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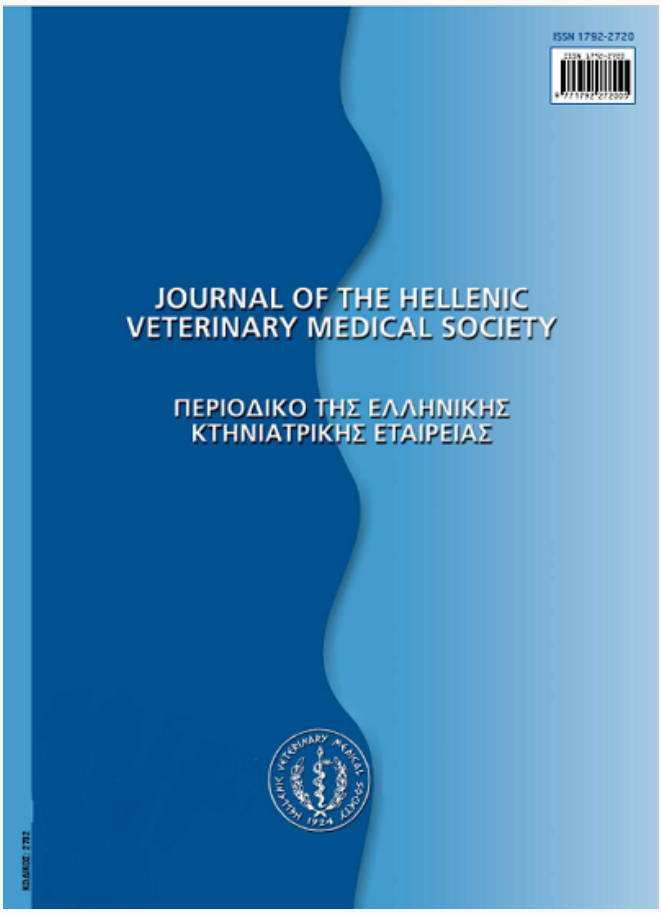

Τρόφιμα ως πιθανή πηγή μόλυνσης των θηλαστικών με τον ιό γρίπης H5N1

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ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2007, 58(3): 220-231
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The following paper was presented in part during the 1st International Influenza Symposium organized in Athens, on February 23rd 2007, in the amphitheatre of the Foundation of Biomedical Research of the Academy of Athens (FBRAA), under the auspices of the Ministry of Rural Development and Food, the FBRAA and the Hellenic Veterinary Medical Society.

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Τρόφιμα ως πιθανή πηγή μόλυνσης των θηλαστικών με τον ιό γρίπης H5N1

Pensaert M., Kyriakis C.S., Van Reeth K.

ΠΕΡΙΛΗΨΗ. Τα πρόσφατα κρούσματα γρίπης ανθρώπων και άλλων θηλαστικών από τον υψηλής λοιμογόνου ικανότητας ιό της γρίπης των πτηνών H5N1 έχουν προκαλέσει ερωτήματα σχετικά με την ασφάλεια της κατανάλωσης των πτηνοτροφικών προϊόντων. Ο σκοπός αυτής της ανασκόπησης είναι η συλλογή και παρουσίαση πληροφοριών αναφορικά με τον κίνδυνο της προσβολής των ανθρώπων από τον ιό H5N1 μέσω της στοματικής οδού. Η παρουσία του ιού στα εδώδιμα πτηνοτροφικά προϊόντα και οι αλλαγές εισόδου του ιού μέσω του γαστροεντερικού σωλήνα (GI), καθώς και οι πιθανότητες της εισαγωγής του μέσω του γαστροεντερικού σωλήνα (GI), από απόψεως λοιμογόνου ικανότητας, είναι τα κύρια σημεία εστίασης. Η μετάδοση του H5N1 από τα πτηνά στον άνθρωπο είναι σπάνια και έχει συνδυαστεί με την πολύ στενή επαφή του ανθρώπου με τα ασθενή ζώα. Είναι γενικά αποδεκτό ότι οι πλέον πιθανοί οδοί εισόδου του ιού είναι η στοματοφαρυγγική και/ή οι ιστοί του αναπνευστικού σωλήνα όπου μπορεί να συμβεί η αντιτύπωση του ιού και να οδηγήσει σε κλινικά συμπτώματα. Όμως, ο μικρός αριθμός περιστατικών σε ανθρώπους σε σύγκριση με το μεγάλο αριθμό των ανθρώπων που έχουν έρθει σε επαφή με ασθενή από τον ιό H5N1 ζώα, αποδεικνύει καθαρά ότι δεν υφίσταται εύκολα αποδεκτή θύρα εισόδου. Η πιθανότητα εισόδου του ιού μέσω του GI σωλήνα έχει προταθεί, αλλά μέχρι τώρα δεν έχει αποδειχθεί ότι ο ιός μπορεί να αντιτυπωθεί στο έντερο του ανθρώπου. Η ύπαρξη διάρροιας σε μερικούς ασθενείς και η εύρεση του ιϊκού RNA σε ληφθέν υλικό (swabs) από το έντερο και το ορθό τριών ασθενών δεν επιτρέπει να εξαχθεί, με ασφάλεια, συμπέρασμα ότι ο GI σωλήνας μπορεί χρησιμεύει ως θύρα εισόδου ή όργανο στόχου. Περαιτέρω, τα περιστατικά της νόσου σε ανθρώπους δεν έχουν καθαρά ή οριστικά συνδεθεί με την κατανάλωση κρέατος πτηνών. Είδη αιλουροειδών, αφετέρου, έχουν φυσικά και πειραματικά μολυνθεί μετά την κατανάλωση προσβεβλημένων πτηνών. Όμως, επίσης και σε αυτά τα θηλαστικά, δεν επιβεβαιώ-

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Food as a possible source of H5N1 influenza virus infection in mammals

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ABSTRACT. The recent infections of humans and other mammals with the highly pathogenic H5N1 avian influenza virus have raised questions about the safety of the consumption of poultry products. The aim of this review is to collect and present information regarding the risk of infection in humans with H5N1 virus via the oral route. The presence of virus in edible poultry products and the chances for viral entry via the gastrointestinal (GI) tract, from a pathogenetic point of view, are the main focus points. The transmission of H5N1 from poultry to humans is a rare event which has been associated with very close contact with infected animals. It is generally accepted that the most likely candidate routes for the virus entry are the oropharygeal and/or respiratory tract tissues where virus replication may occur and leading to clinical symptoms. However, the low number of human cases, compared to the high number of humans that have been exposed to H5N1 virus infected animals, shows clearly that a readily accessible portal of entry does not exist. The possibility of virus entry through the GI tract has been proposed, but, so far, no proof that the virus can replicate in the human intestines has been shown. The presence of diarrhoea in several patients and the detection of viral RNA in the intestines and rectal swabs of 3 patients do not allow one to safely conclude that the GI tract can serve as a portal of entry or a target organ. Furthermore, the occurrence of disease has not clearly or definitely been associated with the consumption of poultry meat in any human case. Feline species, on the other hand, have been naturally and experimentally infected after the consumption of infected poultry. However, also in these mammals, it was not established that the intestine is the initial portal of entry of the virus. In conclusion, the possibility that the intestinal tract serves as a portal of entry in mammals remains unlikely, since there is no convincing evidence that the GI tract tissues can support virus replication. It can, however, not be excluded that food containing

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θηκε ότι το έντερο ήταν η αρχική θύρα εισόδου του ιού. Συμπερασματικά, η πιθανότητα ο εντερικός σωλήνας να χρησιμεύει στα θηλαστικά ως θύρα εισόδου παραμένει απίθανο, μιας και δεν υπάρχει καμία πειστική απόδειξη ότι οι ιστοί του GI σωλήνα μπορεί να υποστηρίζουν την αντιτύπωση του ιού. Όμως, δεν μπορεί να αποκλειστεί ότι τρόφιμα τα οποία περιέχουν τον ιό μπορεί να αποτελέσουν πηγή μόλυνσης όταν περνούν τους στοματο-φαρυγγικούς ιστούς, οι οποίοι έχουν ταυτοποιηθεί ως σημεία-στόχοι για την αντιτύπωση του ιού.

Λέξεις ευρετηρίασης: τρόφιμα, γρίπη πτηνών, θηλαστικά

virus may be a source of infection when passing oropharyngeal tissues, which have been identified as target sites for virus replication.

Key words: food, avian influenza, mammals

1 INTRODUCTION

The recent H5N1 avian influenza epidemic in poultry in several Asian countries has led to the occurrence of direct bird to human transmissions of the virus. This unexpected and unprecedented evolution has given rise to an increasing global concern about the zoonotic character and the pandemic potential of the highly pathogenic avian influenza viruses.

Since the H5N1 virus has not adapted to humans so far and considering the low number of human cases compared to the high number of contacts, the transmission of the virus to humans cannot be considered as zoonotic events, but rather as occasional zoonotic incidents. Although occurring rarely, transmission of avian influenza viruses is certainly a concern for public health and better insights in the factors and mechanisms allowing the infection in humans to start are needed when trying to avoid such incidents.

The pathogenesis of H5N1 infections in humans is poorly understood and the exact portal(s) of entry of the virus is (are) not determined. Some information may be found from H5N1 infections in other mammals whether they have become infected naturally or experimentally.

The question which will receive the main attention in the present document is whether ingestion of the virus can lead to infection in mammals including humans. If this is the case, the next important issue may be whether food products derived from infected animals or contaminated during handling, may be a source of human infection. This issue is important from a food safety point of view, but it might also represent a professional hazard (e.g. butchers...). Furthermore, this point has importance when water, which has been contaminated by faecal excretions from infected water birds, is being used for consumption, washing or swimming.

The present contribution is aimed to collect information on the possibilities and restrictions which may influence the occurrence of infection with H5N1 virus via oral ingestion. It mainly considers these features from a pathogenetic point of view and includes information obtained from H5N1 infected mammals, particularly laboratory animals and felines.

2 AVIAN INFLUENZA VIRUSES (AIV)

Avian influenza virus infection in birds has a disseminated course and disease ranges from asymptomatic, to mild respiratory signs, to a rapidly fatal disease. In fatal disease outbreaks, highly pathogenic avian influenza (HPAI) virus subtypes H7 and H5 are involved. As a consequence of the multisystemic pattern of infection in avians, virus is widely disseminated in the body of infected birds. AI viruses in poultry replicate in the intestinal tract and show a definite enterotropism, which contrasts with the classical mammalian influenza viruses when they cause a natural infection in their host. The highly pathogenic AI viruses are thus, in poultry, present not only in all tissues, but also in all products and excretions of infected birds. Transmission of avian influenza viruses between birds occurs via aerosol, but also to a large extent, directly and indirectly, through faeces and faecally contaminated fomites, water or food material.

2.1. Presence of HPAI viruses in organs of poultry

Numerous studies have been performed on the dissemination of HPAI viruses in avians, which serve as important providers of all kinds of food. Tissue tropism, virus titres in different organs and degree of viraemia vary remarkably between different virus strains and among avian species. Most experimental trials have been performed in chickens and less information is available about turkeys or other domesticated birds.

HPAI viruses have been demonstrated in the respiratory and gastrointestinal tracts, bone marrow, muscle tissue, blood and several internal organs (Mase et al. 2005, Swayne and Beck 2005, Muramoto et al. 2006). In one study, the H5N1 strain replicated in thigh and breast muscles to virus titres as high as $10^{7.3}$ EID_{50} per

gram tissue. In internal organs of chickens inoculated with HP H5N1 isolates from Japan, virus titres reached up to $10^{7.5}$ EID_{50} per gram tissue between 2 and 4 days post virus inoculation (Mase et al. 2005).

Intranasal inoculations of turkeys with a HP H5N1 virus from the 1997 Hong Kong outbreak revealed the presence of infectious virus in the upper respiratory tract, lung, heart, brain, pancreas, bone marrow, bursa, thymus, spleen, testicles, adrenal glands and feather follicles, but virus titres were not determined (Perkins and Swayne 2001).

HPAI virus infections in domesticated and wild waterfowl usually remain without clinical signs or lead to disease with a markedly less fulminating character. Duck meat has been found positive for HPAI viruses during routine surveillance of imported poultry. H5N1 virus, for example, was detected in frozen meat of subclinically infected ducks imported from China into South Korea (Tumpey et al. 2002) and Japan (Mase et al. 2005a). These viruses were later used for inoculation trials in ducks, chickens and mice (Tumpey et al. 2003, Mase et al. 2005a). The virus strain recovered from duck meat in South Korea caused 100% mortality in chickens and 22% in mice, while ducks remained healthy even though they contained high virus titres in the brain, lung, kidney (as high as $10^{6.8}$ EID_{50} per gram tissue) and muscle (reaching $10^{5.5}$ EID_{50} per gram) (Tumpey et al. 2002, 2003). The duck meat isolate in Japan was highly pathogenic to chickens, replicated well in the lungs of mice and spread to the brain, but was not as pathogenic in mice as human H5N1 isolates (Mase et al. 2005b).

The H5N1 isolate from duck meat imported into Japan replicated in multiple organs in ducks after experimental inoculation and induced neurological signs in some of the inoculated animals (Kishida et al. 2005). Virus titres in blood amounted to $10^{0.7}$ to $10^{2.3}$ EID_{50} per ml and those in liver and kidney up to $10^{4.5}$ to $10^{7.5}$ per gram tissue. The above information clearly shows that HPAI viruses may end up in all edible products of ducks, even though the animals remain healthy at slaughter.

Experimental inoculations of geese with a HP H5N1 virus from 1997 showed virus replication in brain, lungs and kidneys (Perkins and Swayne 2002). At day 4 post inoculation, virus titres up to $10^{6.7}$ EID_{50} per gram brain, $10^{2.8}$ EID_{50} per gram lungs and $10^{3.6}$ EID_{50} per gram kidney were found.

HPAI viruses are thus widely disseminated in the body of domesticated poultry including geese and ducks. When infected birds are slaughtered during the height of infection, which is 2 to 5 days after contact with the virus, virus can be present at high quantities in all raw edible tissues and in blood.

2.2. Presence of HPAIV in eggs

Virus has been isolated from albumen, yolk samples and the shell surface of eggs during the 1983 HPAI (H5N2) outbreak in Pennsylvania (Cappucci et al. 1985), but not during another HPAI (H7N2) outbreak in 2001-2002 (Lu et al. 2004). HPAI viruses have also been detected in eggs of experimentally infected chickens and turkeys (Moses et al. 1948, Narayan et al. 1969, Beard et al. 1984, Starick and Werner 2003), both on the egg surface and in egg contents. Virus detected on the egg surface probably was the result of faecal contamination during passsage of the egg through the cloaca. Virus detected in egg contents presumably results from viraemia or virus replication in the oviduct, because such eggs were positive particularly when laid 3 to 4 days post inoculation. However, the acute course of the infection in laying chickens leads to a sudden egg drop and to rapid mortality so that the risk that infected eggs are laid or contain high virus quantities is very low.

No information is available on the presence of virus in eggs from AI virus infected ducks.

2.3. Presence of AIV in excretions and secretions

Considering the sites of AI virus replication and the generalised character of the infection with HPAI viruses, different secretions and excretions carry virus sometimes even in high quantities. Chickens inoculated with a highly pathogenic H5N2 virus excreted virus from the nares, mouth, conjunctiva and cloaca. Virus titres on day 3 post inoculation amounted to $10^{4.2}$-$10^{6.5}$ EID_{50} per ml of oropharyngeal secrete and to $10^{4.5}$ EID_{50} per ml faeces (Swayne and Beck 2005).

Chickens inoculated with a HP H5N1 virus excreted virus quantities up to $10^{4.6}$ EID_{50} per ml in the oropharynx and $10^{4.5}$ EID_{50} per ml faeces within 2 to 3 day post inoculation (Tian et al. 2005). In another experiment titres up to $10^{7.5}$ EID_{50} per ml faeces were found in H5N1 inoculated chicks killed at 24 hours post inoculation (Rimmelzwaan et al. 2006).

Ducks and geese experimentally or naturally infected with H5N1 HPAI viruses have also displayed high virus quantities both in oropharyngeal and cloacal swabs (Perkins and Swayne 2002, Tian et al. 2005, Ellis et al. 2004).

These data show that faecal material can contain very high quantities (titres) of HP AI viruses. Faecal material can, therefore, be an important source of avian influenza viruses in different avian species. Faeces excreted from acutely infected birds may readily contaminate all types of food products and water.

3. HPAI VIRUSES IN HUMANS

3.1. Some epidemiological data

It is generally accepted that the classical human influenza virus subtypes (H1N1, H3N2...) do not replicate outside the respiratory tract and that the digestive tract does not play a role in the infection or transmission processes. Only a few rare, and mainly fatal cases of human influenza have revealed low amounts of infectious virus in the blood, internal organs, brain and cerebrospinal fluids. This is also true for the pathogenesis of influenza in other mammalian natural hosts, such as the pig. Transmission of these classical human influenza viruses among humans thus occurs by inhalation of infectious airborn droplets and possibly indirectly through contact and self inoculation of upper respiratory tract or conjunctival mucosae.

Transmissions of HPAIV to humans by H5N1 and H7N7 viruses have occurred and the exact mode of virus entry has, particularly for H5N1, not yet been determined.

Transmission of H7N7 to humans has been reported during epizootics with this virus in poultry and epidemiological evidence shows that it is the result of (self?) inoculation or contact with the conjunctiva. It generally leads to a mild conjunctivitis without respiratory involvement except for one fatal case of pneumonia in the Netherlands (see table 1).

With H5N1, transmission to humans has occurred after close contact with sick or dying poultry and generally had taken place within a week before the onset of disease. Risks were associated particularly with plucking and preparing of diseased poultry, handling fighting cocks and playing with infected poultry, including asymptomatic ducks. In general, no significant risk has to be related with exposure to sick persons or eating or preparing poultry products. However, two cases in humans were reported after eating raw duck's blood, but direct contact with poultry can not be excluded in these cases. Therefore, modes of transmission, such as oral ingestion of contaminated food and water, have been considered in exceptional cases. Swimming in water or rivers contaminated by infected duck faeces, leading to direct intranasal inoculation or conjunctival exposure or possibly to ingestion of virus, have been suggested. Contamination of hands from infected fomites followed by self inoculation and even the use of poultry faeces as fertilizer have also been considered.

3.2. Clinical signs with H5N1

H5N1 infection in humans is characterised by severe influenza-like syndromes with fever, lethargy, cough, shortness of breath and pneumonia. However, besides respiratory symptoms, many patients have complained about gastro-intestinal signs, such as diarrhoea, vomiting and abdominal pain. In some cases, diarrhoea was the first sign preceding other clinical manifestations. Mortality rates are up to 89% in humans younger than 15 years. Death is caused by a progressive respiratory failure followed by multi-organ dysfunction. There have been a few reported cases of central nervous disorders.

3.3 Virology and viral replication

In all cases of human infection with highly pathogenic H5N1 AI viruses in Southeastern Asia, the repiratory tract was clearly the major site of virus replication and of viral pathology (Uiprasertkul et al. 2005). Most patients died as a result of acute respiratory distress syndrome followed by multi-organ failure, but, unlike in poultry, there is no firm evidence for a systemic infection or for infection of the digestive tract. In one fatal H5N1 case in a boy in Thailand, positive-stranded viral mRNA was not only detected in the lung, but also in the small and large intestines, suggesting productive viral production in the gastrointestinal tract (Uiprasertkul et al. 2005). No viral RNA was found in plasma, adrenal glands, brain, bone marrow, kidneys, liver and pancreas. The spleen only contained negative-stranded genomic RNA and no positive-stranded messenger RNA, showing that there had been no influenza virus replication in that organ. On the other hand, examination of the intestines did neither reveal viral antigen-positive cells nor histopathological changes and the patient did not show diarrhoea. In the lungs, viral antigen-positive type II pneumocytes were present and characteristic lesions, such as diffuse alveolar damage, interstitial pneumonia, focal hemorrhage and bronchiolitis, were observed.

In Vietnam 2 fatal cases of H5N1 infection showed severe diarrhoea and encephalitis without respiratory disease (de Jong et al. 2005). Only one of the patients was virologically examined and the virus was isolated from throat and rectal swabs, serum and cerebrospinal fluid. Also, several other H5N1 infected cases showed diarrhoea in the process of the disease. Viral RNA was sometimes detected in intestinal samples by RT-PCR.

Some of these data thus suggest that the gastro-intestinal tract may be involved in the H5N1 infection in humans. However, intestinal viral antigen-positive cells have not been demonstrated until now. While the presence of viral nucleic acid in the intestines could be an indication of virus replication in the intestines, it might also be the result of virus which has been swallowed after throat infection.

In table 1, a list of human cases caused by AI viruses until January 2007 is presented.

Table 1. Avian influenza viruses isolated from humans since 1996

Year	Subtype-pathotype	Location	Number		Total Number		Symptoms
			infected	dead	infected	dead	
1996	H7N7-LP	US	1	0			Conjuctivitis
1997	H5N1-HP	Hong Kong	18	6			Influenza-like
1998-99	H9N2-LP	Hong Kong/China	2	0			Influenza-like
2003	H5N1-HP	Hong Kong	2	1			Influenza-like
2003	H7N7-HP	The Netherlands	83	1			Conjuctivitis
2004	H7N3-HP	Canada	2	0			Conjuctivitis
2003-07	H5N1-HP	Vietnam	93	42	261	157	Influenza-like
		Thailand	25	17			
		Indonesia	74	57			
		Cambodia	6	6			
		China	21	14			
		Turkey	12	4			
		Iraq	3	2			
		Azerbaijan	8	5			
		Egypt	18	10			
		Djibouti	1	0			

*Situation on January 1, 2007; HP: highly pathogenic, LP: low pathogenic

So far, definitive evidence of H5N1 virus replication in the intestines of humans is thus still lacking and based on the above information, it must be stated that the role of the gastrointestinal tract as a possible portal of entry or site of replication for H5N1 virus in humans is doubtful. If oral uptake of H5N1 virus could be responsible for initiating an infection with H5N1 virus, it does not necessarily imply that the lower gastrointestinal tract allows virus to enter in the body. Virus ingested with food could pass, attach to and replicate in tissues in the oropharynx and upper respiratory tract (nasal mucosa). In all H5N1 infected humans, both pharyngeal and nasal swabs yield high viral high loads, which shows that not only the respiratory tract, but also the oropharynx can be a very important target tissue (Beigel et al. 2005, de Jong et al.

2005). It is, therefore, possible that initiation of viral infection after oral virus uptake is a consequence of throat infection rather than of a primary infection of the lower gastrointestinal tract. Also, it is not excluded that presence of intestinal viral RNA or rectal infectious virus is associated with viraemia, considering that infectious H5N1 virus or viral RNA have been demonstrated in the serum of some patients (Beigel et al. 2005, de Jong et al. 2005).

4. HPAIV IN OTHER MAMMALIAN SPECIES

4.1 Natural infection routes

Tigers and leopards: Feeding raw infected chicken carcasses from a local slaughterhouse to tigers and leopards in Thai zoos led to outbreaks of respiratory disease and deaths (Keawcharoen et al. 2004, Thanawongnuwech et al. 2005). This occurred at the time when many chickens in the area were dying with respiratory disease and with neurologic signs due to H5N1 virus. These feline species showed a generalised course of disease. Clinical symptoms included fever, respiratory distress and neurological signs and lesions were present in the lungs, heart, thymus, stomach, intestines, liver, lymph nodes and brains. Virus replication was demonstrated in bronchiolar epithelium, hepatocytes and cerebral neurons. These observations show that H5N1 virus infection can occur in these feline species after oral uptake of H5N1 infected avian carcasses. The site of viral entry upon the oral ingestion of H5N1 virus in tigers and leopards was not determined and no virological examinations of the intestines were performed. The authors suggested that the positive staining for virus nucleoprotein in hepatocytes may have resulted from a heavy virus load that had passed through the digestive tract after carcasses were eaten (Thanawongnuwech et al. 2005). While the intestines as portal of entry was neither proven nor excluded, involvement of the liver, may well have been the result of oropharyngeal or respiratory infection followed by generalisation of the infection rather than initiation in the intestinal tract itself.

Cats: Recently, confirmed natural cases of infection with HP H5N1 virus, presumably as a consequence of feeding on infected wild birds, have been reported in 3 cats and in a stone marten in Germany (source: WHO) http://www.who.int/csr/don/2006_03_09a/en/index.html.

A diagnosis of H5N1 infection was made, but no other virological studies were performed.

Dogs: A fatal H5N1 infection also occurred in a dog in Thailand in 2004 after the ingestion of carcasses from H5N1 infected ducks (Songserm et al. Fatal influenza A H5N1 in a dog: Emerging infectious diseases vol. 12 Nov 2006). The dog showed high fever, panting and lethargy approximately 5 days after consuming the carcasses and he died the next day. Necropsy findings included bloody nasal discharge, severe pulmonary congestion and oedema, and congestion of the spleen, kidneys and liver. H5N1 virus was isolated from the lungs, liver, kidney and urine and immunohistochemistry revealed virus positive cells in the lung alveoli, in the liver, in tubular epithelium and in glomeruli of the kidneys.

Samples from the intestinal tract (duodenum, jejunum, ileum), as well as brain, trachea, heart, spleen and pancreas tested negative in all virologic examinations. Similarly, microscopic lesions were restricted to the lungs, liver and kidneys.

Pigs are also susceptible to avian H5N1 influenza virus in nature. Serological evidence of H5N1 infection was found in a very small population of pigs (8 of 3.175 pigs tested, or 0,25%) in Vietnam in 2004, where H5N1 has hit hardest, and no virological examinations were performed (Choi et al. 2005).

4.2 Experimental infections

Cats-oral inoculations: Infection of 3 domestic cats was experimentally established by oral feeding of chick carcasses from chicks that had been inoculated with H5N1 virus 24 hours earlier (Rimmelzwaan et al. 2006). The liver and lung homogenates of the infected chicks used for feeding contained more than 10^9 ID50 per gram tissue. In all 3 cats, virus replicated not only in the respiratory tract, but also in multiple extra respiratory tissues including the central nervous system. Viral antigen expression and virus-associated ganglioneuritis were observed in the submucosal and myenteric plexi in duodenum and ileum. Virus was detected consistently and at high titres in pharyngeal and nasal swabs, while virus titres were highly variable in rectal swabs. This study clearly showed that H5N1 infection in felids could be established via oral uptake but, again, whether the infection was initiated in the oropharyngeal or gastrointestinal tissues was not determined. The authors suggested that lesions and viral antigens found in the neural plexi of Meisner and Auerbach indicated uptake of virus via the intestinal lumen. The suggestion was made because myenteric plexi were not involved in cats that, in a similar experiment, had been inoculated intratracheally with the same virus strain. Other possible routes of entry were also considered. The neurotropism observed with H5N1 virus in cats was striking and neural pathways of virus spread and dissemination must not be ignored, as further discussed for H5N1 infections in mice and ferrets.

Cats-intranasal and intratracheal inoculations: Intratracheal H5N1 virus inoculations by the use of a catheter were performed in 3 cats as part of the experiments with orally inoculated cats described above (Kuiken et al. 2004, Rimmelzwaan et al. 2006). These cats showed clinical signs similar to the cats that had been orally fed carcasses of infected chicks. Lesions were found in the respiratory tract and extra respiratory tissues including the brain, but not in the intestinal plexi. Virus was isolated from several internal organs tested, which showed that the infection had a generalised character. In these cats, infectious virus was readily detected in pharyngeal and nasal swabs and also in rectal swabs, but the latter were less frequently positive and virus was present at much lower titres. This experiment also demonstrated that HP H5N1 virus can be very pathogenic for cats. Furthermore, horizontal transmission occurred to 2 sentinel cats brought in contact with the inoculated ones after 48 hours.

Pig-intranasal inoculations: In three different experimental studies, pigs have been inoculated intranasally with a high dose ($\geq 10^6 EID_{50}$) of Asian HP H5N1 influenza viruses (Choi et al. 2005; Isoda et al. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals, Arch. Virol. (2006) 151:1267-1279; Shortridge et al. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong, Virology (1998) 252:331-342). Six of the total 8 H5N1 isolates tested were shown to replicate in pigs. Despite the extreme virulence of viruses for poultry, the pigs showed only mild or no clinical signs. Virus titres in nasal swabs were moderate and there was no virus transmission between experimentally inoculated and in-contact pigs. In one study, H5N1 virus was isolated from the respiratory tract (tonsils, trachea and lungs) of all 4 pigs examined and from the liver of 2 of 4 pigs, but not from the intestines, blood, spleen or kidney (Choi et al. 2005). Unfortunately, all these studies were performed on a very limited number of pigs and detailed studies of the cell and organ tropism of H5N1 in pigs are still lacking.

Ferrets-intranasal inoculations: Ferrets have been intranasally inoculated with H5N1 viruses isolated in Hong Kong in 1997 (Zitzow et al. 2002) and in other Southeast Asian countries in 2003 and 2004 (Govorkova et al. 2005, Maines et al. 2005). H5N1 isolates from humans induce more severe respiratory disease, fever, lethargy and weight loss than common human H3N2 human influenza viruses. Diarrhoea and neurological signs were seen following exposure to the H5N1 isolates and many infections were fatal. The H5N1 isolates of human origin caused a systemic infection in ferrets, with high virus titres in the blood, spleen and liver. Only some H5N1 isolates were isolated from the intestine, at virus titres that were at least 100 to 1.000 times lower than those in the respiratory tract. Inflammatory lesions and viral antigen-positive cells, however, were only found in the lung alveoli and bronchioli and in neurons in the meninges, choroid plexus and brain parenchyma. Virus containing cells were not detected in the intestine, despite the presence of diarrhoea in some ferrets. While the human H5N1 viruses had an obvious neurotropism, virus was also isolated from the brains of ferrets inoculated with normal human H3N2 influenza viruses, which do not spread beyond the respiratory tract in humans, and failed to induce neurological signs in ferrets. Because influenza virus titres in the olfactory bulb of ferrets peaked early in infection, the authors suggested that the virus reaches the central nervous system via the olfactory nerves and ethmoid plate after intranasal inoculation. The H5N1 virus was also isolated from the brains of ferrets without detectable viraemia or replication in internal organs, so that it is unlikely that the virus reaches the nervous system via the circulation. Some pathogenetic events in these ferrets inoculated with H5N1 resemble those in cats, particularly the involvement of extra-respiratory organs and the strong neurotropic character. It should be mentioned that involvement of the central nervous system has also been reported in 2 human cases of H5N1 (de Jong et al. 2005).

Mice-intranasal inoculations: Viruses isolated from humans in Hong Kong in 1997 showed two distinct phenotypes in mice (Gao et al., Lu et al. 1999). Some viruses were low pathogenic: they replicated exclusively in the respiratory tract, were generally non lethal, and animals had cleared the virus by 6 to 9 days post inoculation. Other viruses were highly pathogenic: they replicated in multiple organs in addition to the respiratory tract and caused mortality in the mouse model. Most human isolates from 2003 and 2004, in contrast to isolates directly obtained from avians, were also highly pathogenic for mice (Maines et al. 2005). One of such highly pathogenic viruses was isolated from the colon of infected mice, at a low virus titre ($10^{2.5}$ PFU per gram) (Gao et al. 1999). In another mouse infection study with similar H5N1 isolates, the stomach, duodenum and large intestine tested negative for influenza viral antigen positive cells (Dybing et al. 2000). It is clear, at any rate, that the respiratory tract and nervous system are the major sites of H5N1 replication in mice. According to some studies, viraemia is unlikely to contribute to the dissemination of virus to other organs in mice, and this is clearly different in chickens

(Tanaka et al. 2003, Nishimura et al. 2000). One of the Hong Kong/97 H5N1 isolates was found to replicate first in epithelial cells of the nasal mucosa, bronchi and alveoli, thereafter spread via extensions of the nervus vagus and/or nervus trigeminus to the brain stem and later to the cerebral cortex (Tanaka et al. 2003). Virus spread to the central nervous system may also be virus dose-dependent, as a less virulent Hong Kong/97 (H5N1) isolate was isolated from the brain after intranasal inoculation with a high virus dose, but not with a low virus dose.

Macaques-multiple inoculation sites:

Macaques are considered among the most reliable animal models to study the pathogenesis of influenza in humans. In experimental infection studies, cynomolgous macaques were inoculated with a human Hong Kong/97 H5N1 isolate via intratracheal and oropharyngeal and intraconjunctival inoculation routes (Rimmelzwaan et al. 2001, Kuiken et al. 2003). The clinical signs -fever and acute respiratory distress- of H5N1 infection in macaques resembled those in humans. Unlike in cats, mice or ferrets, neurological or gastrointestinal symptoms did not occur in H5N1-inoculated macaques. Another difference with the situation in cats, mice or ferrets, was the lack of evidence for replication of H5N1 outside the respiratory tract. The lungs were clearly the major site of H5N1 virus replication. Multiple organ dysfunction was observed without evidence of virus replication in the brain, spleen, liver or kidney, and was probably due to diffuse alveolar damage and acute respiratory distress syndrome. To date no investigations have been reported as to whether or not H5N1 can replicate in the intestines of macaques.

5. GASTRO-INTESTINAL TRACT AS A POSSIBLE ROUTE OF ENTRY FOR AIV IN HUMANS

For the gastro-intestinal (GI) tract to serve as a possible portal of entry for HP H5N1 virus containing food and thus to be considered as a risk factor from a food safety point of view, several conditions would need to be fulfilled:

1. Virus must be present in food concerned

2. Virus must be present in sufficient quantity to be able to start an infection (reach the so-called minimal infectious dose)

3. The gastrointestinal tract must have cells that allow influenza virus attachment and virus entry in the body

1) Virus must be present

From the above data, it can definitely be concluded that HP H5N1 virus strains are widely disseminated in the body not only of infected domesticated avians (chickens, turkeys and ducks), but also in subclinically infected avian species (ducks and possibly several other wild species). All edible products derived from such animals at the height of infection can be considered as carriers of virus in variable quantities. Considering the acute course of the infection in chickens, virus quantities will be highest when the samples (blood, meat,...) are collected at the end of the incubation period and shortly before the animals become sick, which is 2 to 5 days after the first contact with the virus. Raw food derived from such acutely infected animals (whether sick or not) has, therefore, a high chance to contain virus. HPAI viruses, in general, are also excreted in poultry secretions and excretions in high quantities during these acute stages of disease. Excretions, particularly faeces, of live animals can thus contaminate water and food during slaughter and processing. Eggs may or may not contain virus, but quantities are generally low.

2) Virus quantity reaching the intestine must be sufficiently high

From epidemiological data, it can be assumed that entry of HP H5N1 virus and initation of infection in mammals is not a regular event and that several factors play a role. Intensive contact with infected birds in natural infection or high titers to reproduce infection are needed. This means that there is no easily accessible or highly susceptible primary target site available or that very high virus quantities are needed to reach the minimal infectious dose. Several physical or chemical agents may be of influence to either decrease the virus quantity present in contaminated food or to decrease the quantity of virus reaching the intestinal tract as possible portal of entry. All these may minimise the chance that virus uptake in the gastrointestinal tract occurs.

Temperature: Influenza viruses are rather heat labile. Even though HPAI viruses are, until now, not considered as dangerous from a food safety point of view, different agencies involved in the H5N1 epizootics and human health issues, such as WHO and EFSA, have advised to cook poultry meat and eggs to at least 70°C core temperature prior to consumption. This temperature completely inactivates all influenza viruses. The chance that warmed up food contains sufficient residual virus to start infection is very low to non-existing. However, food products like blood pudding that are consumed raw in some countries might contain sufficiently high amounts of virus if collected from birds during the height of the infection, and may satisfy the criterium for minimal infectious dose if virus uptake via the gastrointestinal tract could occur.

Dilution, removal and inactivation in GI tract: Even if virus containing food is consumed raw, this virus is exposed to several steps which may have a virus removing or inactivating effect prior to reaching the stomach and intestines. After being swallowed, the virus will *adsorb* onto tissues in the upper digestive tract, such as oropharynx, oesophagus and the stomach. It will be diluted by fluids during the digestive processes. Its infectivity may be reduced by the extremely *low pH* of gastric secretions and by the effects of *bile*, when passing the stomach and the duodenum respectively. Influenza viruses have a lipid containing envelope and inactivating effects of bile have been shown with several enveloped viruses.

Studies on the acid resistance of influenza viruses have yielded somewhat contrasting results. In one study (Webster et al. 1978), influenza viruses of ducks were relatively more acid stable than human viruses and it was claimed that this permits them to withstand the acid pH of the gizzard and to replicate in the cells lining the intestinal tract, which is the major site of replication in several avian species. In more recent studies (Lu et al. 2003), 4 H7N2 strains were examined for acid stability at pH 2 and infectivity was lost in less than 5 minutes. Still another study (Scholtissek 1985) showed great differences in pH sensitivity among different subtypes. All H3 strains were relatively stable against low pH (5.1-5.4), independent of the species of origin. All H7 and H5 strains were relatively labile at pH 5.5-6.0. H1 strains showed an intermediate sensitivity. Low pH values appear to affect mainly the HA and NA.

Acid stability of AI viruses appears to be a multifactorial issue in which not only the virus strain, but also the virus quantity, the embedding medium, the pH value and the duration of exposure will play a role. Whether influenza viruses will be inactivated by exposure to the low pH of the gastric secretions is thus unpredictable and complete virus inactivation after passage through the stomach cannot be guaranteed. Acid lability of influenza viruses and virus inactivation by the low pH in the stomach has long been presumed to be one of the main reasons why influenza viruses in mammals do not reach the intestines in an infectious state or in sufficient quantity. However, it must be stated that replication of any influenza virus in intestinal tissues in mammals has never been demonstrated.

3) Virus entry and replication in the lower digestive tract

So far, there is insufficient evidence in humans to state that H5N1 virus, if it ends up in the intestinal lumen upon oral uptake or consumption of infected food, could initiate infection via the intestinal tract or could actually replicate in intestinal tissues of humans. The susceptibility of mammalian intestinal cells to H5N1 or other influenza viruses has been barely examined. In an older pathogenesis study in intranasally inoculated ferrets, 6 of 14 influenza viruses examined (including 2 LPAI viruses), were only isolated from one or another part of the intestinal tract (Kawaoka et al. 1987). In the same study carried out in intranasally inoculated pigs, one of 4 influenza viruses tested was also isolated from the faeces whereas the virus could not be demonstrated in the jejunum, ileum or colon.

A first possible barrier to H5N1 infection of human enteric cells may be a lack of suitable receptors on those cells so that the viral HA cannot attach. Receptor studies in mammals, however, have focused exclusively on the respiratory tract and there is no information on the types of receptors in the human intestinal tract. Obviously, receptor specificity of the viral HA is not the only factor responsible for susceptibility. Indeed, it is generally accepted that the host and host cell specificity of influenza viruses is controlled by a variety of viral genes and their interactions and role in different cellular environments (Zambon 2001). Even if an influenza virus succeeds to enter a host cell, it must successfully co-opt with host cell proteases to replicate there. The viral polymerases, which are responsible for the replication and transcription of viral RNA, play a key role during this stage. During the release of newly produced virus particles from infected cells, the viral HA tends to re-bind to receptors on the cell surface and the neuraminidase (NA) helps to break this binding. Like the HA, the NA has also a preference for one of both types of sialic acid receptors and it may also influence the host and cell tropism of influenza viruses.

Oral uptake of H5N1 has led to infection in cats and tigers, but it should be stressed that either the oropharynx or the upper respiratory tract tissues or the intestinal tract or more than one tissue might serve as the portal of entry of the virus. In the orally inoculated cat experiment, it was suggested that the virus may use, as entry site, nerve endings of the intestinal plexi in the intestinal lumen. It is, however, also possible that spread of influenza virus via neural pathways has occurred and that the plexi were reached via the nervous system and not via the intestinal lumen. Cats showed indeed virus replication in the central nervous tissues indicating neural tropism. Such neural tropism of H5N1 virus has been shown in several experimentally inoculated mammals including ferrets and mice and was also confirmed in one human case in Vietnam. The possibility exists that, through this neurotropic characteristics, virus entry and spread in the body occurs via nerves starting from the upper respiratory tract or

tonsils. Neural spread of H5N1 was shown in mice (Tanaka et al. 2003).

6. CONCLUSIONS

It is unlikely that the lower GI tract (stomach and intestines) could serve as a portal of entry for H5N1 virus in humans after consumption of food products derived from infected animals. There is no evidence that the virus replicates in the human intestine or that gastrointestinal symptoms observed in some patients are due to direct effects of the virus in that organ. Even if virus uptake in the GI tract would be possible, e.g. via nerve endings as was suggested for the orally inoculated cats, the chances that these endings would be hit are very slim considering the many barriers which the virus has to overcome to reach the intestinal lumen in a presumably minimal infectious dose. Diarrhoea, which was seen in some of the human H5N1 cases, may thus very well be an aspecific symptom. Similarly, the presence of viral RNA or even the single isolation of infectious virus from rectal swabs in a few human cases do not allow one to conclude that the GI tract is a portal of entry or target organ for H5N1 virus. Viral RNA or infectious virus may have been produced in throat or respiratory tissues and swallowed, or could have reached the intestine after generalization from other infected targets.

Food that contains H5N1 virus can likely be a source of infection when passing oropharyngeal tissues during swallowing, taking into account that these tissues are considered as an important target site for virus replication. While no intestinal replication with any of the influenza viruses has been demonstrated in mammals, the existence of an undisclosed target cell in the intestinal tract cannot be ruled out. So far, there is no evidence that certain H5N1 strains or mutants exist that have a preference for cell receptors in cells lining the intestinal lumen. However, the exact portal(s) of entry of the H5N1 virus in humans has (have) not been sufficiently studied or identified and animal models can be of help.

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