Highly pathogenic avian influenza A/H5N1 viruses pose a threat to human health as they continue to circulate among domestic poultry since 1997. Since 2003, almost 600 human cases of laboratory confirmed cases of A/H5N1 virus infections have been reported to the WHO of which 59% had a fatal outcome (Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human 195 Infection with Avian Influenza, A Virus, 2008; WHO, 2012). At present, only a few isolated clusters of human-to-human transmission of influenza A/H5N1 viruses have been reported (Ungchusak et al., 2005; Wang et al., 2008). However, it is feared that these viruses will adapt to humans and become transmissible in the future. Adaptation to a new host may take place through accumulation of mutations in one or more gene segments or by exchanging gene segments with mammalian influenza A viruses (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009). Pigs have especially been proposed as mixing vessels for the emergence of reassorted influenza viruses since porcine trachea epithelial cells have receptors for both avian and human influenza A viruses (Ito et al., 1998; Kida et al., 1994; Ma et al., 2009; Suzuki et al., 2000). However, since respiratory tract cells of various other species, including humans, express receptors for avian and mammalian influenza A viruses, they may serve as mixing vessels as well (Shinya et al., 2006; van Riel et al. 2010).

Co-infection of a single cell with two or more different influenza A viruses is required for reassortment between influenza A viruses to take place. Although genetic reassortment has been studied as an outcome of co-infection in vitro and in silico (Greenbaum et al., 2012; Huang et al., 2008; Lubeck et al., 1979; Rabadan et al., 2008), the frequency and conditions required for co-infection of a single cell with two influenza A viruses is largely unknown.

In the present study, we performed semi-quantitative analysis of the frequency of co-infection with influenza A viruses in vitro. To this end, influenza A viruses were constructed with a disrupted start codon of the neuraminidase (NA) gene by reverse genetics technology. Furthermore, most of the NA coding region was replaced by genes encoding eGFP or mCherry (Clontech) as described previously (Rimmelzwaan et al., 2007, 2011). Viruses were constructed with the haemagglutinin (HA) gene of influenza A virus A/Indonesia/5/2005 virus (H5N1) from which the multiple basic cleavage site was removed. These viruses were designated influenza A/H5–GFP and influenza A/H5–mCherry virus, respectively. Furthermore, a virus was constructed with the HA gene of influenza A virus A/PR/8/34 (H1N1) expressing eGFP (A/H1–GFP). All other gene segments were derived from A/PR/8/34 (H1N1). Fluorescent proteins mCherry and eGFP were chosen since these proteins have different excitation and emission spectra, but are similar in size (Shaner et al., 2005). Viruses were propagated in the presence of exogenous NA from Vibrio Cholerae (3.5 mU ml \(^{-1}\); Sigma-Aldrich) as described previously (Rimmelzwaan et al., 2007, 2011) and the presence of the eGFP or mCherry gene was confirmed by the expression of these proteins in cells displaying cytopathologic effect after inoculation under limiting dilution conditions in the presence of exogenous NA. Virus titres of stocks were determined by performing virus titrations in the presence of exogeneous NA as described previously (Rimmelzwaan et al., 2007).

Co-infection experiments were performed using Madin–Darby canine kidney cells (MDCK) and adenocarcinoma human alveolar basal epithelial cells A549 cells (80–90% ...
confluent) seeded on 24-well glass plates (MatTek Corporation). All conditions were tested in triplicate. Both A549 and MDCK cell lines were used since MDCK cells express receptors with both the NeuAc(α2,3)Gal- and NeuAc(α2,6)Gal-linked sialic acid, while A549 cells mainly express NeuAc(α2,3)Gal receptors (Rimmelzwaan et al., 2007). Cells were inoculated at an m.o.i. of 0.1, 1 and 3 TCID₅₀ per cell with one or two reporter viruses and after 16–18 h incubation at 37 °C/5 % CO₂, the presence of double-infected cells was assessed with a Zeiss LSM 700 confocal microscope in combination with Zeiss LSM ZEN 2010 software (Carl Zeiss B.V.). To this end, 25 pictures were made of each well at ×20 magnification and all pixels representing eGFP only, mCherry only or both fluorescent signals were counted and analysed using ZEN 2010 software.

First, A549 and MDCK cells were inoculated at an m.o.i. of 1 with virus A/H5–mCherry in combination with influenza viruses A/H1–GFP or A/H5–GFP or with each virus alone. Double-infected cells could be clearly distinguished from uninfected cells and cells infected with one influenza A virus only (Fig. 1). In addition, double infection did not have an effect on the number of eGFP- or mCherry-expressing cells observed after inoculation with one virus only. Thus, the frequencies of cells expressing eGFP after inoculation with A/H1–GFP or A/H5–GFP virus only were similar to those inoculated with A/H1–GFP or A/H5–GFP and A/H5–mCherry virus (Fig. 2a). Thus, infection with either virus should be considered independent events. Furthermore, the relative frequencies of cells expressing eGFP measured by flow cytometry using FACSCantoII (BD Biosciences) resembled those determined by confocal microscopy and ZEN 2010 software (Fig. 2b). Lower frequencies of infected cells were detected after inoculation with influenza A/H5–GFP, which is most likely caused by differences in brightness and photostability of eGFP and mCherry (Shaner et al., 2005).

However, the infection rates of A549 and MDCK cells after inoculation (m.o.i. of 1) with A/H1–GFP virus [mean (%) eGFP+ cells of 1.0 and 5.3, respectively] were lower than those after inoculation with A/H5–GFP virus (12.5 and 46.2 %), which corresponds with previous findings (Rimmelzwaan et al., 2007). The results obtained with confocal microscopy confirmed these findings (Fig. 2b). In addition, we generated an influenza A/H1–mCherry virus, which gave only very low numbers of mCherry positive cells and was therefore not used in the analysis. The differences in infection rate between these two viruses might be due to differences in entry or post-entry events. Of interest, double-infected cells were readily detected after co-infection of A549 and MDCK cells, both after inoculation with A/H5–mCherry virus and influenza A/H1–GFP or A/H5–GFP virus. By confocal microscopy, the proportion of double-infected cells was highest after inoculation of MDCK cells with influenza A/H5–GFP and A/H5–mCherry viruses (mean 8.4 %; SD 1.2 %) (Fig. 2f). The combination of A/H1–GFP and A/H5–mCherry resulted in fewer double-infected cells, indicating that the low infection rates of A/H1–GFP limited the number of double-infected cells. Also after inoculation of A549 cells the proportion of double-infected cells was lower regardless of the combination of viruses that was used (mean range between 2.2 and 3.0 %) (Fig. 2c–f). Thus, although A549 cells were less susceptible to infection with A/H1–GFP virus than with A/H5–GFP virus the proportion of double-infected cells was similar after infection with both A/H5 viruses. The relative overall poor susceptibility to infection of A549 cells with either subtype of influenza virus may have limited double infection of these cells. The proportion

Fig. 1. Examples of cells co-infected with influenza A/H5–GFP and influenza A/H5–mCherry virus. A549 and MDCK cells were inoculated with an m.o.i. of 1 and after 16–18 h of incubation, cells were analysed by light (a and e) and confocal microscopy (b–d and f–h) to detect mCherry-expressing cells (b and f), eGFP-expressing cells (c and g) and cells expressing both eGFP and mCherry (d and h). Magnification, ×800.
of double-infected cells was found to be dependent on the m.o.i. that was used (Fig. 3a–d). Especially inoculation of MDCK cells with A/H5–GFP and A/H5–mCherry at a high m.o.i. gave rise to a relatively high number of double-infected cells, which confirms that these cells were more susceptible to infection with A/H5 virus than to infection with the A/H1–GFP virus. A549 cells were less susceptible to infection with A/H1 and A/H5 virus than MDCK cells and the m.o.i. in the range 1–3 had limited effect on the number of double-infected cells that could be detected.

Thus, the use of viruses that express different reporter genes allowed the visualization and quantification of double infections in cell lines. Using this unique approach it is possible to assess the likelihood of double infections with various influenza viruses to take place. Co-infection of cells with two or more influenza A viruses is a prerequisite for the emergence of reassorted influenza viruses that can acquire properties of the parental strains, including adaptation to a new host. However, the approach does not allow the prediction of whether reassortant viruses emerge after double infection, since this may be dependent on other factors like the compatibility of packaging signals of the respective gene segments (Hutchinson et al., 2010). Since the reporter viruses used lack a functional NA, a possible contribution of this protein to initiate infections has not been taken into consideration. Also a role for secondary rounds of replication in causing double infections can be excluded. Furthermore, it has been shown that NA can reduce viral super infection by removing sialic acid receptors on infected host cells (Huang et al., 2008).

In conclusion, double infection of cells with viruses carrying the HA of human and avian influenza A viruses of the H5N1 subtype readily takes place provided that the cells express the appropriate receptors for both viruses. Under real life conditions this may result in the emergence of reassorted viruses. The last three pandemics were all caused by reassorted viruses and therefore insight into the possibility of selected influenza A viruses to cause double infections may contribute to our understanding of the risk for reassortment to take place and potentially pandemic


