Sialic acid-dependent interactions between influenza viruses and *Streptococcus suis* affect the infection of porcine tracheal cells

Nai-Huei Wu, Fandan Meng, Maren Seitz, Peter Valentin-Weigand and Georg Herrler

Bacterial co-infections are a major complication in influenza-virus-induced disease in both humans and animals. Either of the pathogens may induce a host response that affects the infection by the other pathogen. A unique feature in the co-infection by swine influenza viruses (SIV) and *Streptococcus suis* serotype 2 is the direct interaction between the two pathogens. It is mediated by the haemagglutinin of SIV that recognizes the α2,6-linked sialic acid present in the capsular polysaccharide of *Streptococcus suis*. In the present study, this interaction was demonstrated for SIV of both H1N1 and H3N2 subtypes as well as for human influenza viruses that recognize α2,6-linked sialic acid. Binding of SIV to *Streptococcus suis* resulted in co-sedimentation of virus with bacteria during low-speed centrifugation. Viruses bound to bacteria retained infectivity but induced only tiny plaques compared with control virus. Infection of porcine tracheal cells by SIV facilitated adherence of *Streptococcus suis*, which was evident by co-staining of bacterial and viral antigen. Sialic-acid-dependent binding of *Streptococcus suis* was already detectable after incubation for 30 min. By contrast, bacterial co-infection had a negative effect on the replication of SIV as indicated by lower virus titres in the supernatant and a delay in the kinetics of virus release.

INTRODUCTION

Bacterial co-infections are a major cause of increased morbidity and mortality associated with influenza epidemics in the human population (McCullers, 2014). Streptococci have a prominent position among bacterial pathogens exacerbating symptoms of influenza virus infections. The aggravating effect of bacterial co-infection is also known for infections by swine influenza viruses (Shope, 1931). In fact, the porcine respiratory disease complex (PRDC) is a multifactorial syndrome of swine characterized by severe respiratory disease after infection by two or more infectious agents (Opriessnig et al., 2011). Swine influenza viruses (SIV) and *Streptococcus suis* are prominent members associated with PRDC.

While it is generally accepted that bacterial co-infection may increase the severity of viral diseases, the molecular events responsible for the pathogenesis of viral–bacterial co-infections are poorly understood. The pathogenic effects resulting from co-infections usually have a multifactorial basis, which makes it difficult to elucidate the network of interactions responsible for disease (Joseph et al., 2013). The complex picture of molecular events involved in the disease caused by viral–bacterial co-infections is also evident in the interplay between influenza viruses and streptococci (Short et al., 2012). Several factors have been reported to contribute to the pathogenesis of co-infections. Furthermore, the same factor may play a different role depending on the viral or bacterial strains involved. An interesting example in this respect is the importance of sialic acid. *Streptococcus pneumoniae* contains a neuraminidase that desialylates cell surface components (King, 2010). In this way, the bacterial adherence is facilitated and the released sialic acids can be used as a nutrient source (Siegel et al., 2014). The neuraminidase of influenza viruses can enhance this effect. Several streptococci lack a neuraminidase. Some of them contain sialic acid as a terminal sugar component of the polysaccharide in the capsule surrounding the bacteria. For group B streptococci it has been shown that the terminal sialic acid is attached to the capsular polysaccharide in an α2,3-linkage (Jennings et al., 1983) that is recognized by Siglecs and sialoadhesins (Chang & Nizet, 2014). By binding to these host cellular lectins, group B streptococci affect the immune response of the host, e.g. by inhibiting leukocyte activation or by modifying the phagocytotic properties of macrophages. In this way, the...
bacteria may indirectly affect infection by other pathogens like influenza viruses. A direct interaction between influenza viruses and streptococci has recently been reported for SIV (H1N1 subtype) and Streptococcus suis serotype 2 (Wang et al., 2013). This is a highly virulent serotype of Streptococcus suis in swine and in addition has a zoonotic potential as indicated by occasional epidemics in humans (Feng et al., 2014). In contrast to group B streptococci, Streptococcus suis serotype 2 contains terminal sialic acid residues connected to the capsular polysaccharide in an

Fig. 1. Immunofluorescence analysis of bacterial attachment at 8 h post-virus-infection. NPTr cells (2 × 10⁵ cells per well) were inoculated with SIV (H1N1 or H3N2) followed 2 h later by Streptococcus suis infection (wt or noncapsulated mutant Δcps) for 2 h. At 8 h post-virus-infection, Streptococcus suis was immunostained (a; green); SIV was stained by monoclonal antibodies directed against the nucleoprotein (a; red). The amounts of adherent bacteria were determined and compared by relative frequency of fluorescence signals per field (b). Results are expressed as mean ± SEM and significance is indicated by asterisks (statistical analysis with unpaired Student's t-test, **P<0.01 and *P<0.05). The experiment was repeated at least three times with duplicate samples. Bars, 100 μm.
a 2,6-linkage (Van Calsteren et al., 2010). This capsular sialic acid is recognized by the haemagglutinin of SIV resulting in the binding to encapsulated bacteria. Pre-infection of porcine tracheal (NPTr) cells by SIV (H1N1 subtype) was found to increase both adhesion and invasion of S. suis serotype 2 (Wang et al., 2013). This effect was sialic-acid-dependent.

Here, we characterized the interaction between SIV and Streptococcus suis in more detail by including SIV strains of the H3N2 subtype and by showing that H1N1 and H3N2 viruses may have different efficiencies in their interaction with Streptococcus suis. Furthermore, we report that while SIV facilitates adherence of Streptococcus suis, bacterial co-infection has a negative effect on the virus infection as evidenced by a delay in the growth kinetics and lower virus titres in the supernatant of infected cells.

**RESULTS**

*In vitro infection of SIV enhances Streptococcus suis adhesion to respiratory epithelial cells*

A recent study reported that pre-infection by H1N1 influenza virus enhances infection of newborn pig trachea cells (NPTr) by Streptococcus suis serotype 2 in a sialic-acid-dependent manner (Wang et al., 2013). We were interested (i) in the mechanism mediating this interaction and (ii) in whether there are differences between the different haemagglutinin subtypes of SIV prevalent in pigs, H1 and H3. Two SIV strains, sw/Bad Griesbach/06 (H1N1, SIV-H1N1) and sw/Herford/07 (H3N2, SIV-H3N2), were used to compare subtype differences.

We used Streptococcus suis serotype 2 strain 10 (wt) and a noncapsulated mutant (Δcps) for the co-infection studies to determine the interaction between Streptococcus suis and SIV. NPTr cells were first infected by either of the two different SIV strains. After having removed non-adherent virus by a washing step, Streptococcus suis was applied as a secondary infectious agent. At 2 h post-virus-infection, cells were incubated with or without Streptococcus suis. Two hours later, non-adherent bacteria were washed away and cells were further incubated at 37 °C. At 8 h post-SIV-infection, cells were analysed were harvested at different times post-virus-infection and titrated by determining the TCID$_{50}$ on Madin–Darby canine kidney (MDCK) cells. Results represent the mean values of virus titres ± SEM pooled from three independent experiments with duplicated samples. Asterisks indicate significant differences between cells infected by SIV alone and cells co-infected by SIV and Streptococcus suis wt at 24 h p.i. (statistical analysis with unpaired Student's $t$-test, ***$P<0.001$, **$P<0.01$ and *

### RESULTS

**In vitro infection of SIV enhances Streptococcus suis adhesion to respiratory epithelial cells**

A recent study reported that pre-infection by H1N1 influenza virus enhances infection of newborn pig trachea cells (NPTr) by *Streptococcus suis* serotype 2 in a sialic-acid-dependent manner (Wang et al., 2013). We were interested (i) in the mechanism mediating this interaction and (ii) in whether there are differences between the different haemagglutinin subtypes of SIV prevalent in pigs, H1 and H3. Two SIV strains, sw/Bad Griesbach/06 (H1N1, SIV-H1N1) and sw/Herford/07 (H3N2, SIV-H3N2), were used to compare subtype differences.

We used *Streptococcus suis* serotype 2 strain 10 (wt) and a noncapsulated mutant (Δcps) for the co-infection studies to determine the interaction between *Streptococcus suis* and SIV. NPTr cells were first infected by either of the two different SIV strains. After having removed non-adherent virus by a washing step, *Streptococcus suis* was applied as a secondary infectious agent. At 2 h post-virus-infection, cells were incubated with or without *Streptococcus suis*. Two hours later, non-adherent bacteria were washed away and cells were further incubated at 37 °C. At 8 h post-SIV-infection, cells were analysed.
by immunofluorescence microscopy. For encapsulated *Streptococcus suis* (wt), the adhesion of bacteria to NPT cells was significantly increased when cells had been pre-infected with either SIV-H1N1 or SIV-H3N2 virus (Fig. 1a, top panels, b). Most *Streptococcus suis* wt (green) cells were associated with SIV-infected cells (red). On the other hand, *Streptococcus suis Δcps* was detected on NPT cells independently of virus infection, and in the case of co-infection, bacterial antigen was not associated with virus-infected cells (Fig. 1a). It should be noted that no binding of *Streptococcus suis* wt (red) was detected on SIV-infected cells at 4 h post-infection (p.i.) (not shown) indicating that bacterial attachment was not induced by residual virus from the inoculum.

**Effect of bacterial co-infection on the replication of SIV in NPT cells**

In order to determine the effect of *Streptococcus suis* on SIV infection, NPT cells were co-infected as described above for the results shown in Fig. 1. The course of infection was monitored by determining the amount of infectious virus released into the supernatant at different time points. The replication kinetics of the SIV strains is shown in Fig. 2. At 24 h post-virus-infection, the titre of the SIV-H1N1 virus in the *Streptococcus suis* wt co-infected sample was about 20 to 30-fold lower compared with SIV mono-infection or co-infection by *Streptococcus suis Δcps* (Fig. 2a). Furthermore, the replication rate of this virus was slower when co-infected with *Streptococcus suis* wt (Fig. 2a). When NPT cells were infected by SIV-H3N2 at the same m.o.i. (0.07), co-infection by *Streptococcus suis* wt resulted in an approximately 10-fold reduction of the amount of infectious virus in the supernatant (Fig. 2b). As the SIV-H3N2 virus replicated faster than the SIV-H1N1 virus (compare SIV mono-infection at 8 h p.i.), the replication kinetics were also determined at a 10-fold lower m.o.i. (0.007). Under these conditions, the difference in the virus titre at 24 h p.i. was about 250-fold lower in the sample co-infected by *Streptococcus suis* wt as compared with SIV mono-infection or co-infection by *Streptococcus suis Δcps*, and the virus replicated somewhat slower than in the two control groups (Fig. 2c). No reduction of viral replication was observed when heat-inactivated streptococci were used (data not shown). Analysis by unpaired Student’s *t*-test indicated that the amount of infectious SIV in the supernatant was significantly lower when cells were co-infected with *Streptococcus suis* wt than in SIV mono-infection at 24 h p.i. (*P*<0.001 in Fig. 2a, *P*<0.05 in Fig. 2b and *P*<0.01 in Fig. 2c).

**Sialic-acid-dependent binding of SIV to Streptococcus suis**

The reduced infectivity detectable in the supernatant of co-infected cells might be due to reduced virus release or to direct binding of SIV to the bacterial agent. Therefore, we further analysed the interaction between SIV and *Streptococcus suis* with a co-sedimentation assay, which could provide evidence whether a direct interaction between virus and streptococci may occur during co-infection.

Virions and bacteria were incubated for 1 h at 4°C and then subjected to low speed centrifugation that is sufficient for pelleting the bacteria but not the virus. The supernatant was analysed for haemagglutination (HA) activity (Fig. 3a) and for the presence of infectious virus (Fig. 3b). Both SIV-H1N1 (sw/Bad Griesbach/06) and SIV-H3N2 (sw/Herford/07) were found to co-sediment with *Streptococcus suis* wt as indicated by the lack of detectable HA activity (shown for the H1N1 virus in Fig. 3a) and by the reduction of the infectivity (Fig. 3b) in the supernatant after the centrifugation step. By contrast, these parameters were only marginally affected when the *Streptococcus suis Δcps* mutant instead of the *Streptococcus suis* wt strain was used for the assay or when the bacteria were pretreated with neuraminidase (Fig. 3a, b). This result demonstrates the direct binding between SIV and *Streptococcus suis* and reflects the co-infection situation.

We further analysed whether the decrease of virions in the supernatant was paralleled by an increase of virions in the pellet fraction. As shown in Fig. 3(c), substantial numbers of infectious viral particles (>500 p.f.u. ml⁻¹) were detected only in the pellet fractions of the samples where SIV-H1N1 or SIV-H3N2, respectively, had been co-sedimented with *Streptococcus suis* wt. The difference between the two samples suggests that there are differences between the two viruses in their efficiency of interaction with *Streptococcus suis* wt. Only a few infectious viral particles were detected in the Δcps co-sedimented sample. The infectivity was significantly reduced in the pellet fraction when the wt bacteria were treated with neuraminidase prior to the co-sedimentation step (Fig. 3c).

Interestingly, a striking variation in the size of the plaques was observed when the virus was co-sedimented with *Streptococcus suis* wt (Fig. 3d, wt ~NA). While some plaques were large, many plaques were very small (<1 mm). By contrast, only a few tiny plaques were visible when the *Streptococcus suis Δcps* mutant was used for co-sedimentation (about 12-fold less) and the proportion of tiny plaques was also reduced (about fourfold) when the wt bacteria had been pretreated with neuraminidase (Fig. 3d, wt + NA).

**Encapsulated Streptococcus suis inhibits HA activity of different influenza viruses**

To analyse the interaction between SIV and *Streptococcus suis* in more detail, we performed a comparative HA inhibition (HI) assay. The wt strain of *Streptococcus suis* and the mutant lacking a capsule (Δcps) were analysed for their ability to prevent different strains of influenza A viruses from agglutinating chicken erythrocytes. In addition to the SIV-H1N1 and SIV-H3N2 used in the former experiments, we included three other H1N1 SIV strains: sw/SH/1,
a classical SIV, as well as the strains sw/Bad Griesbach/06 and sw/Potsdam/81 derived from the avian-origin SIV lineage introduced into the European swine population in 1979. *Streptococcus suis* showed HI activity towards all porcine H1N1 viruses (Fig. 4). The human influenza virus strain WSN, which recognizes α2,6-linked sialic acid (Leung et al., 2012), was also inhibited by *Streptococcus suis* wt. By contrast, the mouse-adapted H1N1 virus PR8, which recognizes α2,3-linked sialic acid (Koerner et al., 2012; Tate et al., 2011), was not inhibited in its HA activity by *Streptococcus suis* wt (Fig. 4). In the case of the H3N2 viruses, *Streptococcus suis* was found to have HI activity towards the two swine viruses (sw/Herford/07, sw/Damme/06) but only marginal inhibitory activity towards the avian strain (dk/K5672), which is consistent with the preference of avian influenza viruses for α2,3-linked sialic acid. Finally, two recombinant viruses, R1 and R2, were analysed that differed by only two mutations in the receptor-binding site of the HA protein conferring a binding preference for either α2,3- or α2,6-linked sialic acid.
Matrosovich et al., 2007). R1 that recognizes α2,6-linked sialic acid was inhibited by Streptococcus suis wt, whereas R2 was unaffected (Fig. 4).

These results indicate that encapsulated Streptococcus suis (wt) inhibits the HA activity of influenza viruses that have a binding preference for α2,6-linked sialic acid. Consistent with this conclusion, lack of a capsule (Δcps in Fig. 4) or pretreatment of the encapsulated Streptococcus suis (wt) with neuraminidase (not shown) resulted in a loss of the HI activity.

Detection of α2,6-linked sialic acid on the surface of encapsulated Streptococcus suis serotype 2 (wt) strain 10 by lectin staining

We applied lectin staining to demonstrate the sialic acid linkage type present on the capsular polysaccharide of strain 10 of Streptococcus suis serotype 2 (wt). As shown in Fig. 5 (upper panels), the bacteria (stained in red) were recognized by Sambucus nigra agglutinin (SNA; green) that is specific for α2,6-linked sialic acid. In contrast to SNA staining, Maackia amurensis agglutinin II (MAA II; red, lower panels) that is specific for α2,3-linked sialic acid did not interact with Streptococcus suis (green). The sialic acid-dependent binding of SNA to encapsulated Streptococcus suis (wt) was demonstrated by including neuraminidase (NA)-treated wt bacteria and mutant Streptococcus suis lacking a capsule (Δcps) as negative controls. The experiments were performed at least three times.

**Fig. 4.** Inhibition of the haemagglutinating activity of influenza viruses by Streptococcus suis. Encapsulated Streptococcus suis (wt) and a deletion mutant lacking the capsule (Δcps) were analysed for their ability to inhibit the haemagglutinating activity of influenza viruses from different host species and subtypes. The hemagglutination-inhibition activity was determined in HI-units (HIU). The error bars indicate SEM. The experiments were performed at least three times.

**Fig. 5.** Expression of sialic acid on Streptococcus suis. Sialic acids of the capsular polysaccharide in Streptococcus suis were detected by lectin staining: MAA II (red) for α2,3-linked sialic acids and SNA (green) for α2,6-linked sialic acids. Streptococcus suis was stained either in red to contrast SNA staining (green) or in green to contrast MAA II staining (red). The sialic-acid-dependent binding of SNA to encapsulated Streptococcus suis (wt) was demonstrated by including neuraminidase (NA)-treated wt bacteria and mutant Streptococcus suis lacking a capsule (Δcps) as negative controls. The experiments were performed at least three times.
Co-infection by influenza virus and Streptococcus suis

(a)

SIV H3N2 + Streptococcus suis wt

SIV H3N2 + Streptococcus suis wt+NA

SIV H3N2 + Streptococcus suis wt Δcps

(b)

Number of adherent bacteria on virus-infected cells (bacteria number per cell)

R1+ Streptococcus suis wt

R2+ Streptococcus suis wt

Number of adherent bacteria on virus-infected cells (bacteria number per cell)

R1+wt  R1+Δcps  R2+wt  R2+Δcps
It should also be noted that no binding of Δcps was detectable when adhesion was restricted to 30 min at 4°C. The polysaccharide of the streptococci. To determine whether adhesion ability of Streptococcus suis is affected by prior SIV infection or not.

Porcine tracheal cells (NPTr) were infected by SIV sw/Herd/07 (H3N2 subtype) followed at 16 h p.i. by incubation with Streptococcus suis. In order to prevent bacterial replication, Streptococcus suis and cells were incubated for 30 min at 4°C in the presence of gentamicin. The result obtained by immunofluorescence microscopy with H3N2 SIV-infected cells is shown in Fig. 6(a). Very few encapsulated (wt) or noncapsulated (Δcps) Streptococcus suis were observed on the surface of non-SIV-infected NPTr cells (not shown). Prior SIV-H3N2 infection enhanced adhesion of Streptococcus suis wt but not that of the Δcps mutant to NPTr cells; most of the Streptococcus suis wt detected was associated with SIV-infected cells. When Streptococcus suis wt was pretreated with neuraminidase, the bacteria number found on SIV-infected NPTr cells was strongly reduced (Fig. 6a). The sialic acid-dependent adhesion of Streptococcus suis wt was also observed when cells were pre-infected with H1N1 virus (not shown). Thus, the adhesion of Streptococcus suis wt to respiratory epithelial cells was enhanced after SIV infection in a sialic-acid-dependent manner.

It should also be noted that no binding of Δcps was detectable when adhesion was restricted to 30 min at 4°C (Fig. 6a). However, the binding of Δcps to uninfected cells was detected in the previous experiment (Fig. 1), where the conditions (incubation for 2 h) were favourable for streptococci adherence and multiplication.

To analyse the importance of the haemagglutinin of influenza viruses in the sialic-acid-dependent adhesion of Streptococcus suis to virus-infected cells, we included the two viruses R1 and R2 that differ only by two point mutations in the receptor-binding site of the haemagglutinin resulting in preferential recognition of 2,3-linked sialic acid. The effectiveness of the sialic-acid-dependent binding is evident from the quantification shown in Fig. 6(b) (bottom panel). About 30 bacteria were found attached to each R1-infected cell.

Taken together, our results indicate that SIV and Streptococcus suis affect each other during co-infection of NPTr cells. Encapsulated Streptococcus suis delayed infection by each of the two SIV strains and reduced the amount of infectious H1N1 virus detectable in the supernatant. Moreover, adhesion of encapsulated Streptococcus suis was increased via the capsular sialic acid that is recognized by the HA protein expressed on the surface of SIV-infected NPTr cells.

**DISCUSSION**

Influenza viruses bind to sialylated macromolecules, often with a preference for either 2,3- or 2,6-linked sialic acid (Matrosovich et al., 2013). Recognition of this linkage type may vary substantially depending on the context of the oligosaccharide containing this sugar in a terminal position (Sauer et al., 2014; Stevens et al., 2006). In a recent report about viral-bacterial co-infection of NPTr cells, Wang et al. (2013) analysed the effect of SIV of the subtype H1N1 on Streptococcus suis serotype 2. Here we extended this analysis by including viruses from a different subtype and by also addressing the effect of the bacteria on virus infection. The polysaccharide of Streptococcus suis serotype 2 contains a rhamnose residue (Wang et al., 2013) that is absent from glycoconjugates of vertebrate cells and thus not included in
the glycan arrays that have been applied to characterize the binding properties of influenza viruses (Sauer et al., 2014; Stevens et al., 2006). Our data indicate that the capsular sialic acid of Streptococcus suis is recognized efficiently, because encapsulated bacteria competitively inhibited the HA by all human and porcine viruses analysed that have a preference for α2,6-linked sialic acid. The differences in the HI titre between different virus strains indicate that the haemagglutinins of the respective viruses recognize the capsular polysaccharide with different efficiencies. Strain-dependent differences in the interaction of SIV and Streptococcus suis were not reported by Wang et al. (2013).

The sialic-acid-dependent interaction between SIV and Streptococcus suis was also demonstrated by a co-sedimentation assay. Interestingly, bound viruses retained infectivity and induced plaque formation. However, many of the plaques were distinctly smaller in size compared with the plaques of control virus. This finding suggests a delayed replication. It remains to be shown whether viruses attached to bacteria are internalized with a delay. An alternative explanation is that viruses first have to be released from the bacterial surface by the viral neuraminidase and this enzymic action may be responsible for the delay in the replication. However, it should be noted that the amount of infectious virus that could be recovered in the supernatant fraction after a washing step was low. Additional incubation of the bacterial pellet at 37 °C which allowed the viral neuraminidase to cleave the bacterial sialic acids did not result in an increased amount of virus released into the supernatant (data not shown). Additional application of bacterial neuraminidase from Clostridium perfringens was also not effective in this respect (data not shown). The interaction between SIV and Streptococcus suis appears to be very tight or even irreversible.

The effect of SIV on Streptococcus suis was also evident when the latter was added to SIV-infected NPTr cells. When Streptococcus suis was added at 2 h p.i. under conditions that permit bacterial multiplication, adherence of Streptococcus suis was substantially enhanced and the bacteria were predominantly found attached to virus-infected cells at 8 h p.i. This result confirmed the data reported by Wang et al. (2013) for H1N1 virus. We have shown that enhanced adherence to SIV-infected cells also occurs with H3N2 virus.

From previous studies it is known that mutants of Streptococcus suis lacking a capsule bind more efficiently to cultured cells than do encapsulated wt bacteria (Benga et al., 2004). The same result was obtained with uninfected NPTr cells. This binding was observed after incubation for 2 h at 37 °C. As shown in our study, no binding of the capsular mutant was detected when adhesion was restricted to 30 min at 4 °C. However, these restrictive adhesion conditions are sufficient for binding of Streptococcus suis (wt) to SIV-infected cells. Therefore, adherence of Streptococcus suis wt to SIV-infected cells is more efficient than binding of streptococci lacking a sialylated capsular polysaccharide and, thus, infection of pigs by SIV is expected to significantly facilitate colonization by Streptococcus suis. Streptococci that lack sialic acid cannot make use of this strategy to enhance bacterial infection. These pathogens have found different ways to use co-infection with influenza viruses for enhancement of infection. For example, it has been shown that the sialic acid released by the viral neuraminidase can be used by streptococci as a metabolite to support bacterial colonization (Siegel et al., 2014); furthermore, release of sialic acids from the cell surface may render cellular ligands accessible for bacterial adhesion (Nita-Lazar et al., 2015). Furthermore, influenza virus infection may support subsequent bacterial infection also by factors not related to sialic acid. In mouse infections, bacterial infection was enhanced even after clearance of influenza virus infection (Smith et al., 2013).

While SIV has a positive effect on Streptococcus suis (serotype 2) infection, bacterial co-infection had a negative effect on SIV-infection. Although the infectivity titre determined at 48 h p.i. in the co-infected samples was similar to that of cells infected by H1N1 virus only, there was a more than 10-fold difference at 24 h p.i. For the H3N2 virus, the difference was less pronounced. However, this virus replicates faster than the H1N1 virus. When H3N2 virus was applied at a 10-fold lower m.o.i., there was a more than 100-fold difference in the infectivity at 24 h p.i. between the co-infected and mono-infected samples. The difference in the amount of virus released may — at least in part — be explained by the direct binding of virions to bacteria. However, it cannot be excluded that the bacteria bound to the surface of infected cells have a detrimental effect on virus budding resulting in a reduced number of virus particles released into the supernatant. The importance of the direct binding between influenza viruses and streptococci is also evident from a comparison with different co-infection partners. When mice were infected by influenza virus (PR8) and Streptococcus pneumoniae, which lacks sialic acid on the capsular polysaccharide, influenza virus infection was even enhanced under co-infection conditions compared with mono-infection (Smith et al., 2013). Our findings do not exclude the possibility that factors other than direct viral–bacterial interactions may also play a role in the outcome of infection; for example, the increased production of local proinflammatory mediators in co-infected NPTr cells (Wang et al., 2013) may affect the replication efficiency.

Streptococcus suis serotype 2 has zoonotic potential (Feng et al., 2014). As mentioned above, it shows a sialic-acid-dependent interaction not only with SIV but also with human influenza viruses (Fig. 4). Therefore, the possibility has to be considered that infection of humans by Streptococcus suis may be enhanced under conditions of co-infection with human influenza viruses. In this context it is interesting to note that group B streptococci have a similar capsular polysaccharide with terminal sialic acids. However, these sialic acid residues are connected to galactose via an
z2,3 rather than an z2,6-linkage (Jennings et al., 1983) and thus are not preferred ligands for human influenza viruses. Our analysis of the co-infection of porcine tracheal cells by Streptococcus suis serotype 2 and SIV of both H1N1 and H3N2 subtypes shows that SIV has a beneficial effect on the bacteria whereas Streptococcus suis has an inhibitory effect on virus infection. However, viral–bacterial co-infection has to be considered as a multifactorial interaction between the two partners. Sialic-acid-dependent interaction is only one factor among these complex events. There may be other factors that enhance virus infection under conditions of bacterial co-infection. Studies with Madin–Darby canine kidney (MDCK) cells have demonstrated that Streptococcus suis releases a protease that is able to proteolytically activate influenza viruses and thus to enhance infectivity (Wang & Lu, 2008). Under appropriate conditions, such an enhancement may also occur in NPTr cells. Furthermore, the innate immune response may be different in mono- and co-infected cells and thus may affect SIV infection. Such factors have to be analysed for Streptococcus suis in the future to get a more comprehensive picture of how infection by the individual pathogens is altered under conditions of co-infection.

METHODS

Cells, influenza viruses and bacteria. Newborn pig trachea cells (NPTr; provided by François Meurers, INRA, Nouzilly, France) and Madin–Darby canine kidney cells (MDCK II) (Punyadarsaniya et al., 2011) were maintained in Eagle’s minimal essential medium (EMEM; Gibco) supplemented with 5 % (NPTr) or 10 % (MDCK II) FBS (Biochrom). Cells were maintained at 37 °C in a humidified 5 % CO2 atmosphere.

Five SIVs, four human influenza viruses and one avian influenza virus were used in this study. SIV of H1N1 subtypes A/sw/Potsdam/15/1981 (sw/Potsdam/81) and A/sw/Bad Griesbach/IDT5604/2006 (sw/Bad Griesbach/06), and H3N2 subtypes A/sw/Herford/IDT5932/2007 (sw/Herford/07) and A/sw/Damme/IDT5673/2006 (sw/Damme/06) have been described in a previous study (Meng et al., 2013). The SIV strain A/Swine/Shanghai/1/2005 (sw/SH/1) of the H1N1 subtype has only been used by Chengping Lu (Nanjing Agricultural University, China). Two H1N1 subtype strains of human influenza virus were used: A/Puerto Rico/8/34 (PR8) was provided by Wolfgang Garten (Philips-University Marburg, Germany) and A/WSN/33 (WSN) was obtained from Roland Zell (University of Jena, Germany). The recombinant influenza viruses, R1 and R2 (Matrosovich et al., 2008), were provided by Mikhail Matrosovich (Philips-University Marburg, Germany). The avian influenza virus strain of H3N2 subtype A/Sentinel duck/Lake Constance/SA632/2008 (dk/5672) was provided by Martin Beer (Friedrich-Loeffler-Institut, Insel Riems, Germany). Virus stocks were propagated in MDCK cells (SIV, WSN, R1 and R2) or 10-day-old specific-pathogen-free embryonated chicken eggs (PR8 and dk/5672) propagated in MDCK cells (SIV, WSN, R1 and R2) or 10-day-old avian influenza virus strain of H3N2 subtype A/Sentinel duck/Lake Constance/SA632/2008 (dk/5672) were added and incubated at 37 °C. After removal of the enzyme, viruses were washed three times with cool PBS and then subjected to different assays.

Lectin staining. Lectin staining of bacterial surfaces was performed with Sambucus nigra agglutinin (SNA) or M. amurensis agglutinin II (MAAII). For detection of z2,6-linked sialic acids, FITC-labelled SNA (Vector laboratories) was used and biotinylated MAAII was applied to determine z2,3-linked sialic acids on the surface of bacteria that had been untreated or pretreated with neuraminidase. The binding of biotinylated lectins was visualized by incubation of the samples with streptavidin-Cy3 (Sigma-Aldrich) followed by fluorescence microscopy.

HA and HI assay. The haemagglutinating activities of influenza viruses and the HI activities of Streptococcus suis were determined by HA and HI assays, modified from a World Health Organization and the World Organisation for Animal Health protocol (OIE, 2012; World Health Organization, 2011). To evaluate the HA activity, serial dilutions were prepared from Streptococcus suis (wt or the deletion mutant Δcps), followed by addition of 1 % chicken erythrocytes in 96-well U-bottom plates and incubation at room temperature. To determine the HI activity, Streptococcus suis was applied at different concentrations, pretreated or non-pretreated with neuraminidase (described above). Influenza A virus (8 HAU) was added and incubated at room temperature. Afterwards, 1 % chicken erythrocytes were added and the HI titre was determined.

Co-sedimentation of SIV and Streptococcus suis. To determine whether SIV would bind directly to Streptococcus suis, a co-sedimentation analysis was performed. SIV was incubated with Streptococcus suis which had been pretreated or non-pretreated with neuraminidase as described above. Following 1 h of incubation at 4 °C, supernatants and pellets were collected carefully. Streptococcus suis (1 × 105 c.f.u.) was mixed with 1 × 106 TCID50 ml−1 of SIV (bacteria/virus ratio of 1000) and rotated at 4 °C for 1 h. Supernatants and pellets were collected to evaluate the infectivity of SIV by determining the TCID50 or by plaque assay on MDCK cells, respectively. In addition, pellets were carefully washed with PBS to remove unbound SIV prior to the plaque assay.

A larger amount of virus was required to analyse the supernatant for HA activity. For this purpose, 100 μl of SIV (1.8 × 109 TCID50 ml−1) was mixed with 2.7 × 10−5 c.f.u. of Streptococcus suis at 4 °C. After 1 h of incubation, the supernatants were collected and analysed by HA assay.

Virus titration. The infectivity of the viruses was evaluated by a TCID50 assay or plaque assay on MDCK cells as previously described (Meng et al., 2013; Punyadarsaniya et al., 2011).

Co-infection of NPTr cells by SIV and Streptococcus suis. SIV (H1N1 or H3N2 virus) was inoculated onto NPTr cells at a m.o.i. of 0.07 (H1N1 and H3N2) or 0.007 (H3N2 only). After 1 h of incubation at 37 °C with infection medium (EMEM containing 1 μg ml−1 acetylated trypsin), cells were washed twice with PBS and further maintained at 37 °C. At 2 h post-virus-infection, NPTr cells were infected with Streptococcus suis (wt or Δcps) at an m.o.i. of 100. Following 2 h of incubation with infection medium, cells were washed with PBS and fresh infection medium was added. Supernatants were harvested at 4, 8, 24, 48 and 72 h post-virus-infection and titrated by TCID50 assay on MDCK cells. NPTr cells were fixed with 3 % paraformaldehyde at 4, 8, 24 and 48 h post-virus-infection for immunofluorescence analysis.
To evaluate the adherence ability of *Streptococcus suis* after infection by influenza viruses, NPTr cells were infected with SIV-H3N2, R1 or R2 at an m.o.i. of 0.02. At 16 h post-virus-infection, cells were incubated with *Streptococcus suis* wt strain, wt strain pretreated with neuraminidase or the Δcps mutant plus gentamicin (final concentration 50 μg ml⁻¹) at 4°C for 30 min. The same result was obtained when the antibiotic was omitted (not shown).

**Immunofluorescence analysis (IFA).** NPTr cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. Antibodies against influenza A virus nucleoprotein (AbDSerotec; mouse) and rabbit anti-*Streptococcus suis* antisera (Benga et al., 2004) were used as first antibodies, followed by incubation with Alexa Fluor 488 anti-mouse IgG (H+L) antibody (Invitrogen) and Alexa Fluor 568 anti-rabbit IgG (H+L) antibody (Invitrogen), respectively. All antibodies were diluted in 1% BSA and incubated for 1 h at room temperature. Finally, nuclei were stained with DAPI and the samples were embedded in Mowiol. IFA was performed using a Nikon Eclipse Ti fluorescence microscope and the NIS Elements AR software (Nikon). Furthermore, the analySIS 3.2 software (Soft Imaging System) was used to quantify bacterial attachment by analysing the area of bacterial fluorescent signals on the cell surface.

**Statistical analysis.** Data in the figures are shown as the means ± SEM. All statistical analyses were carried out using Prism 5 software (Prism 5 for Windows; GraphPad Software).

**ACKNOWLEDGEMENTS**

This work was performed by N. W. and F. M. in partial fulfilment of the requirements for the PhD degree from the University of Veterinary Medicine Hannover. N. W. was a recipient of a Georg-Christoph-Lichtenberg scholarship from the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology, and Translational Medicine (HGNI). F. M. was a recipient of a fellowship from China Scholarship Council. This work was supported by a grant from Deutsche Forschungsgemeinschaft (He1168/15-1) and from Bundesministerium für Bildung und Forschung (FluResearchNet, project code 01KI1006D). We are grateful to Michaela Schmidtke (University of Jena, Germany), Ralph Dürrwald (IDT Biologika GmbH, Dessau-Rosslau, Germany), Chengping Lu (Friedrich-Loeffler-Institut, Insel Riems, Germany), Martin Beer (Friedrich-Loeffler-Institut, Insel Riems, Germany) and Hilde Smith (Wageningen UR, Netherlands) for kindly providing viral and bacterial strains.

**REFERENCES**


