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# Avian influenza, migratory birds and emerging zoonoses: Unusual viral RNA, enteropathogens and *Cryptosporidium* in poultry litter

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## KEYWORDS

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**Abstract** The last decade has witnessed the emergence of several new viral infectious agents, most notably avian influenza H5N1, SARS and West Nile Virus. The emergence of these agents is heavily associated with zoonotic animal hosts, as well as migratory pathways of infected bird vectors. The environmental survival and persistence of nucleic acid associated with these viral agents may be important for both the detection as well as the occurrence of related diseases. Our hypothesis suggests that nucleic acid from such emerging viruses may enter into a virus-parasite surrogate relationship to aid in viral persistence. We suggest that *Cryptosporidium* and other gastrointestinal parasites, including *Giardia*, could be a) a reservoir of genetic material and a environment where assortment between that genetic variation can occur and, b) a source of zoonoses through infection of the 'target' animal (including humans). One example which illustrates this may be the uptake dsRNA from rotavirus into cryptosporidial oocysts, as this parasite has previously been shown to contain dsRNA viral-like particles. The importance of such a surrogate relationship is discussed and its implications for human and animal health highlighted.

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## Outbreaks in birds worldwide

The sporadic outbreaks of highly pathogenic (HP) H5N1 avian influenza viruses in domestic poultry in Far East Asia since 1997 [1] and in particular its tendency to mutate and spread in poultry as well as in humans has been reported extensively [2]. A resurgence of outbreaks of avian

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influenza (A/H5N1) has been reported recently in poultry in Vietnam, South Korea, Japan, Thailand and Hungary, as well as cases of human infection in Indonesia and China, following a period of relatively low activity. This is not unexpected, as similar trends were observed annually in 2004, 2005 and 2006 in Far East farms. The potential environmental sources are continually and intensively being monitored worldwide and thus H5N1 still poses a serious threat to public health and spreading alarm of an imminent pandemic in the West.

(HP) H5N1 was reported in Europe in 2006, mainly in wild birds [3]. In February 2007, there was an avian influenza outbreak on a large turkey farm in Suffolk, UK [4]. On 12 November 2007, a second outbreak of (HP) H5N1 in poultry occurred on another farm in Suffolk and was confirmed on the same day by the Department for Environment, Food and Rural Affairs (DEFRA) [5]. The affected farm contained approximately 5000 turkeys, 1000 geese and 4500 ducks. It was a free-range farm, where birds were kept indoors at night but in paddocks during the day, thus potentially exposing them to wild birds, for which the area is well known.

In Vietnam, 52 outbreaks of avian influenza in wild birds and poultry were notified, between 6 December 2006 and 17 January 2007, affecting 41 communes in 19 districts of seven provinces in the southern part of the country (Ca Mau, Bac Lieu, Hau Giang, Kien Giang, Vin Long, Soc Trang, Tra Vinh). The outbreaks in December and January 2006 occurred in provinces where immunisation should have been carried out, although a number of them occurred on farms that were unregistered and therefore had been missed by vaccination teams. Infections also occurred on some registered farms, either because of incomplete immunisation coverage or vaccine failure. Vaccination of domestic poultry is continuing. So far, around 2.1 million birds have been vaccinated (including approximately 0.3 million chickens and 1.8 million ducks), but full coverage has not been achieved as yet. Single outbreaks of avian influenza in domestic poultry have recently also been reported in Japan – on a chicken farm on the island of Kyushu, and in Thailand on a duck farm in Phitsanulok province, where there had been no cases of H5N1 infection since 2004 and July 2006, respectively. Other outbreaks have also been reported in South Korea (<http://www.eurosurveillance.org>).

## Worldwide situation in humans

According to the WHO, as of the 19 June 2008, as detailed in Fig. 1, the cumulative number of confirmed human cases of avian influenza A H5N1, reported to the WHO was 385 of which there were 243 deaths (63% mortality) ([http://www.who.int/csr/disease/avian\\_influenza/country/cases\\_table\\_2008\\_06\\_19/en/index.html](http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_06_19/en/index.html)).

In Egypt, two human cases in two members of one family were reported in December 2006. Both patients died in the same month ([http://www.who.int/csr/don/2007\\_01\\_18/en/index.html](http://www.who.int/csr/don/2007_01_18/en/index.html)). On 18 January 2007, a report by the WHO revealed that samples from these two patients had genetic markers previously observed to be associated with some reduced susceptibility of influenza viruses to oseltamivir (see WHO Report, 2007 for further details). Both patients had been on treatment with oseltamivir, but only for two

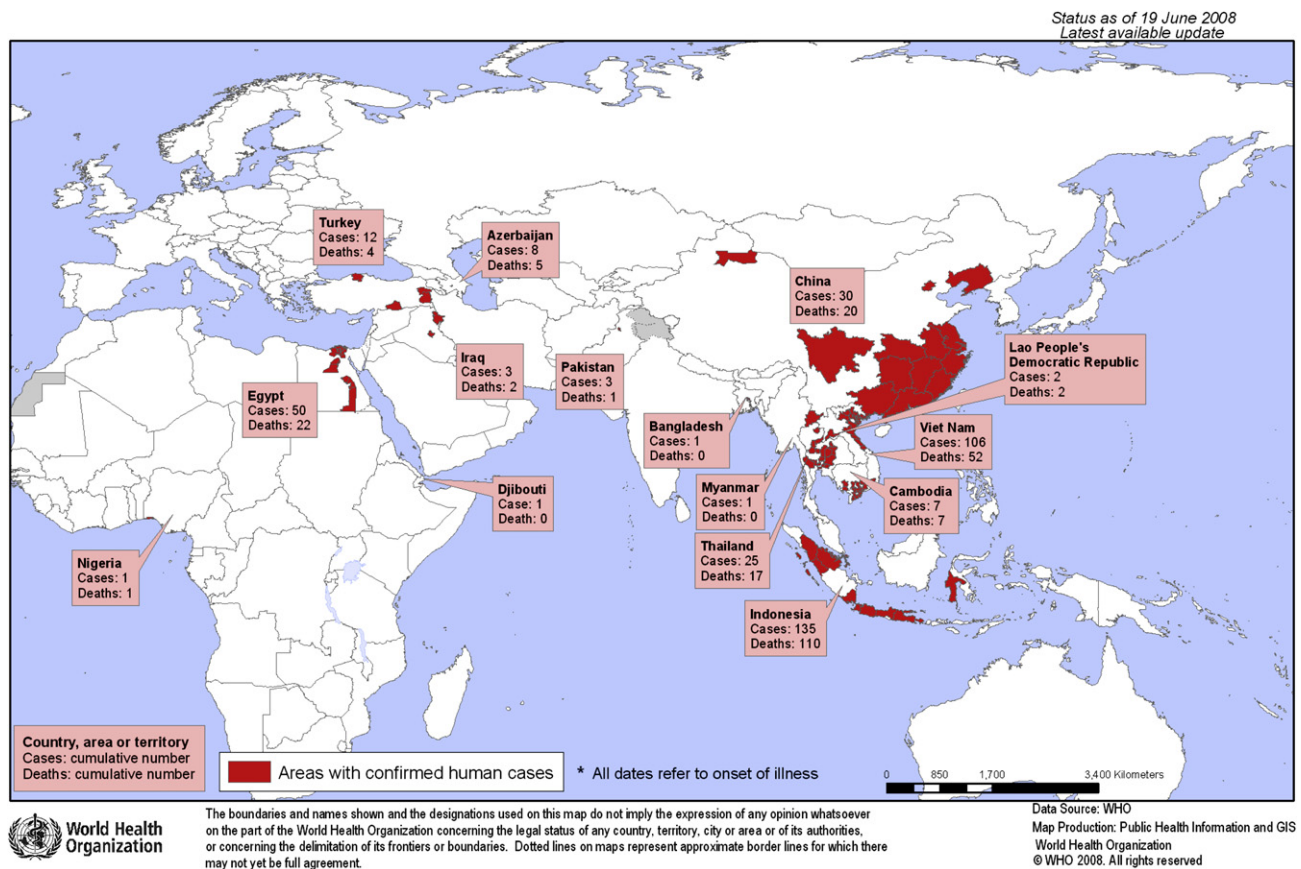
days before the samples that yielded the viruses were taken. This genetic marker of resistance had previously been identified in Vietnam in one patient in 2005. These markers are not associated with any known change in the transmissibility of the virus between humans, and antiviral resistance is uncommon in Egypt, therefore the public health implications at this time are limited. Monitoring of the levels and type of antiviral resistance is important and is being carried out. An update on the epidemiology of human infection and viral ecology of avian influenza H5N1 has been recently reviewed [6]. So far in 2008, there have been 34 human cases reported from Bangladesh (1), China (3), Egypt (7), Indonesia (18) and Vietnam (5), which have accounted for 26 deaths (see Fig. 2).

## Current perspectives of H5N1 vs human health

To date, transmissions have nearly always occurred after close contact between humans and infected birds. However, the situation in birds remains a cause for concern. Despite integrated efforts in the veterinary sector in some countries (vaccination, surveillance, increased bio-security), outbreaks of H5N1 are still occurring regularly or intermittently in poultry in China, Egypt, Indonesia, Thailand and Vietnam, where humans live in close contact with domestic animals. Furthermore, cases of H5N1 infection have been suspected or reported in birds in countries where people have close contact with poultry but the lack of effective surveillance means it is impossible to determine whether or not H5N1 is present in poultry. The fact that the H5N1 virus has been spreading since 2003, without causing the emergence of a pandemic strain, does not necessarily mean it will not do so. It is believed that the avian strain that contributed to the influenza pandemic in 1918–1919 was present in birds for a number of years before the pandemic emerged [7] and the origin of the 1918 flu pandemic is itself an enigma and still remains so. That is why the elimination of the H5N1 virus in domestic poultry remains crucial. Molecular technology has stepped in to seek some answers to this riddle. Molecular characterization of the H5N1 virus from poultry in Asia [8,9] and the complete genome of human H5N1 isolates from Thailand, indicated that the virus might have adapted to allow spread more efficiently among avian species.

## Background work in Northern Ireland

Apart from migratory wild birds, contaminated straw and water used in poultry houses are also among the most likely sources that have been suggested for the entry of viruses in France, Germany or the UK, during the outbreaks of avian influenza virus in recent years. Insofar as poultry houses are concerned, our group [10] has been intensively monitoring raw ingredients of mushroom composts, particularly straw and poultry litter for altogether different purposes. In the course of this study comprising several strands of investigation, we found that the compost straw and poultry litter carried an unusual compendium of dsRNAs associated with mushroom virus (X) disease [11]. Hybridization and sequence analyses of such segmented RNAs have suggested that they could have arisen from more than a single virus



**Figure 1** Areas with confirmed human cases of H5N1 avian influenza since 2003\*. WHO source: [http://gamapserver.who.int/mapLibrary/Files/Maps/Global\\_H5N1inHumanCUMULATIVE\\_FIMS\\_20080619.png](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1inHumanCUMULATIVE_FIMS_20080619.png).

and some of these elements could be associated with fungal hosts such as *Cryptonectria* spp. [12]. To elucidate the aetiology of these unknown viruses, we closely monitored farm materials and their wastes for bacteria, faecal parasites (e.g. *Cryptosporidium* spp.) and viruses. We found a number of novel eubacteria [13] and the occasional oocysts of cryptosporidia in mostly wet straw/compost wastes that were being processed alongside pig slurry solids and in water samples. Other workers have found similar organisms, including *Pseudomonas aeruginosa* [14]. Wheat straw used in composts also contained phytopathogenic bacteria of the genus *Pseudomonas*, some of which carried a unique dsRNA phage (phi-6), from which, we have isolated 'defective' virus-like particles (VLPs), by zonal centrifugation [15].

### Basis of our hypothesis

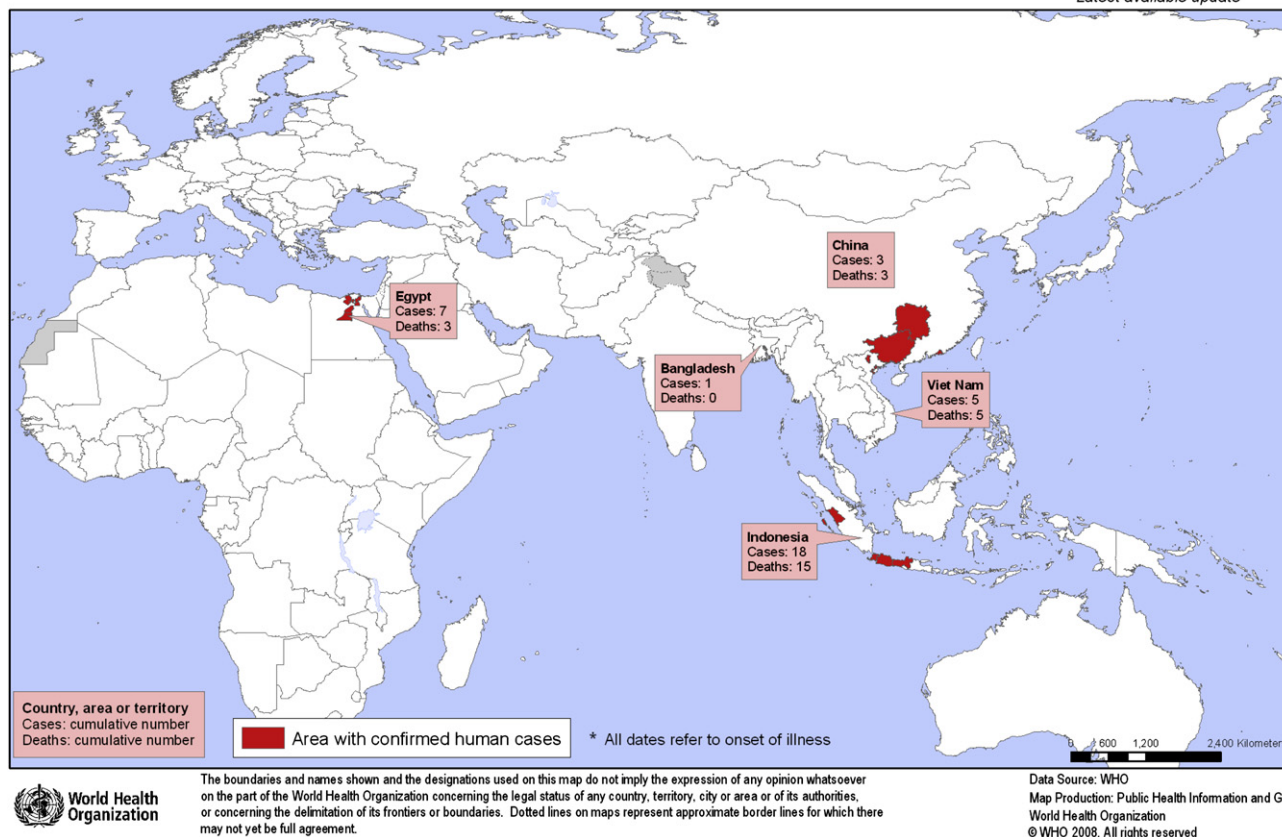
Given that the avian influenza H5N1 virus is a negative-sense ssRNA virus and prompted in part, by our observations concerning (ds) and (ss) RNAs, which are indicative of the presence of viral forms, coupled with the imminent threat of avian influenza virus flu, we set out to review our plant and animal sanitary risk assessments and screen for viral RNA components in straw, poultry litter, water and the wastes arising from poultry farms, particularly of those located in the vicinity of the mouth of rivers in County Antrim, Northern Ireland as it enters into the province's

largest watercourse, Lough Neagh (Grid Reference 134867; Ordnance Survey (NI) 1:50,000; sheet 14; Grid letter Grid J). The basis for extending our investigations to include avian influenza virus components was due to the fact that Lough Neagh is a niche for flocks of mute swans (*Cygnus olor*) from north-western continental Europe, which are partially migratory or nomadic [16]. Following treatment of Lough water, including filtration and chlorination, this water is used as a source of potable drinking water, as well as being utilized for animal farming, particularly poultry and/or pig farms. In addition the Lough itself is frequently used for recreational purposes, including wind-surfing and water/jet-skiing. To date, there has been limited data on the carriage of faecal pathogens in mute swan populations, with the exception of two reports concerning salmonellae in the mid 1970s [17, 18]. Although our previous report did not suggest that these wild swans are carriers of avian influenza virus, from the risk assessment point of view, these may be regarded as a potential source or may serve as a model for other migratory wildfowl.

### Preliminary experimental evidence

Selective enrichment techniques [19] and molecular methods [20] were used for the isolation and identification of enteric pathogens, which were found in poultry litter or in water samples on farms, but which only occasionally present in fresh composts. Enteric pathogens were

Status as of 19 June 2008  
Latest available update



**Figure 2** Areas with confirmed human cases of H5N1 avian influenza since 1 January 2008\*. WHO source [http://gamapserver.who.int/mapLibrary/Files/Maps/Global\\_H5N1Human\\_2008\\_FIMS\\_20080619.png](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1Human_2008_FIMS_20080619.png).

infrequently isolated from composted wastes [21] and faecal material from wild swans [22]. However, the test parameters, load levels and sampling regimes including timing and seasons may in future determine the true nature of the presence or absence of zoonotic pathogens. Most pathogenic bacteria have relatively short periods of persistence in different environments, such as composts, waste and the water environments (ranging from a few days to months), however it should be noted that some *Salmonella* spp and other zoonotic pathogens have survived nearly 20 years in soils and in lake beds [23]. Such observations have not been followed up and the viral ecology has been, by far, either an overlooked or ignored subject. For instance, many indicator pathogens for achieving sanitation in an environmental niche remain enshrined and reliant upon mainly bacterial and occasionally fungal species mortality, survival or persistence data.

#### Lack of comprehensive inventory of viruses, sources of contaminants (straw, water, faecal droppings)

Despite the rapid advancement of molecular techniques for pathogen detection in plant, animal or human and environmental samples [24], viruses have been the least studied of all pathogens and much less understood for their

survival, persistence and regeneration in compost or their wastes [25]. We do not know the host range, their survival and as to how host organisms would support or maintain viral ss/dsRNA in harsh environments, such as those encountered by avian flu virus. Niche/host generalist (e.g. gram negative bacteria such as *Escherichia coli*, *Pseudomonas* spp., etc) and specialist (viral or bacteriophage) interactions are known [26], but viral ecology and maintenance of "novel host" use has been by far less investigated and scarcely understood. Since World War II we have witnessed that RNA viruses are prone to mutations during their attachment to proteins that change or expand their host ranges and may well offer, in part, a plausible explanation as to why some ~50 RNA viruses including human immunodeficiency virus, Ebola virus, SARS coronavirus and several hantaviruses have shifted from non-human hosts to humans [27,28]. Interestingly, BLASTn alignments of deduced RdRP (RNA-dependent RNA Polymerase) sequences of unknown ss/dsRNAs [15], that we have isolated from poultry farms, exhibit the highest similarity with RdRPs of PRD1 phage (host: *Salmonella typhimurium*), fowl plague virus and those of dsRNA carrying *Cryptosporidium baileyi* (poultry biotype). Previously, we reported on the prevalence of bacterial faecal pathogens from unseparated slurry, as well as separated solid and liquid fractions of slurry, from a commercial pig farm in Ireland [29]. In recent years, strict European Union legislation (EC no.1774/2002)

has been invoked for controlling the disposal of wastes carrying animal residues in feedstuff, as well as detailing rules about food or farm wastes containing animal by-products not intended for human consumption [30]. Farm wastes pose the 'silent' danger of human food-borne disease outbreaks and have health implications worldwide.

### Preliminary molecular surveillance for avian flu viral RNA elements

In the light of the above, one has to bear in mind that processed and unprocessed animal farm wastes are in circulation and they, eventually can enter watercourses and thus pose a serious zoonotic pathogen threat. Therefore, in a preliminary study, we re-assessed the RT-PCR screening procedures for the occurrence of viral elements, vis-à-vis, bacterial and parasitic faecal pathogens from a major watercourse in Northern Ireland frequented by migratory wild swans. The same water source, following appropriate treatment, is used for several purposes including water for farm animals, human consumption as well as for water recreational activities. Our initial assays using methods of [31] revealed that *C. baileyi* was present in the samples tested. Using a 'One-Step RT-PCR' kit and the primers described by [8,32] for amplifications of target sequences from RNA extracts from undifferentiated water samples (incorporating parasitic oocysts), we obtained amplifications of at least three of the eight segments of influenza virus [33]. Interestingly, BLAST alignments of deduced RdRP (RNA-dependent RNA Polymerase) sequences exhibited highest similarity with RdRPs of PRD1 phage (host: *S. typhimurium*), phi-6 phage (host: *Pseudomonas phaseolicola*), fowl plague virus and those of dsRNA carrying *C. baileyi* (poultry biotype). However, it has to be stressed that none of the samples tested positive for the presence of all segments of avian influenza H5N1 [33].

*Cryptosporidium* spp. have emerged as an important parasitic infections in both the immunocompromised and the immunocompetent human host. Waterborne transmission of these protozoa is a major risk factor in the acquisition of this infection by avian or human populations, for which there is presently no effective antimicrobial treatment [34]. Therefore it is important to know the oocyst load originating in vectors close to water sources, including swans and their progression in poultry bird stocks.

The contribution by these birds to the environmental contamination of surface water associated with their habitat is probably small. Nevertheless, the parasitic pathogen could serve as a 'surrogate' for variant viral elements. However, it is not known as to the potential 'synergistic' effects emerging from the compendium of viral elements of different descriptions, that we obtained in our separate strands of investigations. Classic examples of animal virus evolution are well documented [35] in that 'defective' viral particles in conjunction with helper viruses could trigger the evolution of new viral diseases and their successful assemblage is analogous to the mechanisms of in vitro synthesis of bacteriophage Q $\beta$  RNA and the outcome will, of course, eventually depend on host reactions. A number of undiscovered candidates may exist to act as helper viruses including the defective RNA elements of

bacteriophage in the straw. Furthermore, the detection of avian cryptosporidia from the water frequented by wild birds raises the prospects of 'intermediary hosts' that may play a role in viral disease development.

While we cannot assert the parasitic transmission as the potential route of the viral disease at this juncture, we suggest that cryptosporidia can be used as an environmental 'surrogate' indicator for viral presence. Previously RNA polymerase complexes have been purified from *Cryptosporidium parvum* [36]. Western blot analysis revealed the association of the complexes with two different proteins, encoded by large and small segments of viral double-stranded RNAs. Each complex was found to contain only double-stranded RNA, both double- and single-stranded RNA, or only single-stranded RNA. Maximum RNA-dependent RNA polymerase activity was observed within the complexes containing both double- and single-stranded RNAs. These complexes possessed both transcriptase and replicase polymerase activities. Virus-like particles with a diameter of 31 nm were copurified with RNA polymerase complexes, and buoyant density and polymerase studies suggest that *C. parvum* harbors a putative double-stranded RNA virus which separately encapsidates the large and small RNA segments. The mechanism of replication and other characteristics of this virus are similar to those of the viruses of the family Partitiviridae, previously identified only in fungi and plants.

In our studies too, *Cryptosporidium* oocysts tested by RT-PCR assays exhibited specific viral molecular signatures of a compendium of viruses (ss, dsRNA species) and thus reiterates the prospects of cryptosporidia oocysts being used for 'surrogate' detection of viral genetic imprints for monitoring, surveillance and vigilance of environmental samples for risk assessments. As our findings represent an initial risk assessment study of only one flock of migratory swans, a more comprehensive study comprising medical, veterinary, molecular and aquatic science researchers is now required involving larger population sizes and from a greater geographical area, particularly from surfaces waters utilized for water extraction.

### Bird faecal material may still hold clues to some of sporadic viral outbreaks

Reed et al. (2003) [37] observed that aquatic waterfowl are asymptomatic carriers of essentially all hemagglutinin and neuraminidase combinations of influenza A virus. Avian influenza strains do not usually replicate well in humans, but they can undergo genetic re-assortment with human strains that co-infect pigs. This can result in new strains with a marked increase in virulence for humans. Wild birds can acquire enteropathogens, such as *Salmonella* and *Campylobacter* spp., by feeding on raw sewage and garbage, and can spread these agents to humans directly or by contaminating commercial poultry operations. Our preliminary study on poultry and pig farms has indicated the potential dangers as these animal agriculture units are in proximal distances in Ireland. Conversely, wild birds can acquire drug-resistant enteropathogens from farms (e.g. epidemiology of antibiotic resistant campylobacters, [38]) and spread these strains along migration routes. Birds

contribute to the global spread of emerging infectious diseases in a manner analogous to humans travelling on aircraft. A better understanding of avian migration patterns and infectious diseases of birds would be useful in helping to predict future outbreaks of infections due to emerging zoonotic pathogens [39]. The World Health Organization considers that direct contact with infected poultry or with surfaces and objects contaminated by their faeces is the main route of human infection with H5N1. Most human cases have occurred in rural or periurban areas where many households keep small poultry flocks, which often roam freely, sometimes entering homes or sharing outdoor areas where children play. Infected birds shed large quantities of virus in their faeces, so opportunities for exposure to infected droppings or to environments contaminated by the virus are frequent in these circumstances.

## Conclusions and hypothesis testing

Many of the global emerging infectious diseases continue to be of viral aetiology, including H5N1, SARS and more recently H1N1. Social trends in travel behaviour, global migration due to war, insurrection or for economic purposes, as well as global warming, distort the well-established "classical" mechanisms of transmission of such viral agents and now require us to reconsider new avenues and vehicles of transmission of such agents. Our hypothesis suggests that *Cryptosporidium* and other gastrointestinal parasites, including *Giardia*, could be a) a reservoir of genetic material and an environment where assortment between that genetic variation can occur and, b) a source of zoonoses through infection of the 'target' animal (including humans).

We urgently need to test whether or not such parasites can allow the uptake and integration of novel viral agents, particularly the dsRNA viruses, either through passive uptake or through some symbiotic relationship between virus and parasite, to aid these both in persistence and future infectivity of future hosts. Given the urgency of combating emerging infectious viral diseases, we encourage other groups to examine this hypothesis as a novel route of transmission, for the sake of global public health.

## Conflict of interest

None of the listed authors have got any conflicts of interest with this manuscript.

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