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H9 avian influenza reassortant with engineered polybasic cleavage site displays a highly pathogenic phenotype in chicken

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In the field, highly pathogenic avian influenza viruses (HPAIV) originate from low-pathogenic strains of the haemagglutinin (HA) serotypes H5 and H7 that have acquired a polybasic HA cleavage site. This observation suggests the presence of a cryptic virulence potential of H5 and H7 low-pathogenic avian influenza viruses (LPAIV). Among all other LPAIV, the H9N2 strains are of particular relevance as they have become widespread across many countries in several avian species and have been transmitted to humans. To assess the potential of these strains to transform into an HPAIV, we introduced a polybasic cleavage site into the HA of a contemporary H9N2 isolate. Whereas the engineered polybasic HA cleavage site mutant remained a low-pathogenic strain like its parent virus, a reassortant expressing the modified H9 HA with engineered polybasic cleavage site and all the other genes from an H5N1 HPAIV became highly pathogenic in chicken with an intravenous pathogenicity index of 1.23. These results suggest that an HPAIV with a subtype other than H5 or H7 would only emerge under conditions where the HA gene could acquire a polybasic cleavage site and the other viral genes carry additional virulence determinants.

INTRODUCTION

Highly pathogenic avian influenza viruses (HPAIV) lead to devastating losses in the poultry industry worldwide and pose a threat for initiating a new pandemic due to repeated transmission to humans. HPAIV have been shown to originate from low-pathogenic precursors of the subtypes H5 and H7 only (Alexander, 2000; Garten & Klenk, 2008). Their haemagglutinin (HA) specifies a polybasic cleavage site (Garten & Klenk, 1999; Garten & Klenk, 2008) and thus is susceptible to proteolytic activation by the ubiquitous protease furin (Stieneke-Gröber *et al.*, 1992). Consequently, infection of poultry with HPAIV leads to systemic viral spread and the lethal course of the disease. In contrast, the activation cleavage of HA from low-pathogenic avian influenza viruses (LPAIV) is restricted to trypsin-like proteases with monobasic specificity (Garten & Klenk, 2008). Infection with LPAIV results in a subclinical outcome or mild disease which may lead to drops in egg production, delays in growth, or exacerbation of underlying conditions or other disease (Suarez, 2010). Recent H9N2 viruses have an HA cleavage site (HACS) with dibasic (R-S-S-R) or tribasic (R-S-K/R-R) motifs (Aamir *et al.*, 2007; Guo *et al.*, 2000; Liu *et al.*, 2004; Perk *et al.*, 2006a, b; Xu *et al.*, 2007) resembling the minimal furin motif R-X-R/K-R (Vey *et al.*, 1992). These viruses are of lower pathogenicity in birds, but appropriate mutations could alter the HACS to be recognized by furin, potentially resulting in HPAIV.

Amongst LPAIV, H9N2 strains have attracted particular attention. Since the mid-1990s, infections in poultry, mainly chickens, have occurred in many countries and have reached panzootic proportions (Alexander, 2007). Furthermore, G1-like strains like the prototype strain A/Quail/HongKong/G1/97 (H9N2) carry six internal protein genes closely related to clade 0 H5N1 HPAIV isolated in Hong Kong 1997 (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). Therefore, those strains were presumed to be the donors of the internal protein genes of clade 0 viruses (Guan *et al.*, 1999, 2000, 2003; Guo *et al.*, 2000). This observation suggests the potential for H9N2 strains to transform into highly pathogenic viruses. Besides widespread prevalence in ducks, quails and chickens, H9N2 viruses were also transmitted to pigs and humans (Butt *et al.*, 2005; Cong *et al.*, 2007, 2008; Ducatez *et al.*, 2008; Guo *et al.*, 2000; Lin *et al.*, 2000; Peiris *et al.*, 1999). Some human H9N2 isolates have acquired changes in HA at the receptor binding site, like human H3N2 strains (Matrosovich *et al.*, 2001), suggesting further adaptation

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The GenBank/EMBL/DDBJ accession numbers for the ChkEmR66 (H9N2) wild-type sequences are CY076720–CY076727.

to the human host. Moreover, large numbers of undetected subclinical infections with H9 strains have been suggested by serological surveillance studies among farmers and poultry workers reporting positivity rates from 1.0–15.5 % (Jia *et al.*, 2009; Peiris *et al.*, 1999; Wang *et al.*, 2009).

To reveal the cryptic virulence potential of H9N2 LPAIV, we altered the HACS of the H9N2 strain A/Chicken/Emirates/R66/02 (ChkEmR66) by either changing the dibasic motif RSSR at its HACS to the minimal furin motif RSRR or by extending the HACS with a basic amino acid motif from an H5N1 HPAIV. In addition, we generated two reassortants carrying either the HA from HPAIV A/Swan/Germany/R65/06 (H5N1) (SwanGerR65) and the remaining seven gene segments from ChkEmR66, or the ChkEmR66 HA with the polybasic HACS RRRKKR/G and the remaining gene segments from SwanGer65. These recombinant viruses were studied in cell culture and chicken.

RESULTS

Recombinant H9N2 viruses with polybasic HACS

We introduced the polybasic HACS of HPAIV SwanGerR65 (H5N1) into the HA gene of the poultry isolate ChkEmR66 (H9N2) by site-directed mutagenesis (Weber *et al.*, 2007). Furthermore, we changed the dibasic motif RSSR of the ChkEmR66 HACS to the minimal furin cleavage motif RSRR (Vey *et al.*, 1992). Using the appropriate plasmids, we rescued the wild-type ChkEmR66 (GenBank accession numbers: CY076720–CY076727); the two HACS mutants, being the tribasic variant ChkEmR66-H9$_{RSRR}$ and the polybasic variant ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$, and the reassortant viruses ChkEmR66-H5$_{R65}$, SwanGerR65-H9$_{R66}$ and SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ (Table 1).

ChkEmR66-H9$_{RSSRRRRKKR}$ resembles an HPAIV *in vitro*

To investigate whether the cleavage site mutants and reassortants are able to undergo multicycle replication in the absence of trypsin, and thus display the phenotype of an HPAIV *in vitro*, we performed plaque assays on MDCK-H cells in the absence or presence of trypsin. The parental ChkEmR66 and the reassortant SwanGerR65-H9 required trypsin for formation of any visible plaques. Remarkably, the tribasic HACS mutant ChkEmR66-H9$_{RSRR}$, with minimal furin motif (Vey *et al.*, 1992), was also unable to form any plaques in the absence of trypsin. In contrast, the polybasic HACS mutant ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ and the two reassortants ChkEmR66-H5$_{R65}$, which carries the HA from HPAIV SwanGerR65, and SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ formed plaques in the absence of exogenous trypsin (Fig. 1a).

To investigate the activation cleavage of the HA precursor HA0 directly, we performed Western blots from lysates of infected MDCK-H cells. Corresponding to plaque formation, the HA0 of ChkEmR66, ChkEmR66-H9$_{RSRR}$ and SwanGerR65-H9$_{R66}$ remained uncleaved in the absence of trypsin, whereas the HA0 of SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ was processed into the HA1 and HA2 fragments (Fig. 1b).

Growth kinetics in cell culture were in accordance with plaque formation and proteolytic HA activation. The parent virus ChkEmR66 and the tribasic HACS mutant ChkEmR66-H9$_{RSRR}$ were dependent on trypsin, since in the absence of an exogenous protease they reached similar titres at 8 h but stagnated from then on at 4–5 orders of magnitude lower levels than in the presence of trypsin. However, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ was able to replicate independent of trypsin but reached a 10^3-fold lower titre. This impaired growth could be attributed to incompatibility of the engineered polybasic HACS to the H9 HA as indicated by the Western blot from SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$-infected cells (Fig. 1c). Furthermore, decreased virus yield may result from premature irreversible conformational changes of the intracellularly processed mutant H9 HA in the acidic milieu of the trans-Golgi network (Grambas & Hay, 1992).

Taken together, these data demonstrate that the introduction of a polybasic HACS into the HA of ChkEmR66

Table 1. Recombinant viruses with their HACS regions

Basic amino acids modified or inserted into the HACS region of the wild-type ChkEmR66 by mutagenesis are shown in boldface type. Different residues in the SwanGerR65 HA are underlined.

Virus	Features	HACS region
ChkEmR66	A/Chicken/Emirates/R66/02 (H9N2), monobasic cleavage site	NVPARSSR
SwanGerR65	A/Swan/Germany/R65/06 (H5N1), polybasic cleavage site	N<u>S</u>P<u>Q</u>GER<u>RR</u>K<u>K</u>R
ChkEmR66-H9$_{RSRR}$	A/Chicken/Emirates/R66/02 (H9N2), HACS mutant with minimal furin motif	NVPARS**R**R
ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$	A/Chicken/Emirates/R66/02 (H9N2), HACS mutant with cleavage site extended with polybasic motif from SwanGerR65	NVPARSSR**RRRKKR**
ChkEmR66-H5$_{R65}$	A/Chicken/Emirates/R66/02 (H9N2), reassortant with HA gene from SwanGerR65	N<u>S</u>P<u>Q</u>GER<u>RR</u>*K*<u>*K*</u>R
SwanGerR65-H9$_{R66}$	A/Swan/Germany/R65/06 (H5N1), reassortant with HA gene from ChkEmR66	NVPARSSR
SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$	A/Swan/Germany/R65/06 (H5N1), reassortant with mutated ChkEmR66 HA gene carrying an HACS extended with polybasic motif from SwanGerR65 HA	NVPARSSR**RRRKKR**

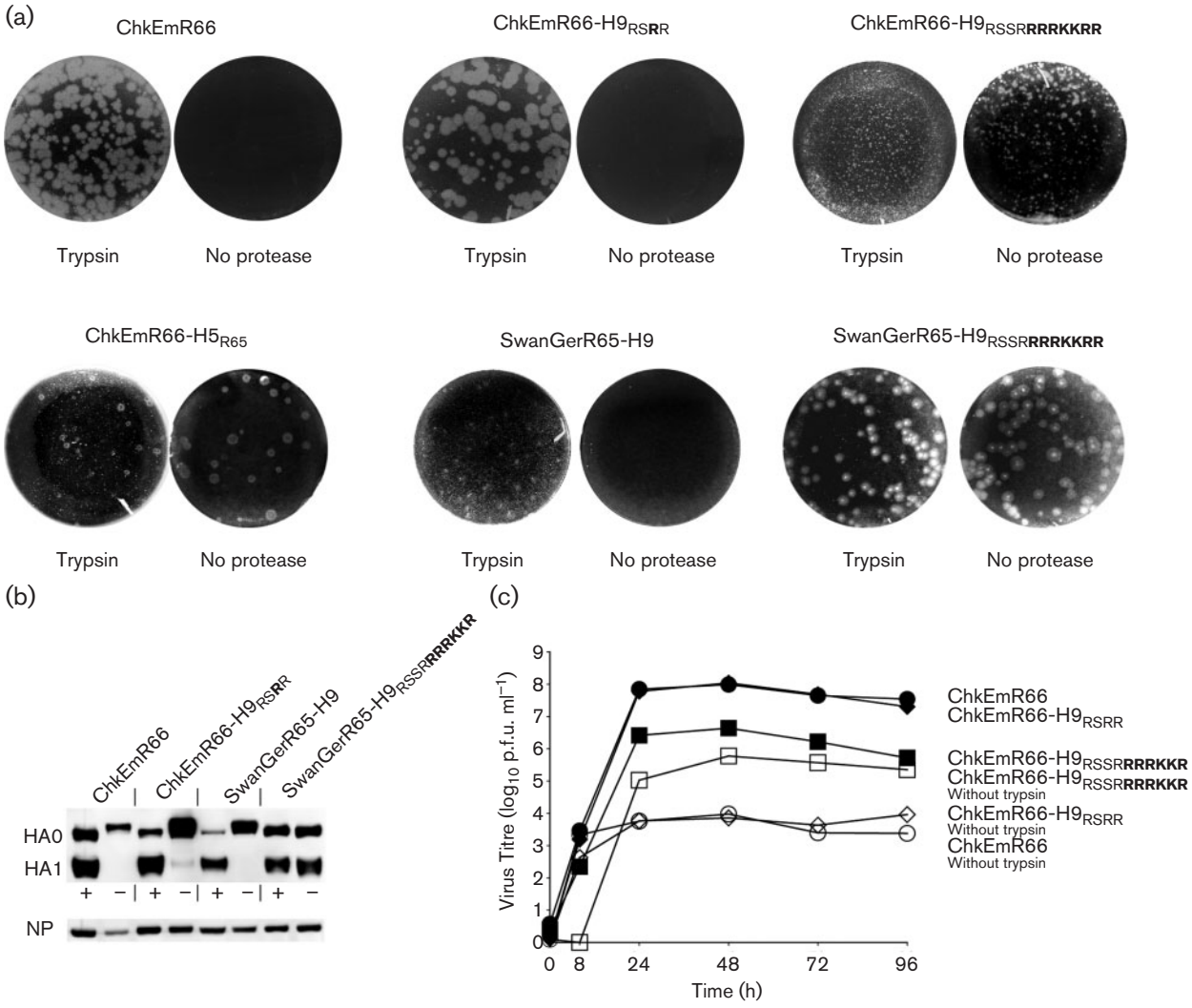

Fig. 1. *In vitro* properties. (a) Plaque assays of ChkEmR66, ChkEmR66-H9$_{RSRR}$, ChkEmR66-H9$_{RSSRRRRKKR}$, ChkEmR66-H5$_{R65}$, SwanGerR65-H9 and SwanGerR65-H9$_{RSSRRRRKKR}$ on MDCK-H cells in the presence and absence of trypsin. (b) Proteolytic HA activation. Western blots from lysates of MDCK-H cells infected with ChkEmR66, ChkEmR66-H9$_{RSSR}$, SwanGerR65-H9 or SwanGerR65-H9$_{RSSRRRRKKR}$ at an m.o.i. of 0.1 in the presence (+) or absence (−) of trypsin. (c) Growth kinetics. MDCK-H cells were inoculated with ChkEmR66 (circles), ChkEmR66-H9$_{RSRR}$ (diamonds), and ChkEmR66-H9$_{RSSRRRRKKR}$ (squares) at an m.o.i. 10^{-3} in the presence (filled symbols) or absence (hollow symbols) of trypsin.

permits proteolytic activation independent of trypsin. In this respect, ChkEmR66-H9$_{RSSRRRRKKR}$ and SwanGerR65-H9$_{RSSRRRRKKR}$ resemble an HPAIV *in vitro*.

Pathogenicity in chickens

In order to investigate their virulence, we infected chickens oculonasally with 10^6 p.f.u. of ChkEmR66, ChkEmR66-H9$_{RSSRRRRKKR}$, ChkEmR66-H5$_{R65}$, SwanGerR65-H9 and SwanGerR65-H9$_{RSSRRRRKKR}$. Animals inoculated with ChkEmR66 or the reassortant SwanGerR65-H9 showed only mild respiratory symptoms. Remarkably, the course of disease in animals infected with the polybasic HACS mutant ChkEmR66-H9$_{RSSRRRRKKR}$ did not differ significantly. In contrast, the reassortant viruses ChkEmR66-H5$_{R65}$ and SwanGerR65-H9$_{RSSRRRRKKR}$ were pathogenic in chickens. ChkEmR66-H5$_{R65}$ caused temporary non-lethal disease with notable symptoms from days 3–9 post-inoculation (p.i.), whereas infection with SwanGerR65-H9$_{RSSRRRRKKR}$ had already led to signs of disease on day 2. By day 7 p.i., all SwanGerR65-H9$_{RSSRRRRKKR}$-infected chickens had died or were in a moribund state and had to be euthanized (Fig. 2).

To investigate viral shedding, we took oral and cloacal swabs from virus-infected animals (16 from each group) on day 2. Among the ChkEmR66-infected chickens, seven animals shed virus orally and 11 animals cloacally with titres ranging from 1.6 to 2.6 $\log_{10}$TCID$_{50}$ ml^{-1}. However,

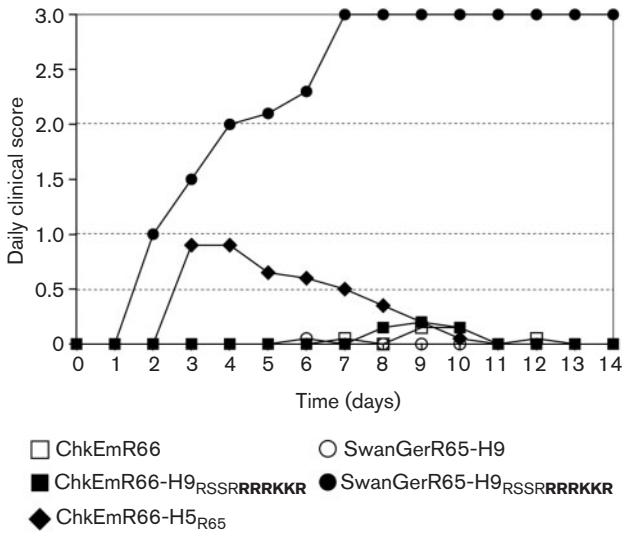

Fig. 2. Virulence in chickens. Daily clinical score after oculonasal inoculation with 10^6 p.f.u. of ChkEmR66 (□), ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ (■), SwanGerR65-H9 (○), ChkEmR66-H5$_{R65}$ (◆) and SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ (●). The birds were observed for 10 days for clinical signs and scored as: 0, healthy; 1, ill; 2, severely ill; or 3, dead. Daily clinical score was calculated from the sum of the individual clinical scores from all birds divided by the number of animals per group (ten chickens).

the polybasic HACS mutant ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ could be detected in cloacal swabs from nine animals with titres ranging from 1.6 to 2.5 $\log_{10}$TCID$_{50}$ ml^{-1}, but not in oral swabs (Fig. 3). Eleven oral swabs (1.6–2.6 $\log_{10}$TCID$_{50}$ ml^{-1}) and eight cloacal swabs (1.6–2.6 $\log_{10}$TCID$_{50}$ ml^{-1}) were positive in ChkEmR66-H5$_{R65}$-infected chickens. Somewhat higher titres were found in the SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$-infected animals as the oral titres ranged from 1.6 to 3.2 $\log_{10}$TCID$_{50}$ ml^{-1} (14 animals) and cloacal titres from 1.6 to 2.4 $\log_{10}$TCID$_{50}$ ml^{-1} (eight animals) (Fig. 3). Taken together, virus shedding on day 2 could be detected to different extents. These data support the conclusion that the polybasic HACS mutation introduced into ChkEmR66 is detrimental for virus replication *in vivo*. However, this disadvantage is counterbalanced in the reassortant SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$.

To assess the virulence of SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ compared with the HPAIV SwanGerR65 according to the OIE criteria (Alexander, 2008), we determined the intravenous pathogenicity index (IVPI). The IVPI of SwanGerR65 is 2.88 and that of SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ was determined to be 1.23. Since this value is greater than the internationally accepted threshold of 1.2 (Alexander, 2008), SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ meets the official definition of an HPAIV. Taken together, these data demonstrate that HPAIV with HA other than H5 or H7 can be generated, provided the other viral proteins carry respective cryptic virulence determinants, e.g. originate from an HPAIV.

Pathology

At necropsy, ChkEmR66-H5$_{R65}$-infected animals showed subcutaneous haemorrhages at the shanks and severe atrophy of the thymus (day 3 p.i.), as well as accumulation of mucinous excretions in their larynx (day 3 p.i.). These symptoms were even more prominent in chickens infected with SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$.

To study the extent of viral spread and tissue lesions, we investigated samples of brain, trachea, lung, heart, kidney, spleen, pancreas, caecum and duodenum on days 3 and 6 p.i. by histopathology and immunohistochemistry. No microscopic lesions or influenza virus antigen were found in organs from chickens infected with ChkEmR66, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ or SwanGerR65-H9. However, ChkEmR66-H5$_{R65}$-infected animals acquired focal lesions in brain, lung, heart and spleen, whereas SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$-infected animals developed multifocal lesions in all organs investigated, coinciding with the presence of influenza virus antigen (Tables 2 and 3, Fig. 4).

DISCUSSION

LPAIV of subtype H9N2 belong to the most widespread avian influenza strains in Asia and the Middle East (Alexander, 2007; Ducatez *et al.*, 2008) and have been transmitted to humans (Butt *et al.*, 2005; Guo *et al.*, 2000; Lin *et al.*, 2000; Peiris *et al.*, 1999). Strains of the G1 group carry genes encoding internal proteins closely related to clade 0 H5N1 HPAIV (Guan *et al.*, 1999, 2000). In this study, we analysed whether the introduction of a polybasic HACS into a Middle-East H9N2 strain would give rise to an HPAIV, thereby revealing a cryptic virulence potential of these H9 subtype strains. These studies are particularly important since it is currently not possible to predict the result of the introduction of a polybasic HACS into an LPAIV with any certainty. Whereas such a mutation does not increase the virulence of the reference strain A/Duck/Ukraine/1/1963 (H3N8) for chickens (Stech *et al.*, 2009), a subsequent study revealed that insertion of a polybasic cleavage site into an H6N1 LPAIV field isolate resulted in a highly pathogenic phenotype (Munster *et al.*, 2010). We show here that the engineered polybasic HACS mutant ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ has a low pathogenic phenotype like its parent ChkEmR66. In contrast, the reassortant SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$, carrying a modified H9 HA with polybasic cleavage site and all the other genes from HPAIV SwanGer65, is highly pathogenic in chickens. These findings demonstrate that HPAIV of subtype H9 would only emerge if two conditions were met: that a polybasic HACS mutation occurred and some or all of the other viral genes linked to the virulence of H5N1 HPAIV were acquired by reassortment. However, the exact distribution and the precise localization of these additional virulence determinants remain to be mapped. Besides the polybasic HACS, several virulence markers were assigned to the HA, neuraminidase, polymerase basic 2, polymerase basic 1,

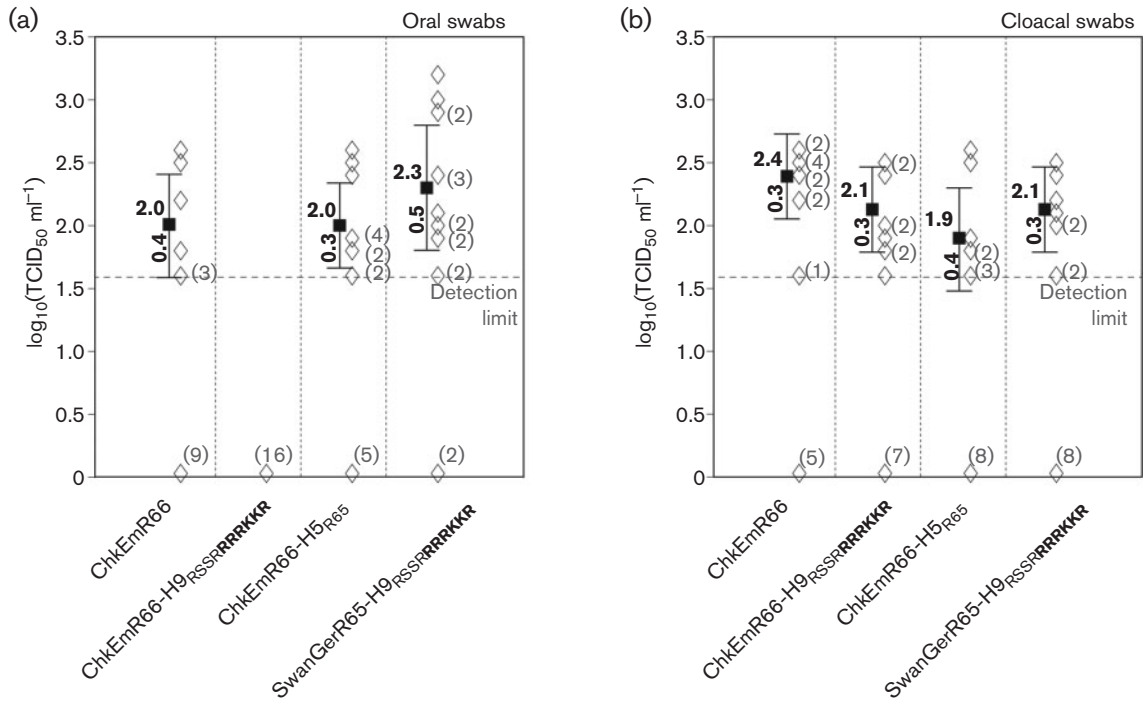

Fig. 3. Virus shedding from chicken. Titres from oral and cloacal swabs taken on day 2 from chickens infected with ChkEmR66, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$, ChkEmR66-H5$_{R65}$ or SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$. Titres are plotted as means of values above the detection limit of 1.6 $TCID_{50}$ ml^{-1} (■) together with standard deviations (error bars) and individual values (◇) along with their number of occurrences given in parentheses.

polymerase acidic, nucleoprotein (NP), matrix 2 and non-structural 1 proteins (Baigent & McCauley, 2001; Banks *et al.*, 2001; Deshpande *et al.*, 1985; Hoffmann *et al.*, 2000b; Ma *et al.*, 2010; Munier *et al.*, 2010; Suarez, 2010; Wasilenko *et al.*, 2008; Zhirnov & Klenk, 2009). The internal protein genes of ChkEmR66 differ from those of the G1 group (Guan *et al.*, 1999, 2000) and H5N1 clade 0 viruses (WHO/OIE/FAO H5N1 Evolution Working Group, 2008) considerably, as the identity values range from 99 to 90 % (3–33 amino acids different per gene segment). These considerable differences correspond to our finding that the virulence of ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ in chicken is not increased compared to its low-pathogenic parent virus. Since both H9N2 LPAIV and H5N1 HPAIV are endemic in Asia and the Middle East, respective reassortment events may occur. If such an H9 reassortant acquires a polybasic cleavage site via recombination or polymerase slippage, as has been inferred for several H5 and H7 HPAIV (García *et al.*, 1996; Khatchikian *et al.*, 1989; Pasick *et al.*, 2005; Perdue *et al.*, 1996, 1997; Suarez *et al.*, 2004), it could become virulent for poultry.

With an IVPI of 1.23 in chickens, SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ meets the official definition of an HPAIV. However, this IVPI is near the lower limit for an HPAIV, and this low value could be attributed to either an incompatibility of the ChkEmR66 H9 HA to the other proteins of SwanGerR65 (H5N1) or an inability of the ChkEmR66 H9 HA to support systemic replication as fully as SwanGerR65 H5 HA does. Correspondingly, the two reassortants SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$ and ChkEmR66-H5$_{R65}$ displayed an organ tropism that was more restricted and showed less abundant viral antigen in the respective organs, in contrast to the homologous SwanGerR65 (Gohrbandt *et al.*, 2010) and other H5N1 HPAIV (Perkins & Swayne, 2001; Pfeiffer *et al.*, 2009). In particular, infection of the endothelium was found exclusively in organs of SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$-infected chicken on day 3 p.i. Inflammatory alterations in affected organs were considerably more prominent compared with SwanGerR65-infected birds (Gohrbandt *et al.*, 2010), which might be attributed to the prolonged course of disease. Compared with ChkEmR66, the HA reassortant ChkEmR66-H5$_{R65}$ displays a more extended tissue spread and occasional replication in brain, indicating that the SwanGerR65 H5 HA itself carries virulence determinants and could contribute to neurotropism. On the other hand, the lower virulence and moderate viral spread of SwanGer-H9$_{RSSR\mathbf{RRRKKR}}$ compared with the homologous HPAIV SwanGerR65 could be attributed to the incompatibility of the ChkEmR66 H9 HA to the engineered polybasic cleavage site and to the remaining seven genes from the H5 strain SwanGerR65.

Table 2. Pathology at day 3 p.i.

Histopathology and IHC detection of influenza virus nucleoprotein in infected chickens. Affected organs are listed and the results of immunostaining are given as: ++, multifocal; +, focal; −, negative. HE, haematoxylin and eosin stain.

Virus and method	Brain	Trachea	Lung	Heart	Kidney	Spleen	Pancreas	Caecum	Duodenum
ChkEmR66, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$, SwanGerR65-H9									
IHC (n=3)	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
Histopathology (HE)	None	None	None	None	None	None	None	None	None
ChkEmR66-H5$_{R65}$									
IHC (n=3)	−/+/−	−/−/−	+/+/+	+/+/+	−/−/−	−/−/+	−/−/−	−/−/−	−/−/−
IHC positive cell types	Neurons	None	Pneumocytes I+II	Cardiac myocytes	None	Macrophages	None	None	None
Histopathology (HE)	Lymphocytic meningo-encephalitis	No lesions	Heterophilic pneumonia	Mixed cellular myocarditits	No lesions	Follicular hyperplasia, apoptotic lymphocytes	No lesions	No lesions	No lesions
SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$									
IHC (n=3)	−/+/++	−/+/+	+/++/+	−/+/++	−/+/+	+/+	−/+/+	−/−/+	−/+/−
IHC positive cell types	Neurons, glial cells, ependymal cells	Mononuclear cells, endothelium	Pneumocytes I+II, endothelium	Cardiac myocytes, endothelium	Tubular epithelium, endothelium	Mononuclear cells, endothelium	Acinar cells	Neurons (PNS)	Macrophages, neurons (intramural ganglia)
Histopathology (HE)	Lymphocytic meningoencepha-litis, glial cell proliferation, ependymal and neuronal degeneration	No lesions	Heterophilic pneumonia, pneumocyte II hyperplasia, infiltration with macrophages	Mixed cellular myocarditits, myocardial degeneration	No lesions	Follicular and retikuoendo-thelial hyper-plasia, degeneration of lympho-cytes and macrophages	Acinar necrosis	Neuronal degeneration, glial cell proliferation	None

Table 3. Pathology at day 6 p.i.

Histopathology and IHC detection of influenza virus nucleoprotein in infected chickens. Affected organs are listed and the results of immunostaining are given as: ++, multifocal; + focal; −, negative.

Virus and method	Brain	Trachea	Lung	Heart	Kidney	Spleen	Pancreas	Caecum	Duodenum
ChkEmR66, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$, SwanGerR65-H9									
IHC (n=3)	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
Histopathology (HE)	None	None	None	None	None	None	None	None	None
ChkEmR66-H5$_{R65}$									
IHC (n=3)	+/+/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/++/++	−/−/−	−/−/−
IHC positive cell types	Neurons, ependymal cells, glial cells	None	None	None	None	None	Acinar cells	None	None
Histopathology (HE)	Lymphoplasma-histiocytic meningo-encephalitits, neuronal degene-ration, glial cell proliferation	No lesions	No lesions	Lympho-plasma-histiocytic myocarditis	Interstitial lymphocytic nephritis	No lesions	Mixed cellular pancreatitis, acinar cell degeneration and necrosis	No lesions	Focal serositis
SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$									
IHC (n=3)	++/++/+	−/−/−	−/++/−	−/−/−	++/++/−	−/+/−	+/++/++	−/+/−	−/+/−
IHC positive cell types	Neurons, glial cells, ependymal cells, choroid plexus	None	Pneumocytes ii, macrophages	None	Tubular epithelium	Single mononuclear cells	Acinar cells	Mononuclear cells	Intestinal epithelium, neurons (intramural), villus stroma, mononuclear cells
Histopathology (HE)	Lympho plasma-histiocytic meningo-encephalitis, glial cell proliferation	No lesions	Heterophilic pneumonia, congestion, oedema, infiltration with histiocytes	Lympho-plasma histiocytic myocarditis and pericarditis	Interstitial lymphoplas-macellular nephritis, tubular necrosis	Depletion, retikulo-endothelial hyperplasia, degeneration of lymphocytes and macrophages	Lympho-histiocytic pancreatitis, acinar degeneration and necrosis	Lymphocyte depletion and necrosis, infiltration with histiocytes	Lymphoplasma-cellular serositis

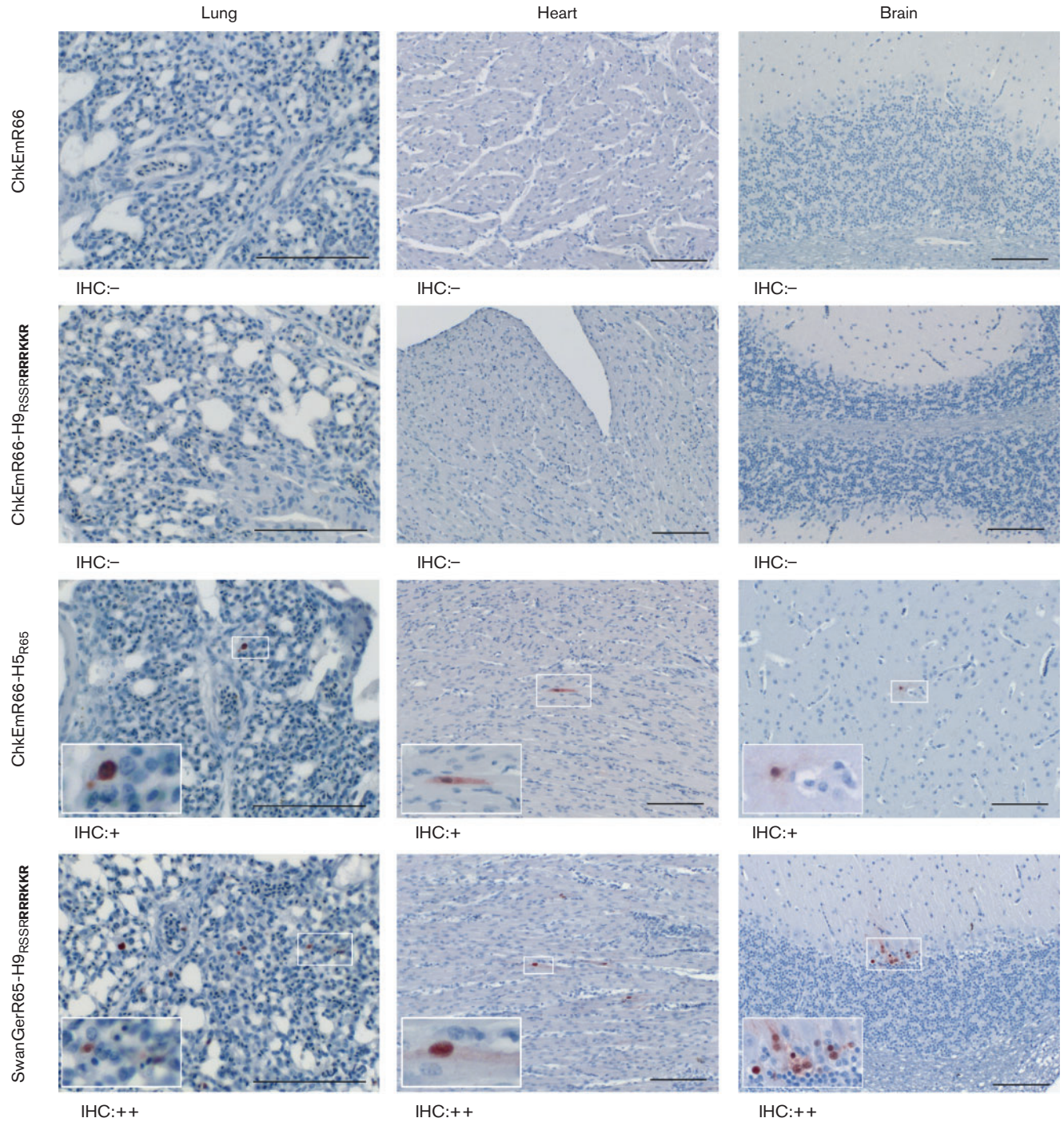

Fig. 4. Viral organ tropism in chicken. Immunohistochemical (IHC) detection of influenza A virus nucleoprotein (indicated by a red stain) in lung, heart and brain from chickens sacrificed on day 3 p.i. with 10^6 p.f.u. of ChkEmR66, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$, ChkEmR66-H5$_{R65}$ or SwanGerR65-H9$_{RSSR\mathbf{RRRKKR}}$. The extent of IHC staining is given as: ++, multifocal; +, focal; –, negative. Insets show enlarged regions with virus-positive cells; the corresponding area is indicated by a rectangle. Bars, 100 μm.

Recently, an H9N2 chicken virus with the same HACS, PARSSR/G, like ChkEmR66 (Table 1), was subjected to site-directed mutagenesis, resulting in two point mutations within the modified HACS (PARKKR/G), followed by ten passages in the air sacs of chicks. Prior to passaging, the HACS mutant required trypsin for *in vitro* replication and

was not lethal in chickens, after either intranasal or intravenous infection. Remarkably, the passaged progeny virus replicates trypsin-independently and exhibits a highly pathogenic phenotype after intravenous injection but is non-lethal after intranasal inoculation (Soda *et al.*, 2011). In other studies, however (Bogs *et al.*, 2010; Munster *et al.*, 2010), the polybasic HACS mutants were generated by insertions and were highly pathogenic following both intravenous or oculonasal infection. Taken together, the polybasic motif apparently has to be acquired by an insertion mutation to provide furin-susceptibility for the HA (Ohuchi *et al.*, 1991) and, above all, the polybasic HACS appears to be essential for initiating systemic spread.

The maintenance of influenza viruses in bird populations would not necessarily require systemic spread within the infected organism but rather viral shedding that promotes efficient transmission to other susceptible animals. In contrast to its parent virus, ChkEmR66-H9$_{RSSR\mathbf{RRRKKR}}$ is not shed orally from chickens, is restricted in viral growth and has a smaller plaque phenotype. Such properties suggest that the polybasic motif introduced is detrimental for virus replication and, thus, indicate that further adaptation in the vicinity of the HACS (Gohrbandt *et al.*, 2010) or in other regions would be essential in order to prevent extinction in the wild. These implications should be the subject of subsequent transmission studies.

In H9 strains, the natural acquisition of a polybasic cleavage site appears rather unlikely since naturally occurring HPAIV observed to date exhibit only HA subtypes H5 or H7 (Alexander, 2000; Garten & Klenk, 2008). However, we demonstrate here that an H9 HA with an artificial polybasic cleavage site can result in a highly pathogenic phenotype, provided it is in the presence of an appropriate genetic background. This finding emphasizes that a polybasic cleavage-site mutation could result from a unique mutability of the H5 or H7 HA (García *et al.*, 1996; Khatchikian *et al.*, 1989; Pasick *et al.*, 2005; Perdue *et al.*, 1996, 1997; Suarez *et al.*, 2004). Overall, the inability of circulating H9N2 or other non-H5/H7 strains to acquire a polybasic HACS to date has remained an apparently insurmountable restriction in the evolution into HPAIV.

METHODS

Cells and recombinant viruses. Human embryonic kidney (HEK293T), Madin-Darby canine kidney (MDCK) and MDCK-H cells were cultivated in minimal essential medium containing 10 % FBS (Invitrogen). MDCK-H cells were originally obtained from Alan Hay at the National Institute of Medical Research, London, UK, and passaged in Frederick Hayden's Laboratory at the University of Virginia Health Sciences Center, USA, using standard laboratory procedures, prior to distribution to Roche Discovery Welwyn (Matrosovich *et al.*, 2003).

Plasmids encoding the gene segments of strains SwanGerR65 (H5N1) and ChkEmR66 (H9N2) have been described (Stech *et al.*, 2008). The HA gene of ChkEmR66 (H9N2) was transformed into MDS42 bacteria (Pósfai *et al.*, 2006). Introduction of polybasic cleavage sites was performed by site-directed Quikchange mutagenesis method (primer sequences available on request). Recombinant viruses were rescued as described (Hoffmann *et al.*, 2000a; Stech *et al.*, 2008) and propagated either on MDCK cells or in 11-day-old embryonated chicken eggs (ChkEm-HA$_{R65}$). The gene composition of recombinant viruses and their HACS were verified by sequencing RT-PCR amplicons obtained from viral RNA (data not shown). All viruses with polybasic HACS or of HPAIV origin were handled under BSL3+ conditions.

Plaque assay and growth curves. Plaque assays were performed on MDCK and MDCK-H cells either in the presence of 2 μg ml^{-1} *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) or in the absence of any exogenous protease. The volume of the inoculum was 400 μl. Growth curves were performed in duplicate on MDCK-H cells in the presence (2 μg ml^{-1}) or absence of TPCK-treated trypsin with an m.o.i. of 10^{-3}. Virus titre in the supernatant was determined by plaque assay on MDCK cells in the presence of 2 μg ml^{-1} TPCK-treated trypsin at 0, 8, 24, 48, 72 and 96 h p.i.

Western blots. MDCK-H cells were infected at an m.o.i. of 0.1 in the presence of 2 μg ml^{-1} TCPK-treated trypsin or without any exogenous protease. After 24 h cell lysates were obtained. Proteins were separated by SDS-PAGE on 10 % polyacrylamide gels and electrotransferred to nitrocellulose membranes. For the detection of HA, in-house polyclonal rabbit anti-H5 (Pavlova *et al.*, 2009) and polyclonal rabbit anti-H9 sera were used. For secondary antibody, we used a goat-anti-rabbit antibody (BioVision) conjugated with HRP (1 : 10 000 for 1 h at room temperature), followed by detection by chemiluminescence (Supersignal West Pico chemiluminescent substrate kit; Pierce).

Animal experiments. The animal experiments were evaluated by the responsible ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) and gained governmental approval (registration number LALLF M-V/TSD/7221.3-1.1-018/07).

Two-week-old white Leghorn specific-pathogen-free chickens (Lohmann, Cuxhaven, Germany) (16 animals per group) were infected oculonasally, observed daily for clinical symptoms and scored: 0, healthy, 1, ill, 2, severely ill and 3, dead, according to the OIE guidelines (Alexander, 2008). Moribund birds which were too sick to eat or drink were euthanized and scored as dead on the next observation day according to OIE guidelines (Alexander, 2008). On day 2 p.i., oral and cloacal swabs (16 animals per group) were taken; the virus titres were determined by $TCID_{50}$ assay (Kalthoff *et al.*, 2008) on MDCK cells in the presence of 2 μg ml^{-1} TPCK-treated trypsin (starting dilution 10^{-1}). After 3 and 6 days p.i., respectively, three chickens from each group were sacrificed for histopathological examination. The IVPI was determined as described by Alexander, (2008).

Histopathology. From sacrificed birds, tissue samples of brain, trachea, lung, heart, kidney, spleen, pancreas, caecum and duodenum were taken on days 3 and 6 p.i. (three animals per group). Tissues were formalin-fixed and processed for paraffin-wax embedding according to standardized procedures. Immunohistochemical detection of influenza virus A NP was performed as described (Kalthoff *et al.*, 2008).

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