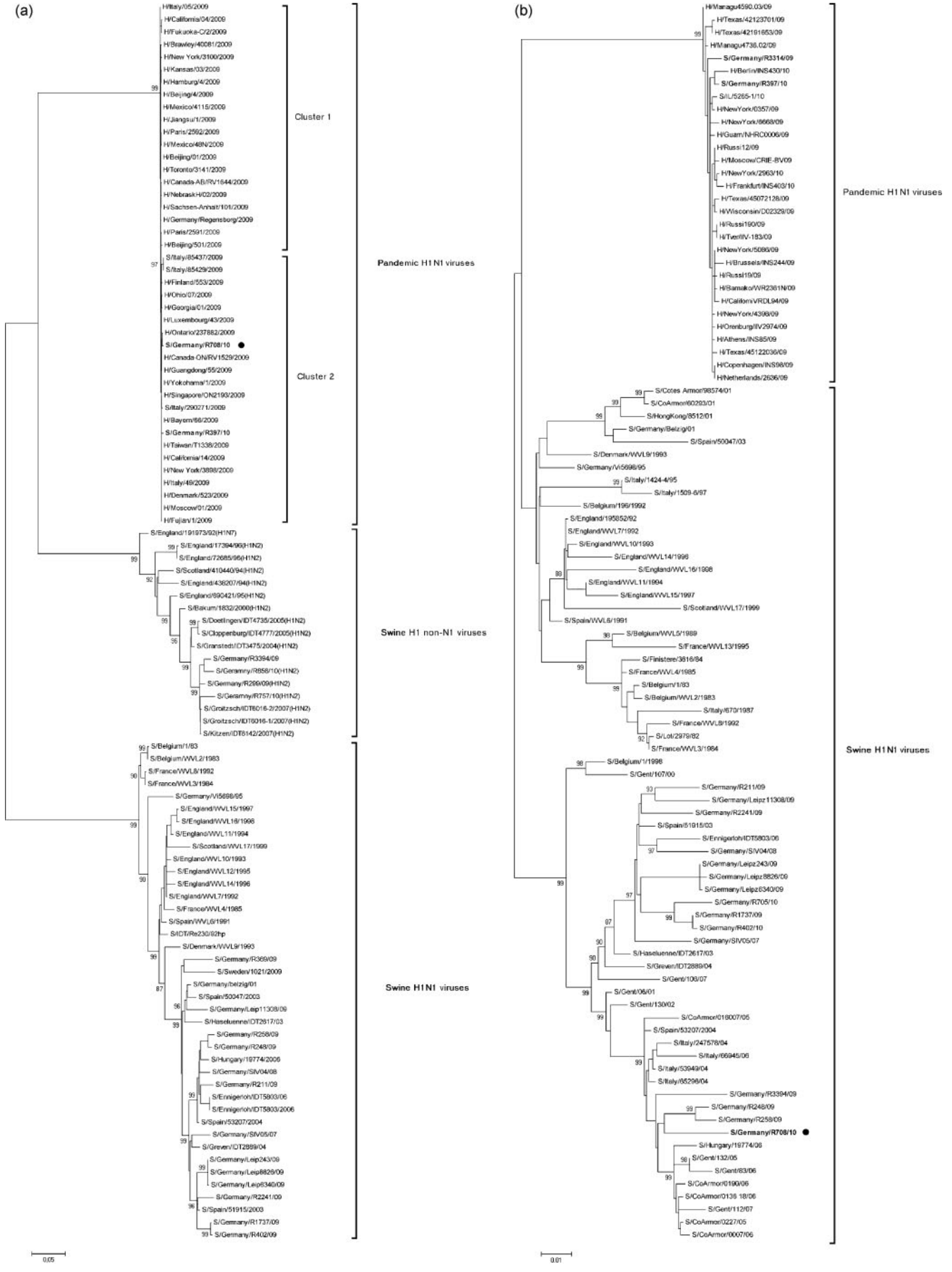
(a)
H/Italy/05/2009
H/California/04/2009
H/Fukuoka-C/2/2009
H/Brawley/40081/2009
H/New York/3100/2009
H/Kansas/03/2009
H/Hamburg/4/2009
H/Beijing/4/2009
H/Mexico/4115/2009
H/Jiangsu/1/2009
H/Paris/2592/2009
H/Mexico/48N/2009
H/Beijing/01/2009
H/Toronto/3141/2009
H/Canada-AB/RV1644/2009
H/Nebraska/02/2009
H/Sachsen-Anhalt/101/2009
H/Germany/Regensburg/2009
H/Paris/2591/2009
H/Beijing/501/2009
Cluster 1
S/Italy/85437/2009
S/Italy/85429/2009
H/Finland/553/2009
H/Ohio/07/2009
H/Georgia/01/2009
H/Luxembourg/43/2009
H/Ontario/237882/2009
S/Germany/R708/10
H/Canada-ON/RV1529/2009
H/Guangdong/55/2009
H/Yokohama/1/2009
H/Singapore/ON2193/2009
S/Italy/290271/2009
H/Bayern/66/2009
S/Germany/R397/10
H/Taiwan/T1338/2009
H/California/14/2009
H/New York/3898/2009
H/Italy/49/2009
H/Denmark/523/2009
H/Moscow/01/2009
H/Fujian/1/2009
Cluster 2
Pandemic H1N1 viruses
S/England/191973/92(H1N7)
S/England/17394/96(H1N2)
S/England/72685/96(H1N2)
S/Scotland/410440/94(H1N2)
S/England/436207/94(H1N2)
S/England/690421/95(H1N2)
S/Bakum/1832/2000(H1N2)
S/Doetlingen/IDT4735/2005(H1N2)
S/Cloppenburg/IDT4777/2005(H1N2)
S/Granstedt/IDT3475/2004(H1N2)
S/Germany/R3394/09
S/Germany/R858/10(H1N2)
S/Germany/R269/09(H1N2)
S/Germany/R757/10(H1N2)
S/Groitzsch/IDT6016-2/2007(H1N2)
S/Groitzsch/IDT6016-1/2007(H1N2)
S/Kitzen/IDT8142/2007(H1N2)
Swine H1 non-N1 viruses
S/Belgium/1/83
S/Belgium/WVL2/1983
S/France/WVL8/1992
S/France/WVL3/1984
S/Germany/Vi5698/95
S/England/WVL15/1997
S/England/WVL16/1998
S/England/WVL11/1994
S/Scotland/WVL17/1999
S/England/WVL10/1993
S/England/WVL12/1995
S/England/WVL14/1996
S/England/WVL7/1992
S/France/WVL4/1985
S/Spain/WVL6/1991
S/IDT/Re230/92hp
S/Denmark/WVL9/1993
S/Germany/R369/09
S/Sweden/1021/2009
S/Germany/belzig/01
S/Spain/50047/2003
S/Germany/Leip11308/09
S/Haseluenne/IDT2617/03
S/Germany/R258/09
S/Germany/R248/09
S/Hungary/19774/2006
S/Germany/SIV04/08
S/Germany/R211/09
S/Ennigerloh/IDT5803/06
S/Ennigerloh/IDT5803/2006
S/Spain/53207/2004
S/Germany/SIV05/07
S/Grevern/IDT2889/04
S/Germany/Leip243/09
S/Germany/Leip8826/09
S/Germany/Leip6340/09
S/Germany/R2241/09
S/Spain/51915/2003
S/Germany/R1737/09
S/Germany/R402/09
Swine H1N1 viruses
0.05
(b)
H/Managua4590.03/09
H/Texas/42123701/09
H/Texas/42191653/09
H/Managua4736.02/09
S/Germany/R3314/09
H/Berlin/INS430/10
S/Germany/R397/10
S/IL/5265-1/10
H/NewYork/0357/09
H/NewYork/6668/09
H/Guam/NHRC0006/09
H/Russi12/09
H/Moscow/CRIE-BV09
H/NewYork/2963/10
H/Frankfurt/INS403/10
H/Texas/45072128/09
H/Wisconsin/D02329/09
H/Russi190/09
H/Tver/IIV-183/09
H/NewYork/5086/09
H/Brussels/INS244/09
H/Russi19/09
H/Bamako/WR2361N/09
H/California/VRDL94/09
H/NewYork/4398/09
H/Orenburg/IIV2974/09
H/Athens/INS85/09
H/Texas/45122036/09
H/Copenhagen/INS98/09
H/Netherlands/2636/09
Pandemic H1N1 viruses
S/Cotes Armor/98574/01
S/CoArmor/60293/01
S/HongKong/8512/01
S/Germany/Belzig/01
S/Spain/50047/03
S/Denmark/WVL9/1993
S/Germany/Vi5698/95
S/Italy/1424-4/95
S/Italy/1509-6/97
S/Belgium/195/1992
S/England/195852/92
S/England/WVL7/1992
S/England/WVL10/1993
S/England/WVL14/1996
S/England/WVL16/1998
S/England/WVL11/1994
S/England/WVL15/1997
S/Scotland/WVL17/1999
S/Spain/WVL6/1991
S/Belgium/WVL5/1989
S/France/WVL13/1995
S/Finistere/3816/84
S/France/WVL4/1985
S/Belgium/1/83
S/Belgium/WVL2/1983
S/Italy/670/1987
S/France/WVL8/1992
S/Lot/2979/82
S/France/WVL3/1984
Swine H1N1 viruses
S/Belgium/1/1998
S/Gent/107/00
S/Germany/R211/09
S/Germany/Leipz11308/09
S/Germany/R2241/09
S/Spain/51915/03
S/Ennigerloh/IDT5803/06
S/Germany/SIV04/08
S/Germany/Leipz243/09
S/Germany/Leipz8826/09
S/Germany/Leipz6340/09
S/Germany/R705/10
S/Germany/R1737/09
S/Germany/R402/10
S/Germany/SIV05/07
S/Haseluenne/IDT2617/03
S/Greven/IDT2889/04
S/Gent/100/07
S/Gent/06/01
S/Gent/130/02
S/CoArmor/018007/05
S/Spain/53207/2004
S/Italy/247578/04
S/Italy/66945/06
S/Italy/53949/04
S/Italy/65296/04
S/Germany/R3394/09
S/Germany/R248/09
S/Germany/R258/09
S/Germany/R708/10
S/Hungary/19774/06
S/Gent/132/05
S/Gent/83/06
S/CoArmor/0190/06
S/CoArmor/0136 18/06
S/Gent/112/07
S/CoArmor/0227/05
S/CoArmor/0007/06
0.01

Fig. 1. Phylogenetic trees comprising the HA (Fig. 1a) and NA (Fig. 1b) genes of the reassortant pandemic H1N1 virus R708/10, sequences of other swine influenza viruses obtained in this study and publicly available Eurasian porcine sequence datasets (GenBank). Analysis was based on full-length coding sequences of the HA and NA genes. Therefore, the HA sequence of pandemic (H1N1) 2009 swine virus R3314/09 was not included (only 1222 nt available). Reassortant virus R708/10 is marked by a circle (●). The trees were generated using a maximum-likelihood approach (PAUP 4.0 and MEGA 5.0 software) based on a GTR+I+G model as suggested by the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) (ModelTest, embedded in TOPALi v2.5). The phylogenetic trees were midpoint rooted. Numbers at nodes indicate maximum-likelihood bootstrap values of 500 replicates under the specified model. Only values >85 are shown. Bar, 0.01 nt substitutions per site. S, Swine; H, human.

multifocal acute heart muscle degeneration were seen in one of them, whereas the other one showed mucopurulent bronchopneumonia. In both piglets follicular hyperplasia was evident in the spleen. The animals were negative for porcine circovirus type-2, but positive for porcine respiratory and reproductive syndrome virus. No clinical symptoms or health problems suggesting an influenza virus infection of the owner, his family and the stockmen who were in contact with the piglets were observed.

Apparently, the pandemic (H1N1) 2009 virus has currently displaced other human seasonal influenza viruses of subtype H1 to become the dominant H1 virus in the human population. However, in the course of the pandemic many countries were reporting co-circulation of seasonal and pandemic human (H1N1) 2009 influenza viruses (World Health Organization, 2010b). Also natural co-infection in humans involving pandemic (H1N1) 2009 and seasonal H1N1 virus was reported (Ducatez *et al.*, 2010). In addition, infection of swine with the human pandemic (H1N1) 2009 virus has been observed in multiple countries (Moreno *et al.*, 2010a; Song *et al.*, 2010; Welsh *et al.*, 2010). In a systematic monitoring programme in Hong Kong, 10 pandemic (H1N1) 2009 viruses were isolated from pigs between October 2009 and February 2010 and, in addition, a reassortant virus carrying the NA of pandemic (H1N1) 2009 in a porcine virus backbone was described (Vijaykrishna *et al.*, 2010).

This prompted us to explore further the genotype of the other two pandemic (H1N1) 2009 viruses derived from pigs in Germany. Instead of sequencing the whole genome, newly developed diagnostic assays were used for genotype characterization (Ducatez *et al.*, 2010 and own primers, sequences available on request). All further six gene segments of both viruses proved to be identical with human pandemic (H1N1) 2009 isolates (not shown).

The detection of a virus consisting of seven pandemic (H1N1) 2009 genome segments plus the NA gene of a Eurasian H1N1 porcine virus demonstrates reassortment compatibility and activity between the pandemic (H1N1) 2009 and other swine H1N1 viruses. In addition, a reassortant virus from pigs in Italy was detected and characterized recently which was derived from pandemic (H1N1) 2009 and carried an NA gene similar to that of H1N2 SIV (Moreno *et al.*, 2010b). Experimentally, this spectrum has also been extended to the highly pathogenic avian influenza virus of the subtype H5N1 by co-infection of cell cultures and virus rescue *in vitro* (Octaviani *et al.*, 2010). Therefore, the continued circulation of pandemic (H1N1) 2009 in humans, its transmissibility to and spread within pig populations, together with new findings on continuing evolution, reassortment and *trans*-species transmission events (AVMA, 2010), should prompt for more systematic investigations of pig populations and for a thorough analysis of porcine influenza viruses.

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