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SCIENTIFIC REPORT submitted to EFSA

A scientific evaluation of pork, pork products and turkey meat as a possible source of foodborne infection with novel H1N1 (nH1N1) influenza virus in humans[1]

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Summary

This report describes the genetic make up and presumed swine origin of the novel (pandemic) 2009 H1N1 influenza virus that has caused a sustained infection and pandemic in humans. Swine are readily infected upon experimental inoculation with human nH1N1 isolates and infected humans have occasionally transmitted the virus to swine in the field. However, swine-to-human transmission has not been reported so far. Turkeys became infected after experimental inoculation via the reproductive tract, but not via the respiratory route, and there have been a few outbreaks of nH1N1 in turkey breeder hens in the field. The infections in swine and turkeys as food-producing animals have raised questions about the possibility that food or food products from these animal species could pose a risk of foodborne infection in humans.

This possibility was analysed on the basis of specific pathogenetic features of nH1H1 infection in swine and turkeys and on the basis of biological and physico-chemical characteristics of type A influenza viruses in general. The different requirements for a virus infecting food-producing animals to cause a foodborne infection in humans were discussed and applied to nH1N1 influenza virus.

So far, reverse zoonosis with humans serving as a source of infection for swine, has been described in several countries, but zoonotic transmission from pigs or turkeys to humans has not been reported. This does, however, not exclude food as a possible source of infection for humans. It was shown repeatedly that nH1N1 infection in pigs only involves the respiratory tract and that there is no viraemia or dissemination to other organs. nH1N1 virus does not reach muscles and thus does not colonize meat. Low titre virus contamination of pork or pork products by respiratory excretions at slaughter or at processing cannot be excluded.

The nH1N1 virus, when ingested, would have to overcome different hurdles upon arriving in the gastro-intestinal tract. Mammalian influenza viruses are susceptible to acid pH and to bile salts, both of which may exert an inactivating effect. There is no evidence that the gastro-intestinal tract of humans can serve as a portal of entry or target organ for influenza viruses of swine and this premise can be extrapolated to nH1N1 virus. So, nH1N1 virus if and when ingested with contaminated food products, particularly when present at low titres or when the food is eaten raw, is highly likely to become inactivated prior to arrival in the intestines.

Virus that has been swallowed, possibly at high titres e.g. with respiratory secretions in infected pigs, does not replicate in the intestines. Furthermore, in contrast to typical enteric human viruses known to cause foodborne infections, the infectivity of influenza viruses is poorly resistant to physical and chemical agents. Influenza viruses are susceptible to heat and threshold values obtained with the frequently studied avian influenza viruses can be applied to all influenza viruses, including nH1N1, because their inactivation kinetics at increasing temperatures are similar. Heating at 70°C and thus moderate cooking inactivates high virus titres within seconds, even when the virus is embedded in meat products or in by-products. While less is known about the pathogenesis of potential nH1N1 infection in turkeys, the same rules would apply.

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Most disinfectants used for disinfecting equipment that is possibly contaminated with influenza virus during food processing, easily destroy these viruses, as do the common lipid solvents, which act on the lipoprotein outer envelope of the virion.

All these factors were discussed and, based on this evaluation, it was concluded that pork or pork products or turkey meat possibly contaminated with nH1N1influenza virus are not a foodborne threat.

Key words: influenza, H1N1, pigs, pork, turkey, food.

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REPORT

1. Origin and characteristics of the novel H1N1 (nH1N1) 2009 influenza virus relative to other influenza viruses.

A novel H1N1 influenza virus was first detected in humans in Mexico and North America in March and April 2009 and subsequently spread worldwide. By 11 June 2009, 74 countries had officially reported 28,774 cases including 144 deaths and the World Health Organization declared the first influenza pandemic in 41 years. Information on the current status of the pandemic can be found at http://www.who.int/csr/disease/swineflu/en/index.html).

Phylogenetic analyses showed that the virus is a reassortant of at least 2 circulating swine influenza viruses (SIVs) (Garten et al. 2009, Smith et al. 2009). Six gene segments were similar to ones previously found in triple reassortant SIVs circulating in pigs in North America, from which the "pandemic H1N1 2009" influenza virus has derived its "classical swine" H1 haemagglutinin (HA). These North American swine viruses already have a mix of avian, human and swine virus genes. The genes encoding the neuraminidase and matrix proteins were more closely related to those in influenza viruses circulating in swine populations in Europe or Asia.

Influenza viruses of H1N1, H3N2 and H1N2 subtypes are endemic in swine populations worldwide (reviewed by Olsen et al. 2006). Some of these SIVs are (in part) of human origin, but they are antigenically and genetically distinct from contemporary seasonal human H1N1 and H3N2 viruses, because the human and swine viruses have evolved in distinct lineages. As an example, the 1918 pandemic H1N1 virus is the progenitor of both the so-called "classical swine" H1N1 virus circulating in pigs in North America and the contemporary seasonal H1N1 viruses of humans. Over the years, the seasonal human H1N1 virus has undergone considerable antigenic drift in its H1. The classical swine H1N1 SIVs have remained relatively stable antigenically, but during the last 10 years a series of reassortment events led to the emergence of "triple reassortant" H1N1 and H1N2 viruses with a higher degree of genetic and antigenic diversity (Vincent et al. 2008). This disparate evolution has resulted in an increasing antigenic divergence between North American swine and human influenza viruses of the H1N1 subtype. Furthermore, the SIVs in Europe differ significantly in their antigenic and genetic make-up from those circulating in North America, while still other variants are circulating in various Asian countries (Brockwell-Staats et al. 2009). The dominant H1N1 SIV in Europe, for example, entered pigs from the avian reservoir around 1979 and is designated as "avian-like" H1N1 SIV. Because the H1 of the novel pandemic H1N1 virus belongs to the classical swine lineage, it shows relatively close similarity to SIVs

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circulating in North America and to earlier (before 1950) human H1N1 viruses, but it is antigenically very different from H1 SIVs in Europe and from contemporary human H1N1 viruses (Gatherer et al. 2009, Morens et al. 2009, Peiris et al. 2009).

One of the most remarkable characteristics of the 2009 pandemic H1N1 virus as compared to the well-known SIVs is sustained human-to-human transmission, and the virological basis for this increased fitness in humans remains unknown. The novel H1N1 virus most likely emerged by genetic reassortment between existing SIVs in pigs, but it had never been reported in swine anywhere at the time of its first detection in humans. This is not surprising given the lack of surveillance for influenza viruses in swine populations, especially in Central and South America and in Asia, where the virus may have originated. The available genetic data suggest that the precursor of the pandemic virus has been circulating undetected for more than a decade and that virus transmission to humans resulted from a single event (Garten et al. 2009, Smith et al. 2009).

The novel H1N1 virus was almost certainly absent from European swine populations in the past, as it has never been reported by the European Surveillance Network for Influenza in Pigs (ESNIP; www.esnip.ugent.be) that operated from 2001 to 2008. Between mid May 2009 and the time of writing, the novel H1N1 virus has been reported on swine farms in several countries. Experimental infection studies have also confirmed the susceptibility of pigs to the novel virus and its capacity for swine transmission. Data on natural and experimental infections of pigs are presented in more detail in Section 4 of this document. Importantly, there is no evidence so far that pigs have played a role in the spread of the virus in the human population.

In summary, the pandemic 2009 H1N1 influenza virus has a new and unique gene segment combination, not previously identified anywhere. Despite its swine origin, the virus spreads readily and globally from human-to-human, requiring no contact with swine, and it is therefore regarded as a human influenza virus. This novel virus has been predominant in the human population since June – July 2009; meanwhile the previously circulating "seasonal" H1N1 and H3N2 viruses have circulated at very low levels.

The nomenclature of this new pandemic virus has been somewhat confusing. In this document we will use the denomination "novel H1N1" (nH1N1) influenza virus. This means the same virus as "pandemic H1N1 2009" influenza virus or "H1N1 variant" (H1N1v), terms that are used in some other documents.

2. General requirements for a virus to be able to cause a foodborne infection in humans

For a virus that infects food-producing animals, several conditions need to be fulfilled in order for it to serve as a candidate to cause foodborne infections in humans. It is the purpose of the present chapter to discuss these conditions so that they can be applied further to pork and pork products derived from nH1N1 infected swine. Similar requirements may apply to meat and meat products from potentially infected turkeys .

Foodborne viral infections in humans regularly occur when food products (such as meat, vegetables ….) are contaminated by a human virus during preparation via a food handler who

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is a carrier and is excreting the virus at the time of handling. These circumstances thus typically involve human pathogens such as hepatitis A virus, norovirus, human enteroviruses and others, but these viruses are out of the scope of this document since they do not cause infections in animals and do not originate from food-producing animals. The present requirements only relate to food products of animal origin containing an animal virus with which the animal was infected at the time of food harvest or at slaughter. Possible contamination during further processing of the organs or meat, e.g. with excretions or secretions containing the animal virus (but not through a human as carrier) is also considered.

The major requirements for such viruses to cause a foodborne infection in humans are summarized here:

The virus must have zoonotic potential, i.e. it can cross the species barrier between animals and humans and infect humans. The zoonotic properties of nH1N1 virus will be discussed later. Throughout this document we will use the word zoonosis in its simplest definition, i.e. an infectious disease that can be transmitted from other vertebrate animals to humans. To designate virus transmission from humans to non-human animals we use the term "reverse zoonosis".

The virus should be able to reach muscle tissue or other edible organs or tissues during its course of infection in the food-producing animal, but not necessarily in every exposed animal. Experimental inoculation and pathogenesis studies have been carried out with nH1N1 in pigs and other food-producing animal species and these will be reviewed to assess the dissemination of nH1N1 in the body. Also, the possibility that meat or edible organs become contaminated with virus at slaughter or during processing will be considered.

The virus must be able to establish infection in humans via the alimentary tract. The available information on the pathogenesis of nH1N1 in humans and/or human models will be reviewed and discussed with emphasis on the gastro-intestinal tract as a potential portal of entry.

The dose of virus taken up with food and reaching the potential portal of entry in the gastro-intestinal tract should be sufficiently high to initiate the infection, i.e. the minimal infectious dose must be exceeded. This is related to the likelihood that a virus (i) will have resistance to physico-chemical barriers such as adsorption to mucosal surfaces, effect of gastric juices, enteric enzymes, acid pH, bile salts and other hurdles influencing its survival within the gastro-intestinal tract and its entry into the body, (ii) will reach its target cells (if they exist), and (iii) will be able to bind via suitable ligands to specific receptors on these cells.

The virus should be able to resist other hurdles related to the effects of food processing (e.g. cooking, salting and curing). Viruses in food that is to be consumed cooked, need to survive the heat treatment. Animal viruses are very variable in their stability to increases in temperatures. Also, the medium in which viruses are embedded may protect or stabilize virus from heat or chemical inactivation.

All these conditions and circumstances will be discussed with respect to nH1H1 influenza virus as a potential cause of foodborne infection via pork and pork products or turkey meat.

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The different events will be analysed in the light of the pathogenesis of nH1N1 virus and of biological and physico-chemical characteristics of the well-known mammalian H1 influenza viruses, since these have been studied in more detail than nH1N1.

3. nH1N1 influenza virus as potential cause of foodborne infection

The different criteria specified in the previous chapter will now be evaluated and discussed.

3.1. Zoonotic properties and transmission of nH1N1

It is known for a long time that swine influenza is a zoonosis, and most of the well-known SIV subtypes and genotypes circulating in North America, Europe and Asia have occasionally been detected in respiratory samples of humans (Myers et al. 2007, Van Reeth 2007, Van Reeth and Nicholl 2009). These zoonotic infections are usually clinically similar to the disease caused by infections with human influenza viruses More than 50% of these human cases reported a recent exposure to pigs. Secondary transmission is only rarely reported, and none of these infections led to sustained human-to-human transmission. There are less than 100 documented cases of SIV in humans since 1958, but serologic studies suggest that zoonotic SIV infections are under-reported. Unfortunately, the serologic data are difficult to interpret, because of partial serologic cross-reactivity between human and swine influenza viruses. The true incidence and the magnitude of the risk of zoonotic SIV infections therefore remain unknown.

In common with the established SIVs, the nH1N1 virus most likely represents a zoonosis. As already explained, there is strong evidence that the virus is of swine origin and that transmission from pigs to humans has been a single event. Unlike the well-known SIVs, the nH1N1 virus spreads readily between humans and the molecular basis for this adaptation to humans is still unclear. Until now, however, the zoonotic potential of the nH1N1 virus remains unproven. Conversely, there are reports or suspicions of human-to-pig transmission of nH1N1 in several countries including Argentina, Canada, Australia, Northern Ireland, the Republic of Ireland, the USA and several others, but the case in Norway is most interesting (Hofshagen et al. 2009). The majority of nH1N1 infected swine herds in Norway also seem to have been infected by humans. As of 26 October 2009, a total of 23 swine herds throughout the country had tested positive for nH1N1. Fifteen of these 23 herds were in contact with people diagnosed with nH1N1 or with influenza-like disease. In another 5 of the 23 herds, contact with nH1N1 infected humans was suspected, while no information was available for the remaining 3 herds.

It cannot be excluded that the virus spreads by aerosol between swine farms, but this has not been demonstrated or confirmed. Furthermore, there are no indications that pigs have played a role in the epidemiology and spread of the virus in the human population, though the virus is now present in swine populations worldwide. nH1N1 virus is therefore considered a reverse zoonosis, i.e. a zoonotic infection that has been transmitted back from humans to its original animal host.

Though proof is lacking, genetic data indicate that the nH1N1 virus most likely has the potential to spread from pigs to humans. Indeed, full genome sequencing of nH1N1 isolates

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from pigs shows a very high similarity to nH1N1 isolates from humans in all 8 gene segments (Hofshagen et al. 2009). Several factors may contribute to the lack of swine-to-human transmission in the field or the lack of reported cases. First, nH1N1 is far less widespread in swine populations than the typical enzootic SIVs. So far there are no reports of nH1N1 infection in most swine-producing regions of Europe. Second, part of the human population was already immune against nH1N1 by the time the virus started to spread to pigs. Third, no virological diagnosis is made in most cases of influenza-like illness in humans in contact with pigs. Finally, nH1N1 infection can be subclinical in swine as well as in humans. The first two factors will also decrease the likelihood of zoonotic spread of nH1N1 virus to humans via food.

3.2. Pathogenesis and infection with nH1N1 in pigs

3.2.1. Experimental infections

Data on the dissemination of virus during infection within the body, leading to the presence of the virus in edible products derived from food-producing animals, are needed in order to further evaluate their risk for food safety.

Early in the human pandemic and before the nH1N1 virus was reported in swine herds worldwide, experimental pig infection studies were performed with nH1N1 isolates from humans. These studies have shown that immunologically naïve pigs are highly susceptible to the novel virus (Itoh et al. 2009, Lange et al. 2009, Vincent et al. 2009, Brookes et al. 2010, Weingartl et al. 2010).

Brookes et al. (2010) inoculated 4-5 week old pigs with the human California/07/09 nH1N1 isolate by intranasal aerosol. The pigs developed mild clinical signs, even milder than those typical of the well-known, enzootic SIVs. nH1N1 viral RNA was detected in nasal swabs of all inoculated pigs and transmission to in-contact pigs readily occurred during the 4 cycles examined. Some infected pigs continued to show intermittent, low level shedding between 10 and 16 days post inoculation. Lower amounts of virus were detected in oral and ocular swabs. Rectal shedding was detected on just 3 occasions from only 2 of 11 inoculated animals, indicating that the virus has no tropism for the alimentary tract. Using virus isolation or immuno-histochemistry, positive samples were found only in the respiratory tract including turbinates, nasopharynx, thoracic trachea, lungs and associated lymph nodes. Virus was undetectable in plasma, spleen, liver, kidney or muscle (*longissimus dorsi and biceps femoris*), indicating the absence of viraemia. The ileum was also negative, confirming that the intestine is not a target organ. This study clearly showed that the nH1N1 virus has a similar infection profile in pigs as the well-known SIVs that are enzootic in swine populations. Transmission readily occurred to contact pigs, which allows one to conclude that the nH1N1 virus can maintain itself efficiently in immunologically naïve pig populations.

Another pig inoculation study was performed with the human Regensburg/D6/09 nH1N1 isolate (Lange et al. 2009) and the results obtained were similar: mild clinical signs of respiratory disease and transmission to “in-contact” pigs but no transmission to “in-contact” chickens. Diarrhoea developed between 3 and 7 days post inoculation in several inoculated and contact infected pigs. This was thought to be due to a “general compromised condition

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induced by the infection" rather than to influenza virus infection of the gastro-intestinal tract. Virus was isolated from nasal swabs up to 11 days post inoculation. Again, plasma samples were negative for viral RNA, demonstrating the absence of viraemia.

In a Canadian study (Weingartl et al. 2009) pigs were inoculated intranasally or intratracheally with 2 different nH1N1 isolates, either the swine-derived Alberta/OTH-33-8/2009 isolate or the human-derived Mexico/InDRE4487/2009 isolate. With both isolates, pigs developed mild clinical respiratory disease and shed virus for 7-8 days after inoculation, as demonstrated by virus isolation from nasal swabs. The course of experimental infection, macroscopic and microscopic lung lesions and the pattern of virus shedding from the upper respiratory tract resembled those of other influenza A virus infections in swine. The authors concluded that the 2 isolates showed differences in the extent of recovery of infectious virus from the lungs. The swine-derived isolate was less frequently isolated from the lungs and at lower titres, while the human-derived isolate was consistently demonstrated at higher titres. RNA copy numbers and numbers of viral-antigen positive cells in the lungs, on the other hand, were comparable in both groups, which cannot be explained. It should also be noted that conclusions about differences in virus recovery were based on the results of 2-3 pigs only. In both groups, muscle, rectal swabs, blood and submandibular lymph nodes tested negative for viral RNA as determined by RT-PCR.

Miniature pigs became infected after intranasal inoculation with the human California/04/09 nH1N1 isolate with efficient replication in the respiratory tract, but the infection remained subclinical (Itoh et al. 2009).

All these studies in pigs allow one to conclude that the human nH1N1 virus causes a specific respiratory infection in pigs. The pathogenetic course is very similar to that of contemporary endemic H1N1, H1N2 and H3N2 SIVs, which usually do not cause viraemia or virus spread to meat or edible organs. In only two limited studies, H1N1 SIV was isolated from blood samples at low titres for a very short period of time (Romijn et al. 1989, Brown et al. 1993). There are, however, no reports of influenza A virus isolation from pork following natural infection by SIVs.

That the presence of nH1N1 virus is indeed limited to the respiratory tract of pigs was recently re-established in a study specifically aimed at examining fresh pork from experimentally infected pigs (Vincent et al. 2009). Pigs were inoculated intratracheally with 2 human-derived isolates, either Mexico/4108/2009 or California/04/2009. Virus was readily recovered from tonsillar tissues and lungs but not from the lymph nodes, serum, spleen, liver, kidney, faeces or muscle.

3.2.2. Natural infections of pigs with nH1N1

Pathogenesis studies in cases of nH1N1 in pigs in the field, which seemed to result from reverse zoonosis, have not been reported, except for virus isolation from nasal swabs. Clinical disease, if present, was mild and similar to that observed in the inoculated or "in-contact" pigs of the pathogenesis studies cited above (Hofshagen et al. 2009, Howden et al. 2009, Weingartl et al. 2010). Also, there is no reason to assume that the pattern of virus dissemination within the body in naturally infected pigs is different from that in experimentally inoculated pigs.

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In conclusion, the pathogenesis studies in pigs show that, during infection with nH1N1, the virus replicates readily in the respiratory tract but does not cause viraemia and fails to colonize meat or edible organs. Pork and pork organs can normally not be considered as a threat for food safety from a pathogenetic point of view. Virus may be present in turbinates, tonsillar tissues, oropharynx, lungs and associated lymph nodes. It cannot be excluded that meat or organs may become externally contaminated by respiratory excretions or contact with infected lungs during slaughter or during meat processing. Some of these tissues can be eaten raw, such as in minced pork. Whether consumption of such contaminated food can lead to an infection in humans upon ingestion depends on whether or not the intestinal tract of humans represents a portal of entry and whether or not the virus can reach cells at the potential entry site in a sufficient quantity to initiate infection.

3.3. Pathogenesis and infection with nH1N1 in avian species

Turkeys

Two foci of natural nH1N1 influenza virus infection occurred in July 2009 in turkey breeder flocks in Chile (Pantin-Jackwood et al. 2010). The virus isolated showed 99.5 % similarity in its HA gene sequence to the human California strain and a 100 % match to the human strain circulating in Chile at that time. The clinical signs were characterized by a sudden egg drop in layers and by altered egg shells. Egg production resumed after about 20 days and returned to normal levels. No mortality was seen. This represented the first detection of nH1N1 transmission to a non-mammalian species. Infected humans had been in contact with the turkeys and, therefore, these cases likely represent an occasional transmission from infected humans to turkeys. Further limited cases of nH1N1 infection in turkey breeding farms have been reported in Canada, the USA and France.

Surprisingly, turkeys were found to be resistant to infection with nH1N1 virus following experimental inoculation via the respiratory route (Terregino et al. 2009, Russell et al. 2009, Swayne et al. 2009). Different human-derived nH1N1 isolates were used in these studies. Turkey poults or adult turkey hens were inoculated oronasally, intranasally or intraocularly – or via a combination of nasal and ocular inoculation routes - with low and high virus doses. Russell et al. (2009) found only 2 buccal swabs weakly positive by PCR. These swabs had been obtained 2 days post inoculation of turkeys with the highest viral dose and tested negative in virus isolation. No virus could be recovered from cloacal swabs or tissue samples and all birds were negative for heamagglutination inhibiting (HI) antibodies. Terregino et al. (2009) also reported the absence of virus recovery by molecular or conventional methods from blood, tracheal and cloacal swabs, lungs, intestine or muscle tissue. Seroconversion was detected in a limited number of birds with the homologous antigen only. In agreement with the previous studies, Swayne et al. (2009) could not detect nH1N1 virus from swabs or from internal tissues of turkeys by virus isolation or PCR. All turkeys were also negative for antibodies to the virus at 15 days post inoculation.

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More recently, the team of David Swayne demonstrated that nH1N1 infection could be established after intrauterine or intracloacal inoculation of turkey hens (Pantin-Jackwood et al. 2010). Turkeys inoculated via both routes had virus in their oviducts and viral antigen was visualized in the epithelia of the ovary and oviduct, but no virus could be isolated from the lungs, spleen, heart and kidney. The birds seroconverted, and the intrauterine inoculation resulted in mild diarrhoea and decreased egg production. According to this experimental infection study, it is possible that turkey breeder hens in the field became infected by infectious humans during artificial insemination.

Based on all these data, one should be vigilant for the potential emergence of nH1N1 variants with an increased ability to infect turkeys via the natural, respiratory route of infection. Experimental studies with the currently circulating nH1N1 viruses, however, have clearly shown that the virus is not likely to be transmitted to meat turkeys in its present form. Viraemia or virus spread to muscle tissue are unlikely to occur after intrauterine inoculation, given the absence of virus recovery from the lungs and internal organs in the experimental study. Therefore and from a practical point of view, turkeys are not considered as representing a food safety issue at this time.

Chickens

Eleven 3-week-old chickens were also inoculated with the human A/Mexico/4108/2009 nH1N1 isolate in the above mentioned study by Swayne et al. (2009), using a similar inoculation method and dose as for the turkeys. None of the chickens became infected or seroconverted.

In another experimental study, chickens in close contact with nH1N1- infected pigs did not become infected and did not show seroconversion (Lange et al. 2009).

Ducks

Two-week-old domestic ducks (n=11) were also included in the study by Swayne et al. (2009) using the same materials and methods as for turkeys and chickens. No virus was detected in these ducks but one intranasally-inoculated animal had seroconverted at 15 days post inoculation, with an HI titre of 16.

Quail

Eleven Japanese quail were also included in the study by Swayne et al. (2009) referred to earlier. Virus was detected in oropharyngeal swabs at 2 and 4 days post inoculation and these quail seroconverted by 15 days. The infected quail showed rhinitis with influenza virus replication in epithelium and macrophages in the nasal cavity as shown by immuno-histochemical analysis. Neither lesions nor viral antigens were found in other respiratory or non-respiratory tissues. The infected quail failed to transmit the virus to contact-exposed quail.

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Based on the available experimental data, we conclude that the avian species examined are not target animals for nH1N1 infection via the natural, respiratory route, though infection cannot be completely excluded. At present, avian species have not presented a risk to animal health or food safety regarding nH1N1.

3.4. Infection and pathogenesis in humans and in animal models used for human influenza

The nH1N1 influenza virus, even though of swine-origin, has become a pandemic human virus that is transmitted between humans in a similar way as the so- called seasonal human influenza A viruses of subtypes H1N1 and H3N2. The nH1N1 virus can now be considered as another widespread human influenza virus and it may or may not replace the seasonal viruses in the future. It remains to be seen whether the nH1N1 virus will become established and enzootic in the swine populations in the future, as it has done in the human population. In that case, it is likely that the nH1N1 virus will evolve along different pathways in swine and in humans, which may lead to an increasing degree of host specificity. This may decrease the risk for transmission from swine to humans. Still, widespread circulation of nH1N1 in pigs will raise questions as to whether pigs could be a reservoir of a novel zoonotic virus and/or a source of foodborne infection for humans. For a foodborne infection, the virus would be expected to use the gastro-intestinal tract of humans as a possible portal of entry. For that reason, it is interesting to analyse if this can be the case for nH1N1 influenza virus. The potential for transmission of nH1N1 virus via food will, therefore, again be considered from a pathogenetic point of view, based on data gathered from human patients and from studies performed in animal models that are frequently used for human influenza.

3.4.1. Humans

The available information from human patients is largely based on disease signs and pathology and indicates that nH1N1 virus causes a typical respiratory infection, similar to seasonal human influenza viruses. Compared to the latter viruses, nH1N1 virus may cause a more severe infection and pathology in the deeper airways, such as the alveoli, and most fatal cases show a primary viral pneumonia (Mauad et al. 2009). It should be mentioned, however, that any pandemic influenza virus is more likely to cause pneumonia than interpandemic, seasonal viruses. This is at least in part due to the lack of immunity to those pandemic viruses (Kuiken and Taubenberger 2008). The exact sites of nH1N1 virus replication have not been studied, but one assumes that they are similar to those of contemporary human H1N1 viruses, and that they include the nasal mucosa, nasopharynx, oropharynx, trachea, bronchi, bronchioli and alveoli. Some nH1N1 patients, fatal cases in particular, develop multiple organ failure, but direct virus-induced lesions in organs other than the lungs are rare (Mauad et al. 2009). Indeed, nH1N1 infection in humans occasionally leads to severe primary pneumonia with acute respiratory distress and multiple organ dysfunction, with liver, kidneys, central nervous system, heart and intestinal tract as the most commonly involved organs. Some patients show vomiting and diarrhoea, but there is no evidence that the gastro-intestinal tract is a target organ for the nH1N1 virus. The lack of evidence for virus replication in the gastro-intestinal tract also applies to the contemporary human H1N1 or H3N2 viruses.

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Replication of the seasonal human influenza viruses outside the respiratory tract is also controversial (Zambon et al. 2001, Kuiken and Taubenberger 2008). Reports of influenza virus in extra-respiratory tissues are usually based on methods such as virus isolation or RT-PCR. With these methods demonstration of virus in the gastro-intestinal tract or in faecal-rectal swabs, may result from occasional spillover from the heavily infected lungs into the circulation and subsequently in the gut or, more likely, from frequent swallowing of high quantities of virus produced in the upper respiratory tract. The only real proof of virus replication in extra-respiratory tissues should come from *in situ* intracellular detection of viral antigen or nucleic acid using suitable methods. Virus replication has been shown directly in brains and heart of humans but not in the intestinal tract (reviewed by Wright et al. 2007). Only the H5N1 highly pathogenic avian influenza virus has so far been detected in intestinal epithelial cells of humans by immunohistochemistry (Korteweg et al. 2008). H5N1 positive intestinal cells were reported in only one of several investigations, and they were rare and diffusely spread. We therefore conclude that convincing evidence for intestinal replication of influenza viruses in humans is still lacking.

In general, epidemiological, pathological and clinical findings with human seasonal influenza viruses in the past and with the nH1N1 virus to-date manifestly point towards the respiratory and oropharyngeal tissues as the major, and possibly only, real targets. As to the possible extra-respiratory spread of influenza viruses in humans, there are virtually no real quantitative data or results of *in situ* viral detection available.

3.4.2. Macaques, ferrets and mice

Experimental inoculation studies on the pathogenesis of nH1N1 infection have been carried out in macaques, ferrets and mice, which are often used as influenza models for humans (Itoh et al. 2009, Maines et al. 2009, Munster et al. 2009). Ferrets are widely accepted as a reliable small animal model for humans, because they develop similar clinical signs and lung pathology as humans in response to infection with seasonal, avian or pandemic influenza viruses. Also their pattern of influenza virus attachment to cells in the trachea and lower respiratory tract resembles that in humans. Macaques are also highly suitable, but not readily available. Mice are less suitable because they are not natural virus hosts and most influenza viruses need to be adapted to mice. The clinical signs, pathology and influenza virus distribution in mice frequently differ from that in the natural virus hosts, and the infection is frequently lethal.

Cynomolgus macaques (Itoh et al. 2009) were inoculated with an nH1N1 virus isolated from humans (A/California/04/09) or with a current seasonal H1N1 virus (A/Kawasaki/UTK-04/09) trough a combination of intratracheal, intranasal ocular and oral routes. Both viruses were isolated from the upper and lower respiratory tract, including the oro-and nasopharynx, tonsil, trachea, main bronchi and lungs. But virus titres and virus isolation rates were markedly higher for the nH1N1 virus than for the seasonal H1N1 virus. The nH1N1 virus was also recovered from the conjunctiva of 1 out of 6 macaques. Macroscopic and microscopic lung lesions were seen with both viruses, but they were most severe with the nH1N1 virus. The lymph nodes (chest), heart, spleen, kidneys or liver tested negative with both viruses, showing that no viraemia had occurred. The gastro-intestinal tract was not examined.

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Ferrets have been investigated in three different experiments:

In one study (Maines et al. 2009), groups of 6 ferrets were intranasally inoculated with one of 3 different nH1N1 isolates obtained from human patients in California, Texas and Mexico respectively. A representative seasonal H1N1 virus was used as control. The nH1N1 isolates caused increased morbidity and replicated to high titres in the lungs (4.1 to 6.0 log10 per gram tissue) in contrast with the seasonal virus. Infectious nH1N1 virus was isolated from 8 of a total of 27 intestinal tissue or rectal swab samples and titres varied from 1.3 to 2.7 log10 per ml or gram. The seasonal H1N1 isolate showed similar shedding kinetics in nasal washes as the 3 nH1N1 isolates, but was not recovered from the lungs or intestinal samples. There was no evidence of viraemia or infectious virus in the brain, kidney, liver and spleen with any of these viruses.

In a second study, Munster et al. (2009) inoculated groups of 6 ferrets with an nH1N1 isolate from a human patient in The Netherlands or with a seasonal H1N1 virus. The nH1N1 virus was more pathogenic than the seasonal virus causing more extensive virus replication in the respiratory tract. Both the nH1N1 virus and the seasonal H1N1 virus were detected in the nasal turbinates, but only the nH1N1 virus was also detected in the lungs. These findings do not agree, however, with the relatively extensive gross lung lesions in ferrets of both groups. No virus was detected in liver, spleen, kidney or brain tissue. The gastro-intestinal tract was not examined.

A third study (Itoh et al. 2009) involved the inoculation of ferrets with 5 different human nH1N1 isolates and 1 seasonal virus isolate. The nH1N1 viruses replicated to higher titres in the trachea and lungs than the seasonal virus. None of the viruses caused marked changes in body temperature or weight.

In *mice,* human nH1N1 isolates replicated efficiently in the lungs without prior host adaptation, which is usually required for seasonal human influenza viruses in this species (Itoh et al. 2009). No virus was detected in extra-pulmonary tissue such as spleen, thymus, brain and intestines.

All these studies in animals used as human models, particularly those in ferrets, yielded very similar results on the pathogenesis of nH1N1. The ferret studies also showed nH1N1 virus transmission between ferrets, though respiratory droplet transmission was inconsistent in one study (Maines et al. 2009) and efficient in both others (Itoh et al. 2009, Munster et al. 2009). There was no viraemia or virus detection in extra-pulmonary tissues, demonstrating the exclusive respiratory tropism of nH1N1 infection, in common with seasonal human influenza viruses. A point of discussion is the recovery of virus from the intestines or rectal swabs in one study (Maines et al. 2009) and the possibility that this organ might serve as a possible portal of entry or target organ for the virus upon ingestion. The absence of viraemia indicates that the virus in the intestine must have originated either from virus-containing respiratory secretions that had been swallowed or else must have replicated locally. The relatively low virus titres and the fact that only 8 of the 28 samples were positive in this study point to swallowed virus and not to intestinal replication as the likely source. However, as long as no virus detection *in situ* is performed, the question as to whether or not nH1H1 virus can

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replicate in the gastro-intestinal tract cannot definitively be answered. The authors of that study claim that detection of nH1N1 virus in the intestinal tissue of ferrets is consistent with the gastro-intestinal involvement observed in some human cases (Maines et al. 2009). Based on the present experimental data, this conclusion is too far reaching, also because gastro-intestinal involvement in natural human cases of nH1N1infection is relatively uncommon and is usually part of a multi-organ dysfunction syndrome.

In an earlier study, Kawaoka et al. (1987) examined the possibility of intestinal replication of various influenza A viruses after intranasal inoculation of both ferrets and pigs using virus isolation. Six of 14 viruses examined, including 2 low pathogenic avian influenza viruses, were isolated at low titres from one or another part of the intestinal tract of ferrets. Gastro-intestinal symptoms were not observed. In the same study, one of 4 influenza viruses tested was also isolated from the faeces of pigs, whereas the jejunum, ileum and colon were negative. The authors concluded that many influenza viruses have the potential to replicate in intestinal tissues, but this conclusion is too far reaching, as they did not demonstrate virus in intestinal cells *in situ*. From these inconsistent results, it is rather clear that the gastro-intestines are not target organs for these type A mammalian influenza viruses.

3.5. Hurdles to be overcome by an influenza virus in order to initiate infection in the gastro-intestinal tract of humans following oral uptake

The structural and chemical composition of all influenza A virus particles is identical independent of the type, subtype, genotype or the species of origin. The virus particles consist of 8 separate RNA gene fragments that are surrounded by proteins and enzymes. All these structures are wrapped in a lipoprotein membrane, called the envelope, which is derived from the plasma membrane of the host cell in which the virus has replicated. Thus, not only the structure of the particle but also the chemical composition of all the influenza A viruses, whether from swine, human, equine or avian sources, are similar and consist of 1% RNA, 70% protein, 20% lipid and 5-8% carbohydrates. The particles have to be physico-chemically intact to be infectious under natural circumstances. When external agents distort this physical integrity of the particle or sufficiently alter its chemical composition, the virus may lose its infectivity and becomes inactivated. Processes that disrupt membrane structures act similarly on all influenza A viruses. Disinfectants inactivating one influenza A virus are also considered to inactivate related influenza viruses. Despite this general principle and their similarity in structure and chemical properties, studies on influenza virus inactivation by chemical or physical agents have often led to highly variable and even contradictory results. This variation is due to the circumstances in which the experiments were performed, such as the initial virus titre, the concentration and efficacy of the agents used, the medium in which the virus is embedded, the stability or consistency of the activity of the agent used throughout the experimentation, the sensitivity and reproducibility of the methods used to examine the rate of virus inactivation, the frequency of sampling and possibly others. Published results are often difficult to compare or interpret because too many variables have been introduced and appropriate controls are sometimes not included. The structural and chemical similarity of type A influenza viruses in general terms supports the conclusion that inactivation data obtained with some agents, such as lipid solvents, heat and several others, apply not only to

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the particular influenza virus examined, but to any influenza A virus, whether of avian, swine or human origin, including the nH1N1 virus.

While influenza virions of the type A influenza viruses can be considered as inert entities with highly similar physicochemical characteristics, this no longer holds true when they become biologically active following attachment to and replication in susceptible cells. Here, differences in genes or gene combinations, enzymes and all kinds of proteins such as ligands are determinative and show marked functional differences. Viral ligands and proteins will determine different properties such as host and cell tropism, virulence, transmissibility and many other biological characteristics influencing virus-host, virus-organ and virus-cell interactions.

A similar exercise with regard to hurdles to be overcome by highly pathogenic avian influenza viruses in order to infect the gastro-intestinal tract of humans or other mammals has been made in a previous scientific report by the EFSA's Scientific Panel on Biological Hazards (2006).

3.5.1. Physical and chemical barriers related to the human gastro-intestinal tract

Natural barriers, both physical or chemical, will be encountered by a virus when taken up orally or by ingestion and prior to finding potential susceptible cells that might serve as a portal of entry in the gastro-intestinal tract in order to initiate infection. The virus must first be sufficiently resistant to these hurdles if it is to be considered as a potential cause of foodborne infection. These physiological hurdles or natural barriers will now be discussed and applied to nH1N1 virus.

Oral uptake of nH1N1 influenza virus with pork can occur (i) when the virus may have colonized muscles or edible organs during a viraemia in infected pigs, (ii) when pork is contaminated by respiratory excretions at slaughter or (iii) when pork is handled and contaminated by infected humans. Presence of virus in muscle after viraemia will be highly exceptional, as explained earlier in the pig pathogenesis studies, where no systemic infection was found. Still, it cannot be totally excluded since earlier studies with European H1N1 SIVs in pigs have shown that a short and transient presence of virus in blood, and thus presumably in muscles and other organs, was possible (Romijn et al. 1989, Brown et al. 1993). Such an event could occur in nH1N1 infected pigs, although it has not been demonstrated to-date.

When a virus enters the gastro-intestinal tract upon ingestion, several hurdles have to be overcome prior to reaching potential cells that may serve as possible site of entry for reaching other organ systems.

a) Adsorption

Virus particles, when taken up with food, may adsorb onto buccal, throat, oesophageal and gastric surfaces and mucins during their passage to the intestines. This adsorption process is not specific and is inefficient to reduce high quantities of ingested virus. It may eliminate a relatively small quantity of virus present in food prior to reaching the intestines as one may expect in the case of influenza viruses. The nett effect of adsorption will be of limited value against typical enteric viruses for which a low infectious dose is sufficient to initiate infection in the intestines as target organ.

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b) Acidity in the gastric lumen

Resistance of infectivity to acid appears to differ among influenza viruses, particularly between mammalian and avian viruses. Because the protocols used in studies on the effect of pH are very different, comparison of the results is often difficult. Selected data will be presented here to provide some insight. This is useful to assess the possible barrier role of stomach acidity when influenza viruses are ingested with food. However, most studies have been performed with avian influenza viruses because, contrary to the mammalian influenza viruses, avian viruses are known to use the digestive tract as one of the portals of entry in their natural host (Reperant et al. 2009).

Four avian H7N7 strains were compared using a starting titre between 5.5 and 4.7 log10 embryo infectious doses and held at pH values of 2, 5, 10, and 12. At pH 2, all 4 viruses were inactivated after 5 minutes exposure time, while no change in infectious titre was observed after 15 minutes with the other pH values (Lu et al. 2003). After treatment of infected chicken manure at pH 2, influenza infectivity was lost within less than 30 minutes. There seems to be some variation in the response to acidity among influenza virus strains. Three avian influenza viruses were found to be stable at a pH between 7 and 8.5, but their infectivity decreased rapidly below pH 6. Still, avian influenza viruses exhibited more stability below pH 6.0 than human influenza viruses (Stallknecht et al. 1990, Webster et al. 1978).

Glathe et al. (1982) found that human and equine influenza viruses, when exposed to pH 3, had lost their infectivity completely, while this was not the case for avian viruses. The latter viruses showed relatively higher stability in their neuraminidase, which explained the retained infectivity. Also Webster et al. (1978) found that influenza viruses of ducks were more acid-stable than human viruses and claimed that this would facilitate their unharmed passage through the gizzard. Contrary to mammalian influenza viruses in their respective host, avian viruses are known to replicate in the intestinal tract of birds and to be transmitted by the faecal-oral route.

Scholtissek et al. (1985) performed a study on the stability of influenza A viruses from different species at low pH. Strains belonging to different HA subtypes were maintained at varying pH values for 1 hour at 20°C. This study revealed differences between HA subtypes. All H3 strains, independent of the species from which they were isolated, lost infectivity between pH 5.1 and 5.4. All H7 and H5 subtypes were inactivated with thresholds between pH 5.6 and 6.0, and H1 strains were intermediate. They concluded that pH stability of the infectivity is a trait of the HA and is dependent on the HA subtype, while it is not significantly influenced by other gene products. The effect of acidity was found to be reversible in the pH range from 6.4 to neutral, but was irreversible below pH 6.0 (Stallknecht et al. 1990, Sato et al. 1983).

The general conclusion of these and other studies was that (1) influenza A viruses are acid-labile viruses and (2) their sensitivity to acid pH shows HA dependency, with avian subtypes being more resistant than those of mammals (Fiszon et al. 1989) With respect to acid sensitivity, influenza viruses are very different from the typical acid-stable, foodborne enteric viruses (e.g. hepatitis A virus, norovirus, human enteroviruses, etc.), which are unaffected

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even at pH 2 to 4 and can therefore pass unharmed through the acid milieu of the stomach, even at low doses, to initiate an enteric infection.

The aspect to be discussed here is whether the acid environment in the stomach of humans inactivates influenza viruses such as nH1N1, if and when ingested. The physiological pH in the gastric environment of humans appears to be around 1.6-1.8, but it may sometimes rise up to pH 6. The acidity in the stomach lumen and in the upper duodenum is highly influenced by eating patterns and fluctuates continuously. The type of food, an empty or filled stomach, the rapidity with which food passes through the stomach, acid-suppression medication and many other circumstances will largely determine the pH in the stomach and the upper duodenum. Because physiological gastric acidity levels are in the range of the acid sensitivity values of mammalian influenza viruses, low quantities of virus will be completely inactivated after passage through this acid environment. This is bound to be the case if ingested pork had been accidently contaminated with nH1N1. The inactivating effect of gastric juices may not be sufficient or reliable when high virus quantities are continuously swallowed during a respiratory infection and particularly when the virus is embedded in organic food material and/or passes quickly through the stomach lumen. This may explain the low influenza virus titres inconsistently found in intestinal contents or faecal swabs of pigs, humans and even ferrets during infection with influenza A viruses (Kawaoka et al. 1987).

c) Bile salts, enzymes

In addition to the pH values of gastric secretions, other factors such as bile, urea, gastric mucins and pancreatic enzymes may affect virus infectivity. Bile and pancreatic enzymes are secreted in the upper duodenum. It has been demonstrated that bile may inactivate enveloped viruses, but specific studies with influenza viruses are rare. One study was performed with the paramyxovirus Newcastle disease virus (NDV), which has a similar structure as influenza viruses. Both NDV and influenza viruses are readily inactivated by lipid solvents. In this study (Lee et al. 1975), a 3 log10 inactivation was obtained when fresh bile from non-immune chickens was added to the NDV. Non-specific factors present in bile, probably the bile salts, caused the *in vitro* reduction of infectivity. Bile salts act as lipid emulsifiers and presumably have a disruptive detergent action on the lipid bilayer of the viral envelope. Bile salts in the duodenal lumen may thus play an additional role in *in vivo* inactivation of ingested enveloped viruses entering the intestines after passage through the stomach. The virus-inactivating effect is not absolute but may have an additive value when a low amount of virus is taken up. The inactivating effect of bile *in vivo* may be less pronounced when the virus is protected by organic material. In the study mentioned, a turkey influenza virus was used as a control virus. After a similar treatment with bile, its infectivity titre was reduced by 1.0 log10.

Meanwhile, typical enteric foodborne viruses generally have no envelope and are resistant to the action of bile (Koopmans et al. 2002).

No information has been found on the effect of pancreatic enzymes on influenza viruses during passage through the duodenal lumen.

In conclusion, this part of the report shows that, in order for an influenza virus to reach the intestines and to be considered as a foodborne pathogen, it needs to overcome several hurdles

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during passage through the stomach and duodenum, each with virus-inactivating effects. While each of these hurdles on its own may not lead to total inactivation, their cumulative action may be considerable. This may also explain why H1N1 influenza viruses, when swallowed during a respiratory infection, are sometimes, but inconsistently and at low titres, detected both in the human intestine and in faecal swabs taken from humans or pigs, despite the lack of evidence that these viruses replicate in or use the gastro-intestinal tract as portal of entry. To-date, no viral antigen or nucleic acid have been demonstrated in the intestinal cells of influenza-infected humans or pigs using *in situ* detection methods.

3.5.2. Biological barriers

As mentioned higher, there is strong evidence that influenza viruses fail to replicate in the intestinal tract of humans. The reasons for this lack of intestinal replication remain unknown: they may relate to a failure of infectious influenza virus to reach the upper part of the intestinal tract in sufficient amounts, as discussed above, or to the failure of intestinal cells to support viral replication – which is the subject of the present section – or a combination of both. Both host and viral factors will likely contribute to the absence of intestinal replication. The role of viral factors is illustrated by the failure of human seasonal influenza viruses to replicate in the duck intestine, a major site of avian virus replication (Hatta et al. 2002). The fact that highly pathogenic H5N1 avian influenza viruses replicate extensively in the gastro-intestinal tract in most bird species, but not in humans, illustrates the role of host factors.

This section briefly discusses some key steps in the replication of an influenza virus that determine cell, tissue and host tropism. Each of these steps may represent a barrier for successful infection of human intestinal cells by influenza virus, including the novel H1N1 virus.

a) Virus attachment to cellular receptors

Influenza infection is initiated by attachment of the viral HA to specific receptors on the host cell. The single known receptors for influenza viruses are sialic acids. The receptor binding specificity of the HA depends on the virus host species: swine and human influenza viruses preferentially bind sialic acid attached to galactose by an α-2,6 linkage (Sia α2-6, "human-type" receptor), whereas avian viruses prefer sialic acid with an α-2,3 binding (Sia α2-3, "avian-type" receptor) (reviewed by Nicholls et al. 2008). In the past, it was believed that pigs were the single animal species with both types of receptors (Ito et al. 1998). Recent studies, in contrast, have demonstrated both receptors in the respiratory tract of pigs and humans, with a similar distribution pattern (Shinya et al. 2006, Nelli et al. 2010, Van Poucke et al. 2010). In both species, the human-type receptor was found on epithelial cells throughout the respiratory tract, while the avian-type receptor was restricted to the lung alveoli. The apparent confinement of the avian-type receptor to the lower respiratory tract is seen as an explanation for the poor replication of avian influenza viruses in the upper airways of humans and the lack of human-to-human transmission.

Preliminary data indicate that the nH1N1 virus also prefers the human-type receptor, in common with seasonal human viruses and the established swine viruses (Maines et al. 2009). This observation is in line with the novel nH1N1 virus's replication in the upper respiratory

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tract of humans, and its high transmissibility between humans. It does not explain, however, why the novel H1N1 virus seems to replicate better in the alveoli of humans than seasonal human H1N1 viruses. It is also unclear why this virus replicates without prior adaptation in the mouse respiratory tract, which mainly contains avian-type receptors. In addition, in several studies influenza virus infection of cultured cells was still possible after enzymatic removal of sialic acids. This illustrates that the sialic acid linkage on a cell surface is not the single or main determinant of influenza infection. In any event, even if the virus attaches to a specific receptor, other subsequent steps of the viral replication cycle may still fail to proceed.

Sialic acid receptor distribution patterns have been studied almost exclusively in the respiratory tract of mammals, because it is the major target organ for influenza virus replication. Data about the human intestines are scarce and somewhat contradictory. In a study by Yao et al. (2007), neither human-type nor avian-type receptors were detectable in intestinal epithelial cells, but the avian-type receptor was found on neurons in the intestines. This contrasts with the findings of Sata et al. (1991), who did detect the avian-type receptor in human intestinal epithelium. Nelli et al. (2010) have recently investigated the relative expression and distribution of Sia receptors in a whole series of pig organs: the trachea and lungs, as well as the small intestine and colon, brain and internal organs. They found both types of receptors on epithelia of small and large porcine intestines, especially on goblet cells. The intestines were the single organ in which the Sia receptor expression differed from that described in humans. It is possible that the reported absence of Sia receptors in the human gut is due to the limitations of lectin staining techniques and there is a need for clarification of this issue. Despite the apparent presence of Sia receptors in the porcine gut, influenza viruses fail to cause a productive intestinal infection in pigs. Of course, this can be due to several other causes: the virus may reach the intestinal tract in insufficient amounts or not at all, or stages of the viral replication cycle other than attachment may fail to occur.

b) Cleavage of the HA and fusion between virus and host cell

To enter cells by membrane fusion and thus initiate replication, each monomer of the HA trimer must be cleaved into two polypeptide chains (HA1 and HA2). The activating enzymes are provided by the host. Mammalian influenza viruses and low pathogenic avian influenza viruses have a single arginine at the cleavage site between HA1 and HA2, and their cleavage is activated by extracellular, trypsin-like proteases. It is believed that these enzymes are restricted to the respiratory tract of humans and the respiratory and intestinal tract of avian species and that this is a major reason for the restriction of these viruses to the respiratory system of humans. This contrasts with highly pathogenic avian influenza viruses, which have a multibasic cleavage site and are cleaved by intracellular, ubiquitous proteases. Yet, information on the proteases that activate HA with a monobasic cleavage site mainly comes from *in vitro* studies (reviewed by Klenk et al. 2008) and our knowledge of the proteases involved in the natural setting in the host is highly incomplete.

c) Ability of the virus to replicate within the host cell

Functional interactions between viral proteins and host proteins are essential for the replication of the viral genome and the synthesis of new viral proteins. Many cells are non-permissive or only partially permissive for a given virus, because they do not contain all

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factors required by the virus. The inherent ability of influenza viruses to replicate in cells of the intestinal tract of humans has never been examined.

3.6. Inactivation of influenza viruses in animal food

3.6.1. Temperature

When discussing the possibility that nH1N1 influenza virus may represent a foodborne pathogen possibly acquired by eating pork, pork products or turkey meat, it becomes clear that most of the criteria earlier set for the specific recognition as a foodborne virus do not apply. One such criterion is that the virus must be resistant to chemical and physical agents, temperature included, so that it can withstand e.g. moderate heating. All type A influenza viruses, whatever their origin or host of isolation, are heat sensitive to common threshold values.

Many experiments on heat inactivation have been carried out with avian influenza viruses. Highly pathogenic avian influenza (HPAI) viruses may cause an intensive viraemia in their avian hosts and the virus colonizes meat and internal organs to high titres. On the contrary, as earlier explained, such colonization does not or only exceptionally occurs in mammals infected with mammalian viruses. However, limited contamination of meat, meat products or edible organs can not be totally excluded here.

Heat sensitivity applies for all animal virus species, but their threshold values differ markedly. It is generally accepted that heat induces structural changes in proteins of the virus particle (Baert et al. 2008). Heat treatment of norovirus-like particles, for example, disrupted secondary, tertiary and quaternary protein level structures (Ausar et al. 2006). At higher temperatures, not only the external proteins that serve as ligands for virus binding to host cells, but also internal viral proteins, including structural enzymes needed to start early metabolic processes during replication, are adversely affected. Such enzymes are part of the influenza virion and presumably make influenza viruses rather heat susceptible. Contrary to the variable action of pH, as mentioned above, heat treatment acts similarly on all influenza viruses. Also, heat inactivation is totally irreversible. The results obtained with thermal inactivation of avian influenza viruses in meat or eggs are thus applicable to pork and pork products or turkey meat potentially contaminated with nH1N1 virus.

In general, several factors may influence thermal inactivation. They include the height of the temperature used, the medium in which the virus is embedded, the protective effect of organic material that may prolong the time for inactivation, the presence of inorganic chemicals such as stabilizing bivalent cations or salts in the case of some virus species and the quantity of the virus present in the material. When virus is suspended in physiologic buffered saline solutions, infectivity is lost at a constant rate during heat treatment (first order kinetics reaction) and the time needed for total inactivation can be predicted. When the virus is embedded in organic material such as meat, organs or egg products, however, a small fraction of the virus may lose infectivity at a slower rate. For this reason and for practical purposes, heat treatment experiments have been undertaken to determine the time/temperature combination at which total loss of infectivity is achieved with the virus embedded in artificially prepared media or in natural products (meat, organs, egg products) or in byproducts

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(manure, faeces, water with or without salinity etc.). Some thermal inactivation values for influenza viruses in animal products reported in the literature are given here. While these examples are not exhaustive, these and other time/temperature combinations are relevant and should be applicable to nH1N1, if needed.

- Three minutes at 70 °C were sufficient to inactivate H5N1 HPAI virus embedded in meat or whole egg (Songserm et al. 2006).
- Swayne et al. (2004) reported on an experiment carried out at low and high temperature simulating industrial pasteurization with avian influenza viruses (high and low pathogenic) in different egg products. The starting titre was 5.5 log10 EID50 per ml and temperatures tested were 55, 57, 59, 61, and 63 °C. The study showed residual infectivity at 55 °C but total inactivation in all media at temperatures of 61 °C and higher. A positive correlation was found between the time required for total inactivation and the type of egg product containing the virus. HPAI virus in dried egg white was most resistant to heat inactivation. The time required to obtain total inactivation was shorter than the time used for standard industrial pasteurization of egg products.
- The effect of heat treatment on H5N1 HPAI virus present in chicken thigh meat (starting titre of 6.8 log10 EID50) and breast meat (starting titre of 5.6 log10 EID50) derived from experimentally infected chickens was studied at 30, 40, 50, 60, and 70 °C after 5, 10, 30, and 60 seconds. The titre remained unchanged until 50 °C and complete inactivation was reached at 70 °C after 1 and 5 seconds for breast and thigh meat respectively (Swayne et al. 2006).
- Another study (Thomas et al. 2007) was carried out with 2 HPAI viruses and 1 low pathogenic avian influenza (LPAI) virus in chicken meat. All viruses completely lost infectivity at 70 or 73.9 °C after less than 1 second.
- Thermal inactivation of HPAI virus H7N7 in chicken meat was studied between 50 °C and 65 °C by Isbarn et al. (2007). Virus, at a titre between 7 and 8 log10 PFU per ml., was mixed with chicken meat. At 50 °C, 1 log10 of infectivity was lost after 10 minutes. Both the incubation at 63 °C for 2 minutes and the 500 MPa pressure treatment at 15 °C for 15 seconds inactivated more than 5 log10 PFU of virus.

More data can be found in a scientific opinion published by the EFSA Scientific Panel on Animal Health and Welfare on "Animal health and welfare aspects of avian influenza and the risk of its introduction into the EU poultry holdings" (2008).

In conclusion, thermal inactivation and cooking are very reliable for inactivating all influenza A viruses present in meat, meat products and animal byproducts. As explained, pork or pork products could exceptionally be contaminated by respiratory secretions containing nH1N1 virus during slaughter or handling. In that case, virus titres are expected to be very low so that cooking at 70 °C, or possibly even at lower temperatures, will ensure complete inactivation. Moreover, if such products were to be eaten raw, as could be the case with minced meat preparations, the physico-chemical and biological hurdles within the gastro-intestinal tract, as

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discussed earlier in this report, would most likely prevent foodborne infection with nH1N1 virus.

It cannot be overlooked that pork products, when eaten raw, pass the oropharyngeal tissues of humans upon ingestion. These tissues could represent portals of entry in humans for mammalian influenza viruses, including nH1N1, if present in food. If such occurs, the infection will proceed toward the respiratory tract as the main or only target organ. This infection route, which may be considered as foodborne, would have little or no epidemiological importance compared to the multiple and more evident aerogenic exposures to the virus leading to the respiratory infection route, in the case of virus transmission from pigs to humans.

3.6.2. Curing and salting of pork

No information is available on the effect of curing and salting on any type A influenza virus, including nH1N1, in pork products potentially contaminated with these viruses.

Different viruses behave in different ways and processing of pork meats for production of cured meats varies largely in different countries or regions. Variations in the production technologies may influence the survival of viruses. The majority of studies on virus survival during processing of meats have been performed with such viruses as classical swine fever virus, African swine fever virus, foot and mouth disease virus and swine vesicular disease virus. These viruses are quite different from influenza viruses in the following aspects: (i) they cause a long lasting and extensive viraemia with high virus quantities colonizing muscles in their host, (ii) they are very resistant to physico-chemical treatments (except for the acid pH lability of foot and mouth disease virus), (iii) they cause diseases that are notifiable and legally controlled on an international basis and (iiii) meat presents a high risk for introducing these viruses in disease free populations.

All these points do not apply to mammalian influenza viruses and thus also not to nH1N1.

Certainly, since fresh pork, as discussed in the present context, is not a foodborne threat, cured or dried or smoked or salted products are equally to be excluded.

3.7. Effect of temperature on influenza viruses in animal by-products

There are no data available on the effect of temperature on the survival of H1N1 influenza viruses in animal byproducts. Again, avian influenza viruses have been studied frequently for their survival in water, faeces and bird carcasses, particularly with the purpose to assess the role of these media in virus transmission over long and short distances. The Scientific Opinion of the EFSA Scientific Panel on Animal Health and Welfare (2008) entitled “Animal health and welfare aspects of Avian Influenza and the risk of its introduction into the EU poultry holdings” presents a recent review of survival data in water, faeces and carcasses for different avian influenza viruses. Values given in this document indicate resistance of avian influenza viruses in faeces despite its chemically and enzymatically aggressive composition. Brown et al. (2007) have analysed the tenacity of a total of eight H5 and H7 LPAI virus strains and of two H5N1 HPAI virus strains in water in standardized experiments. Persistence was found to be (i) inversely correlated with increase in water temperature and salinity, (ii) similar to

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previously published data for other AIV subtypes, (iii) variable between isolates of the same subtype, and (iv) less pronounced for HPAIV H5N1 (range of 17-18 days at 22°C vs. 22-42 days for LPAIVs). However, given some isolate-specific variability, it was difficult to conclude that HPAIV H5N1 was less fit for water-borne transmission and some published data seemed to point to the contrary (Webster et al. 2006). In carcasses, with viral loads in tissues often exceeding 8 log10 EID50 per gram, the virus can retain its infectivity for a variable period of time, which is inversely correlated with environmental temperature. Temperatures above 60-65 °C result in complete viral inactivation. It was concluded that there is no evidence for a difference in the tenacity in faeces, water, carcasses, and the environment between Asian lineage HPAI H5N1 viruses and LPAI viruses.

Even though all influenza viruses have similar resistance to the agents mentioned, many of these data with AIVs hardly apply to enzootic swine influenza viruses because (i) swine influenza viruses very rarely cause viraemia and the viral load, if present, is low in carcasses except for the lungs, (ii) they are not or only exceptionally excreted via faeces, and the viral load in faeces or faecally contaminated water would be far lower than for AIVs (iii) unlike the case for AIVs, faeces or water or other byproducts do not play any role in transmission of mammalian influenza viruses as they typically replicate in respiratory tissues and (iii) swine influenza viruses are not subject to international regulations as is the case for many AIVs.

3.8. Effect of disinfectants on survival of influenza viruses

Some data on the effect of disinfectants may be useful when considering potential cleaning protocols for equipment used during the handling of meat or animal byproducts that could be potentially contaminated with influenza viruses. Attention will also be given to the cleaning of hard, non-porous surfaces.

Influenza viruses are enveloped viruses and any disinfectant based on lipid solvents, affecting lipoprotein membrane structures of the virion, will inactivate them equally and readily. Like all other animal viruses, influenza viruses are rapidly inactivated by chemicals affecting nucleic acids (aldehydes….). So, none of the influenza viruses are resistant to these 2 groups of chemical agents, but the efficacy of other disinfectants varies according to their mode of action or concentration or exposure time.

An overview of the activity of several disinfectants is given by Shadid et al. (2009) and these data are presented in Tables 1 and 2. From Table 1, it can be concluded that all the disinfectants tested were able to inactivate the H5N1 avian influenza virus at the recommended concentration within less than 15 minutes of exposure time. Table 2 shows that soap, detergent and alkali at low concentrations are potent and efficient for inactivating influenza viruses.

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Table 1. Effect of chemical factors on the survival of avian influenza virus H5N1 subtype. (From Shahid et al. 2009)

		Exposure time (minutes)			
Disinfectant	**Concentration (%)**	**15**	**30**	**45**	**60**
Formalin	0.2	----	----	----	----
	0.4	----	----	----	----
	0.6	----	----	----	----
Iodine crystals	0.2	++++	++++	++++	++++
	0.4	----	----	----	----
	0.6	----	----	----	----
Phenol crystals	0.2	++++	++++	++++	++++
	0.4	----	----	----	----
	0.6	----	----	----	----
CID 20	0.2	++++	++++	++++	++++
	0.5	++++	++++	++++	----
	1.0	----	----	----	----
Virkon®-S	0.2	++++	++++	----	----
	0.5	----	----	----	----
	1.0	----	----	----	----
Zeptin 10%	0.5	++++	++++	----	----
	1.0	++++	----	----	----
	2.0	----	----	----	----
KEPCIDE 300	0.2	++++	++++	++++	++++
	0.5	+++	----	----	----
	1.0	----	----	----	----
KEPCIDE 400	0.2	++++	+++	++++	++++
	0.5	----	----	----	----
	1.0	----	----	----	----

++++ = amnio-allantoic fluid from four inoculated chicken embryos showed haemagglutination and virus was identified by haemagglutination inhibition

------ = amnio-allantoic fluid from four inoculated chicken embryos did not show haemagglutination

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Table 2. Effect of soap, detergent and alkali on the survival of avian influenza virus H5N1 subtype. (From Shahid et al. 2009)

		Exposure time (minutes)			
Disinfectant	**Concentration (%)**	**5**	**15**	**30**	**45**
Surf Excel®	0.05	++++	++++	++++	++++
	0.1	----	----	----	----
	0.2	----	----	----	----
	0.3	----	----	----	----
Life buoy®	0.05	++++	++++	++++	++++
	0.1	----	----	----	----
	0.2	----	----	----	----
	0.3	----	----	----	----
Caustic soda	0.05	++++	++++	++++	++++
	0.1	----	----	----	----
	0.2	----	----	----	----
	0,3	----	----	----	----

++++ = amnio-allantoic fluid from all four inoculated chicken embryos showed haemagglutination and virus was identified by haemagglutination inhibition

------ = amnio-allantoic fluid from all four inoculated chicken embryos did not show haemagglutination

In another study (Alpin et al 2009) five disinfectant chemicals were tested for effectiveness against a LPAIV H7N2 on hard, non-porous surfaces. Chemicals including acetic acid (1 and 3 %), sodium hydroxide (2%), and calcium hydroxide (1%) effectively inactivated the virus on metal surfaces. A laundry detergent without bleach, sodium carbonate (4%) and the lower concentration of sodium hydroxide (1%) were not able to consistently inactivate LPAIV on hard, non-porous surfaces.

In general, most disinfectants from a very wide range of chemical classes effectively inactivate AIVs, and similar conclusions may be drawn for mammalian influenza viruses. A comprehensive review on AIV inactivation by physical methods and chemical agents has also been published by De Benedictis et al. (2007).

Also, two on-going EU funded projects that are due to be finalized in early 2010, FLURESIST and RIVERS, should provide further insights into the survival and removal of avian flu viruses in different substrates. FLURESIST addresses "avian influenza virus survival in poultry commodities, poultry manure and the environment" (http://www.fluresist.eu/UK/). RIVERS deals with "Resistance of influenza viruses in environmental reservoirs and systems (http://www.rivers-project.eu/background.html).

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Conclusions

Criteria that need to be met for a virus infecting food-producing animals to cause foodborne infections in humans were applied to nH1N1 influenza virus, potentially present in pork, pork products or turkey meat. Swine are fully susceptible to the virus experimentally and may become infected in the field after contact with infected humans. So far, no contact infections from swine to humans and thus no zoonotic transmission has been reported. This in itself does not exclude food as possible origin of foodborne infection. The infection in swine is purely respiratory with no viraemia or virus dissemination to muscles or edible organs. However, low virus quantity contamination of meat or organs by respiratory secretions from infected animals may be possible at slaughter or at processing.

If ingested with food, the virus has to overcome several hurdles such as acid pH in the stomach and bile salts in the duodenum, which are harmful for mammalian influenza virus infectivity, particularly at low virus titres.

There is no evidence that the human gastro-intestinal tract tissues can serve as portal of entry or target organ for mammalian type A influenza viruses, including nH1N1.

When food or food products are heated, rapid virus inactivation occurs. Moderate cooking, e.g. at 70 °C, inactivates high virus titres within seconds even when the virus is protected by meat or other organic materials or when present in animal byproducts. When eaten raw, contaminated meat could initiate infection in humans after ingestion while passing the oropharyngeal tissues, which could be a portal of entry. In that case, however, the infection is expected to proceed towards the respiratory tract and would play no epidemiological or foodborne role. No information is available on the effect of curing or salting of pork, but since pork is not colonized by nH1N1, this question is not of real interest.

Experimental studies have shown that turkeys are refractory to nH1N1 infection via the respiratory route, but may become infected after reproductive tract insemination. This suggests that infected insemination crews may have been responsible for the few cases of nH1N1 infection in turkey breeder hens in the field. On the other hand, there were no indications for viraemia or virus dissemination to muscles after experimental intrauterine inoculation. Regarding potential contamination of turkey meat during slaughter, all aspects mentioned for pork or pork products apply.

It was concluded that nH1N1 possibly present in these food products does not pose a foodborne threat.

Human and animal influenza viruses belong to the most sensitive viruses regarding inactivation in the environment. The commercially available disinfectants for cleaning of equipment used during handling of meat products and animal byproducts will rapidly destroy influenza viruses, not only the products based on lipid solvents but also most other disinfectants.

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Gaps for research

The above discussion reveals the following gaps and needs for research:

- Increased surveillance for influenza viruses in swine populations worldwide. This will also demonstrate whether or not the nH1N1 virus is becoming established in swine worldwide.
- Thorough characterization of nH1N1 isolates from turkeys, to identify potential variants with better ability to infect avian species.
- Animal experiments with genetically engineered influenza viruses to unravel how different gene combinations or mutations in viruses of swine origin affect their replication in and spread to other hosts.
- Closer monitoring and virological investigations in people in contact with pigs, who present with influenza-like illness.
- Investigation of the intrinsic permissiveness for influenza virus replication in epithelial cells of the intestinal tract of humans or other mammals. This should also include examination of the distribution and relative expression of the avian- and human-type sialic acid receptors in the human gut.

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