Influenza Virus Serology at the Human-Animal Interface – from black and white to shades of grey

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Chapter 1

General Introduction

ZOONOSES

The word 'zoonosis' – from the Greek words 'zoon', animal, and 'nosos', disease (plural: zoonoses) – describes diseases transmissible from animals to humans [1]. Various taxa of pathogens, i.e. viruses, prions, bacteria, fungi, protozoa and helminths are associated with zoonotic diseases. Modes of transmission vary from direct- or indirect contact, to the vector-borne route. A comprehensive literature review showed that 60% of 1.415 human pathogens are zoonotic, of which 12% constitute 'emerging infectious diseases'. Thereof, 75% (n=132) have their origin in animals. Zoonotic diseases are twice as likely to be associated with emergence compared to non-zoonotic ailments. When investigating emergence in relation to different taxa, an increased relative risk was only found for viruses and protozoa, but not for bacteria, fungi and helminthes [2].

Besides the economic impact of zoonoses on the animal sector [3], emerging zoonotic diseases also pose a risk to global public health, e.g. through contact with infected animals or indirectly via contaminated food, water supplies or environments [4]. In line with the 'One Health' approach, the World Health Organization (WHO) closely collaborates with the World Organization for Animal Health (OIE) and the Food and Agriculture Organization (FAO). Together, these institutions ensure that WHO member states fulfill the requirements to ascertain and evaluate public health risks posed by emerging diseases, including zoonoses, in the frame of the International Health Regulations [4]. A rapid response and joint action for efficient containment of spread of emerging zoonotic diseases across country borders is paramount. The handling of the SARS outbreak constitutes an example of successful concerted action [5].

In order to cross the interspecies barrier and subsequently efficiently spread in the human population, animal pathogens have to overcome several complex hurdles [6–8]. The first requires potential zoonotic pathogens to come in close contact with humans. In recent years, geographical-, ecological-, environmental- and human behavioural changes have created ample opportunities for such spill-over events, e.g. by disruption of wildlife habitats due to land clearing to create agricultural space, wildlife hunting, animal trade or changes in farming practices [9]. Secondly, a pathogen needs to tackle the intra-human barriers to establish itself in the new host, e.g. by adaption of receptors to enable cell entry- and exit mechanisms and replication machinery [10]. Efficient human-to-human transmission constitutes the third and last step in the chain of emergence, as was demonstrated during the recent H1N1 swine flu pandemic in 2009.

In this thesis, I present avian influenza as an example of zoonotic diseases, which has succeeded in overcoming the first two steps in the chain of emergence, but has not yet acquired the ability to cause efficient human-to-human transmission.

INFLUENZA A VIRUSES

General characteristics of influenza viruses

Influenza viruses belong to the family of *Orthomyxoviridae*. The genome consists of negative-sense single-stranded RNA segments encoding for eleven structural and non-structural proteins. Influenza viruses separate into three genera, termed A, B and C, on the basis of variation in the nucleoprotein and matrix1 (M1) protein [11,12]. Influenza B viruses cause yearly outbreaks in humans [13,14] and are capable to infect seals [15]. Influenza C viruses cause asymptomatic- or mild upper respiratory disease in humans and pigs [16,17]. In contrast to B and C, influenza A viruses are known to have a large host range spanning terrestrial and marine mammals (e.g. humans, swine, dogs, cats, horses, whales, etc.) [18], as well as a large number of wild- and domestic bird species [19]. Two of the eight gene segments of influenza A viruses encode the surface proteins, hemagglutinin (HA) and neuraminidase (NA). Based on these structures, influenza A viruses are classified into different subtypes. Upon infection, the host's immune system responds by generating antibodies to viral proteins, which are targeted to detect past infections. To date, 18 hemagglutinin- and 11 neuraminidase types are known of [20–22]. With exception of H17N10 [21] and H18N11 [22] of which RNA was recently detected in bats, wild birds constitute the reservoir of influenza A viruses [23]. The role of bats as potential reservoir for potentially zoonotic influenza viruses remains to be elucidated [24] and will be further explored as part of this thesis.

Influenza pandemics in the human population

In addition to yearly, seasonal epidemics, influenza A viruses are associated with four major pandemics in the human population. In the 20th century, the following pandemics claimed the lives of millions of people: A(H1N1) in 1918, the 'Spanish flu', A(H2N2) in 1957, the 'Asian flu', and finally A(H3N2) in 1968, the 'Hong Kong flu'. All subtypes circulated successively, until in 1977, strains of subtype A(H1N1), which appeared to be closely related to viruses circulating in the 1950s, resurfaced. This reintroduction into the human population became known as the 'Russian flu' and was putatively caused due to accidental release from a laboratory [25–27], although a recent study suggests that the re-emergence of the virus was more likely due to vaccine trials [28]. From then on A(H1N1)- and A(H3N2) strains have started to co-circulate and, together with influenza B strains, still cause seasonal epidemics in the human population worldwide to date.

In 2009, a novel subtype A(H1N1) emerged in Mexico [29] and caused the first pandemic of the 21st century, which became known as 'swine flu' [30]. The name referred to its mammalian origin, as A(H1N1)pdm09 resulted from complex reassortment events of Eurasian- and North American swine influenza lineages [31,32]. Similarly, previous pandemic strains emerged through multiple reassortment events between circulating human influenza

viruses, and influenza viruses of animal origin [26,32,33]. After the pandemic had subsided, A(H1N1)pdm09 has continued to circulate as seasonal influenza virus strain [34].

Until the unfolding of the A(H1N1)pdm09 pandemic, it was speculated that only 'novel' subtypes can cause future pandemics, i.e. subtypes which have not circulated in the human population before and hence, which the human population is considered immunologically naïve to [35]. In line with this hypothesis, avian subtypes A(H5N1) or A(H9N2) – which had already caused a number of human infection at the time – were regarded as the most likely candidates to cause the next human pandemic [36–39].

Avian influenza as zoonotic disease

The zoonotic potential of avian influenza (AI) viruses was first recognized in the 1960s and 70s when H7 viruses were isolated from patients presenting with conjunctivitis [40–42]. Later, AI subtypes carrying the hemagglutinin types H4, H6 and H10 could be recovered from experimentally infected human volunteers [43]. Highly pathogenic (HP) AI subtype A(H5N1) was first reported to have crossed the species barrier to humans in 1997 in Hong Kong, causing the death of a three-year old boy [44,45]. About six months after this incident, 17 additional human cases were detected, six of which were fatal [46,47]. Concomitant with the human fatalities, HPAI A(H5N1) caused high mortality (~75%) in poultry populations in Hong Kong. Until then, HPAI A(H5N1) had only drawn attention by causing an outbreak among wild geese in Guangdong, China in 1996 [48]. Concerted international action and targeted surveillance quickly lead to the identification of poultry markets as the source for human infection. Driven by concerns that HPAI A(H5N1) might spread and cause the next human pandemic, mass culling of millions of chickens and ducks in the region was initiated consequently [49]. Despite thorough control measures, precursor viruses of HPAI A(H5N1) kept circulating in the region [50], which ultimately resulted in the reporting of three additional human cases in early 2003 [51]. During late 2003 to early 2004, HPAI A(H5N1) resurfaced and rapidly spread across South East Asia [52], causing large outbreaks in poultry and again accounting for a number of new human infections [53]. In 2005, HPAI A(H5N1) further expanded its geographical range to other Asian-, African-, European and Middle Eastern countries [54].

In addition to HPAI A(H5N1), various other HP and low pathogenic (LP) avian influenza (AI) virus subtypes have expanded the list of zoonotic infections, such as A(H9N2), H7-, H6- and H10 subtypes (Table 1). High- and low pathogenicity relates to the course and severity of disease in infected poultry and does not pertain to course of disease in humans [55]. Serological evidence of infection in humans has been found for even more subtypes (Table 1). With exception of a few fatalities of cases with underlying disease, subtypes other than HPAI A(H5N1) generally elicit only mild symptoms in humans [56,57]. In 2013 however, a novel avian influenza subtype emerged, LPAI A(H7N9), that caused similarly severe disease in humans as HPAI A(H5N1) [58]. From its emergence until now, LPAI A(H7N9) caused a total of 488 human cases with a case fatality rate of 38% [59]. Similar to HPAI A(H5N1), direct or

indirect contact with alive or dead poultry and even merely visiting live poultry markets without poultry contact, were identified as risk factors for human infection [60,61]. In contrast to HPAI A(H5N1), A(H7N9) constitutes a low pathogenic subtype (i.e. it causes no or only mild symptoms in poultry) allowing it to spread largely unnoticed in poultry populations [62]. This characteristic and other factors has made monitoring of the distribution of LPAI A(H7N9) in poultry and assessment of associated public health risk, a challenge [63].

Table 1. Avian influenza virus subtypes associated with human infection according to virological- and serological evidence. For virological evidence, information on hemagglutinin (HA) as well as neuraminidase (NA) is stated. For serological evidence, only HA-type is indicated through dark grey shading. For non-shaded subtypes (H14, H15, H16), screening for antibodies was attempted, but no such evidence could be found [57,64, Sikkema et al., submitted].

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16
N1					HP											
N2							LP									
N3							*									
N4																
N5																
N6																
N7							*									
N8																
N9																

HP, Highly pathogenic; LP, low pathogenic; *low and highly pathogenic variants

AI viruses have caused occasional human infections and have not (yet) acquired the ability to efficiently spread in the human population. However, the knowledge of potential adaptation to a mammalian host requiring only few mutations and thereby gaining airborne transmissibility [65,66], reassortment events triggering the past pandemics [67], as well as extensive trade-related movements of poultry increasing the human-animal interface [68] show that AI viruses still pose a continuous public health threat that warrants vigilance [35,69]. Hence, the implementation and continuous evaluation of surveillance systems at the human-animal interface, despite being challenging, are paramount.

INFLUENZA SURVEILLANCE

Surveillance activities are essential to monitor spread of disease, devise control- and prevention strategies and inform public health policymaking. In Europe, several surveillance

approaches are employed: active or passive, mandatory or voluntary, as well as case-based or aggregated [70].

For human influenza viruses, the Global Influenza Surveillance and Response System (GISRS) has been conducting virological surveillance since 1952. Consisting of more than 140 National Influenza Centers (NICs) worldwide, the main tasks of GISRS include advising on strain selection for vaccine updates based on close monitoring of antigenic changes of circulating influenza viruses and providing strains for vaccine production. A selection of clinical specimen received and tested by NICs is sent to Collaborating Centers of the World Health Organization (WHO-CC) and Essential Regulatory Laboratories to further examine virus properties [71].

In 2004, the GISRS responded to the public health threat posed by HPAI A(H5N1) by establishing the WHO H5 Reference Laboratory Network. The primary aim of this network is to collect data on H5N1 and other influenza viruses potentially relevant for public health isolated from humans as well as animals. Identified strains are provided to WHO for potential vaccine production in the frame of pandemic and pre-pandemic preparedness [72]. On the animal side, AI outbreaks caused by viruses with HA types H5 and H7 constitute notifiable diseases, which require mandatory reporting to the World Organization for Animal Health (OIE). LPAI viruses with other HA types are not notifiable [73].

Monitoring avian influenza in the veterinary sector

In Europe, national surveillance of AI in the veterinary sector is well regulated and is carried out all year round. The monitoring system for AI employs a mixture of active and passive surveillance in domestic poultry as well as in wild birds. Facilitated through early warning systems [74], passive surveillance includes virological testing if HPAI (H5- or H7) is suspected in a poultry flock, e.g. when elevated mortality is observed. Complementary, active surveillance aims to detect subclinical circulation of LPAI viruses using serological methods. For that, targeted periodic serological surveillance is carried out in all poultry species (e.g. chicken, turkey, quail, ducks etc.) and production types (e.g. breeders, layers, broilers). The risk of introduction of LPAI into a poultry population differs per species and production type. Generally, farmed water birds are at higher risk of introduction of LPAI than farms keeping Galliformes [75]. Similarly, outdoor production systems have significantly higher rates of LPAI introduction into domestic poultry flocks than birds kept indoors [76]. If a flock tests seropositive for an AI, virological testing is initiated. Given the potential of LP H5- and H7 subtypes to mutate into highly pathogenic strains and the large economic consequences linked therewith, surveillance in the poultry sector is largely focused on these two HA types.

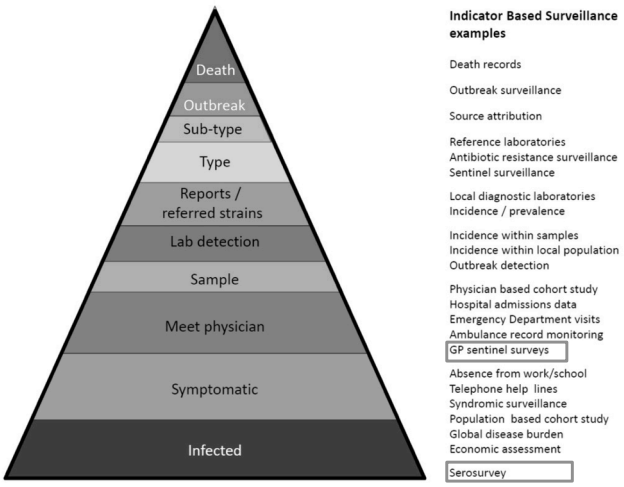
Surveillance in wild birds predominantly targets water- and shore birds, given their role as reservoirs for AI viruses. Oropharyngeal/ tracheal-, cloacal swabs or faeces of clinically healthy, diseased or injured birds are tested for AI virus presence within the active surveillance program. In addition, passive surveillance identifies AI in swabs or organ samples

of ill and dead wild birds. Whereas Asia has a similar surveillance approach as Europe, African countries solely focus on HPAI detection in poultry [77].

Human influenza surveillance

Sentinel surveillance detects patients presenting with influenza-like illness at outpatient facilities as well as hospitalizations of patients with severe acute respiratory syndrome (SARI) at representative surveillance sites. Located halfway in the surveillance pyramid (Figure 1, adapted from [70]), sentinel surveillance, in combination with laboratory detection of the virus, provides an effective means to systematically record (severely) symptomatic influenza cases. However, as influenza virus is only shed for – on average – five days after onset of illness [78], sentinel surveillance based on virus detection methods is also limited by a narrow window of detection In addition, sentinel surveillance misses mild and asymptomatic cases, leading to under-diagnosis and underreporting [71], and is not specifically targeting populations at risk for incursion (such as farmers). These limitations sparked discussions on whether the case fatality rate for human HPAI A(H5N1) cases – currently at 53% [79] – constitutes an overestimate [80], as serological studies suggest that asymptomatic and mild cases do occur, although others argued that reported seroprevalences might be overestimated [81]. A recent project funded by the European Food Safety Authority (EFSA) called 'Flurisk' [82] and a similar venture conducted by the Centers for Disease Control and Prevention in Atlanta [83] assessed the public health threat posed by animal influenza viruses and identified critical data gaps with respect to reporting of non-OIE-notifiable animal influenza viruses. Increased global surveillance of animal influenza viruses in various animal species and production systems as well as closer collaboration between the human and veterinary surveillance systems were advised.

Figure 1. Surveillance pyramid and approaches to address different levels within the surveillance system. (Modified with permission from [70])



SEROLOGY – PROS AND CONS

Antibodies triggered by infection can be detected for months, and therefore, serological methods, such as the hemagglutination inhibition- (HI) or microneutralization (MN)-assay, are valuable tools when acute phase clinical specimens are unavailable or when a laboratory is not equipped for virus isolation or RT-PCR detection [84]. Serological methods constitute an alternative to study exposure on a population level irrespective of severity of symptoms, thereby allow targeting the base of the surveillance pyramid (Figure 1, adapted from [70]). By antibody testing, inferences on attack rate of a virus can be made which in turn allows predicting the course of an epidemic and monitoring effectiveness of control measures. Furthermore, serological methods provide an unbiased estimation of true case fatality rates [85]. Despite HI- and MN-assay being "gold standards" for influenza antibody detection, standardization is limited and variation between different laboratories can be high [86,87]. Another downside is that serological methods may lack HA- and NA-subtype specificity due to cross-reactivity of antibodies triggered by infection with mismatched influenza virus subtypes, such as after infection with a novel pandemic- or an avian subtype.

From a public health perspective, it has long been recognized that early detection followed by rapid tackling of influenza viruses at the animal source reduces economic losses and minimizes the risk of human infection [77,88]. However, in order to achieve this, a more integrated 'One Health' approach by expanding current surveillance systems to subtypes other than H5 and H7 to capture exposure to other subtypes relevant for human health in high risk populations directly at the human-animal interface, is indicated.

OUTLINE OF THE THESIS:

The aim of this thesis was to contribute to the field of emerging disease preparedness, by developing a novel serological screening tool simultaneously targeting multiple AI in animal populations (chicken, bats), thereby providing a means to conduct sero-surveillance studies at the human-animal interface in a standardized and efficient manner. We furthermore identified and addressed key challenges in generating robust evidence of zoonotic infection with animal influenza viruses using serology, as further elucidated in the following chapters:

We first reviewed available virological evidence of human infections with different animal influenza virus subtypes in the available literature, which aided the development of a methodological risk assessment framework for potentially pandemic influenza viruses. This work was carried out within the FLURISK project, dedicated to assess the public health risk of animal influenza viruses (**Chapter 2**). Contact with AI-infected poultry is a main risk factor for human infection, hence monitoring of AI circulation in poultry populations is necessary to efficiently study exposure in high-risk populations. To monitor past circulation of various HP and LPAI types in poultry populations, we developed a multiplex serological screening assay, termed protein microarray, for the use in chickens. This technique, which was also validated for the use in humans, enables simultaneous detection of antibodies against multiple HA types and thereby forms a valuable surveillance tool for studies at the human-animal interface (**Chapter 3.1**). As the protein microarray has been deployed in several large-scale studies screening humans for serological evidence of human- and avian influenza viruses in populations in the Netherlands, Vietnam in China, we addressed the question for how long spotted protein microarray slides are storable under different temperature and humidity conditions without quality loss of antigens (**Chapter 3.2**). A number of sero-surveillance studies at the human-animal interface have been conducted to date and key issues hampering interpretation and comparability of serological results reported by the respective studies were investigated in the following chapter: In **Chapter 4**, we compiled serological evidence of avian and swine influenza virus infections in humans and evaluated the quality and reliability of the published literature by means of a scoring system. We identified quite some heterogeneity between the different serosurveillance studies, e.g. with respect to study design and laboratory methods. We furthermore discussed the issue of cross-reactive antibodies, potentially resulting in false-positive serological results against avian influenza virus antigens. To advance our understanding of cross-reactive antibody responses in non-high risk groups, we studied heterosubtypic antibody reactivity (i.e. reactivity to potentially novel subtypes) against avian influenza viruses in the general population from 13 countries of five continents. We established a measure to capture antibody diversity and examined serological profiles against several human- and avian influenza virus antigens. In this study we addressed the question whether higher antibody diversity is associated with increased cross-reactivity against AI antigens, and furthermore investigated the effect of the onset of the 2009 A(H1N1) pandemic on heterosubtypic responses (**Chapter 5**). Finally, triggered by the recent discovery of novel influenza virus subtypes H17N10 and H18N11 in bats, we explored the role of this taxon as possible reservoir for influenza A viruses. For that, we

adapted the protein microarray platform for the use in bats, and screened for antibodies against previously known human and animal influenza virus subtypes in frugivorous bats from Ghana and touched on the public health implications of our results (**Chapter 6**). In **Chapter 7** we discuss our findings in light of current knowledge in the field.

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CHAPTER 2

INFLUENZA AT THE ANIMAL-HUMAN INTERFACE: A REVIEW OF THE LITERATURE FOR VIROLOGICAL EVIDENCE OF HUMAN INFECTION WITH SWINE OR AVIAN INFLUENZA VIRUSES OTHER THAN A(H5N1)

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ABSTRACT

Factors that trigger human infection with animal influenza virus progressing into a pandemic are poorly understood. Within a project developing an evidence-based risk assessment framework for influenza viruses in animals, we conducted a review of the literature for evidence of human infection with animal influenza viruses by diagnostic methods used. The review covering Medline, Embase, SciSearch and CabAbstracts yielded 6,955 articles, of which we retained 89; for influenza A(H5N1) and A(H7N9), the official case counts of the World Health Organization were used. An additional 30 studies were included by scanning the reference lists. Here, we present the findings for confirmed infections with virological evidence. We found reports of 1,419 naturally infected human cases, of which 648 were associated with avian influenza virus (AIV) A(H5N1), 375 with other AIV subtypes, and 396 with swine influenza virus (SIV). Human cases naturally infected with AIV spanned haemagglutinin subtypes H5, H6, H7, H9 and H10. SIV cases were associated with endemic SIV of H1 and H3 subtype descending from North American and Eurasian SIV lineages and various reassortants thereof. Direct exposure to birds or swine was the most likely source of infection for the cases with available information on exposure.

INTRODUCTION

Influenza virus type A, a member of the family *Orthomyxoviridae*, is an enveloped virus with a negative-sense, single-stranded RNA genome organised in eight gene segments, which encode at least eleven proteins. Antigenic and genetic diversity of two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), is used to classify type A influenza viruses into subtypes; 18 HA and 11 NA subtypes are known to date [1-5]. Water- and shorebirds were identified as reservoirs harbouring all subtypes, except A(H17N10) and A(H18N11) of which RNA was recently detected in bats from Guatemala and Peru, respectively [2,3]. Reservoir animals typically do not display symptoms. In contrast, the diversity of influenza viruses in mammalian hosts is limited to specific subtypes. Human-adapted seasonal influenza viruses since the beginning of the 20th century have had HA subtypes H1, H2 and H3, combined with NA subtypes N1 and N2.

The segmented nature of the genome facilitates the exchange of genetic material if a host is co-infected with two genetically different type A influenza viruses. This reassortment process, also known as antigenic shift if it involves the gene segment encoding the HA, can result in the generation of viruses with surface antigens against which the human population may not have pre-existing, protective antibodies. Additional flexibility is conferred by the accumulation of mutations during replication, potentially resulting in amino acid substitutions that can affect pre-existing immunity if the HA is involved (antigenic drift), host range, virulence, and other factors [6]. If this results in sustained human-to-human transmission of a virus against which a large proportion of the world's human population is immunologically naïve a pandemic can develop resulting in a large number of human cases occurring simultaneously worldwide [7,8]. Such novel introductions of reassorted viruses were at the root of four influenza pandemics in the last 100 years, and claimed the lives of millions of people, namely the 'Spanish flu' A(H1N1) in 1918, the 'Asian flu' A(H2N2) in 1957, the 'Hong Kong flu' A(H3N2) in 1968, and the recent pandemic caused by influenza A(H1N1)pdm09 in 2009 [9,10]. Influenza A(H1N1)pdm09 has replaced previous human seasonal A(H1N1) viruses [11] and, together with A(H3N2) and influenza B viruses, has been causing seasonal influenza epidemics in humans since 2009. With the emergence of the influenza A(H3N2) pandemic in 1968, influenza A(H2N2) viruses ceased to circulate in humans, but H2 subtypes are still present in birds and were also recently isolated from diseased swine [12,13].

The factors that determine whether an animal influenza virus may acquire the ability to efficiently spread among humans are poorly understood [14]. Reassortment is not a necessary prerequisite for human infection, and there is clear documentation of direct transmission and human disease caused by animal influenza viruses, in particular avian (AIV) and swine (SIV) influenza viruses , such as AIV A(H5N1), A(H9N2) and various H7 subtypes, as well as European avian-like SIV A(H1N1) [15-22]. Early detection and in-depth investigation of such events may provide clues for (future) risk assessment of animal-to-human transmissions.

This review was conducted under the framework of the FLURISK project funded by the European Food Safety Authority (EFSA). The main objective of FLURISK is the development of an evidence-based influenza risk assessment framework (IRAF) to assess the potential of animal influenza viruses to cross the species barrier and cause sustained infections in humans. The work presented here aims at describing available evidence for animal-to-human influenza virus transmissions.

METHODS

Search strategy

We performed a literature search using Medline, Embase, SciSearch and CabAbstracts. Search terms included 'influenza', 'influenza virus', 'animals', 'swine', 'birds', 'poultry', 'wild bird', 'water bird', 'waterfowl', 'goose', 'duck', 'chicken', 'turkey', 'environment', 'animal-to-human', 'transmission-to-humans', 'interspecies transmission', 'human', 'case', 'seroprevalence', 'serosurveillance', 'prevalence', 'incidence', 'risk factor', 'exposure' and various subtypes of influenza virus; the terms were used alone or in combinations using Boolean operators. Full search details are available from the corresponding author on request. Only articles published in English were included and the search covered all years available in the respective databases, Medline from 1946, Embase from 1947, SciSearch from 1980, CabAbstracts from 1973, all up to February 2012. The search algorithm automatically discarded duplicates. Newly published evidence that came to our attention between February 2012 and January 2014 was also included. Case counts of avian influenza A(H7N9) and A(H5N1) cases were updated on 31 January 2014 based on the latest figures reported by the European Centre for Disease Prevention and Control (ECDC) [23] and by the World Health Organization (WHO) [24]. Case counts of human infections with swine influenza variant A(H3N2)v were retrieved on 31 January 2014 from the website of the United States (US) Centers for Disease Control and Prevention (CDC) as posted on 18 October 2013 [25. Grey literature was searched in a non- systematic way.

Inclusion and exclusion criteria

Included were papers indicating evidence of human infection with animal influenza viruses (selection criterion 1, Figure 1). Two investigators first screened all papers by title and, when necessary, by abstract. All articles meeting this first criterion were reviewed for details of the methods used to diagnose the infection. Experimental and observational studies describing human infection with animal influenza viruses other than influenza A(H5N1) were included. Articles solely describing human infection with A(H5N1) were excluded, and for influenza A(H5N1) and A(H7N9), the official WHO and ECDC statistics from notifications under the International Health Regulations were used for completeness. Commentaries, reviews, articles dealing with influenza in animals only, studies solely assessing human-to-human transmission of an animal influenza virus (i.e. most of the literature on influenza A(H1N1)pdm09), and articles referring to study subjects described in prior original publications were excluded (selection criterion 2, Figure 1). Studies based on serological

evidence only were excluded to ensure high specificity of the findings. Papers were screened for information on the time period when the study was conducted, total number of people sampled, patient information, nature of exposure (e.g. occupational, recreational), possible exposure to diseased animals, influenza virus subtypes included in testing, number of virologically confirmed cases, information on vaccination history if stated, methods used for confirmation, geographic region and study design. Available data were extracted and summarised in tables. Grey literature such as ProMED, and reference lists from articles were screened for possible additional relevant papers. Virus detection by culture or (real-time) reverse transcription polymerase chain reaction (rtRT-PCR) and sequencing was considered to be definitive proof of infection, listed as virological evidence (Tables 1 and 2).

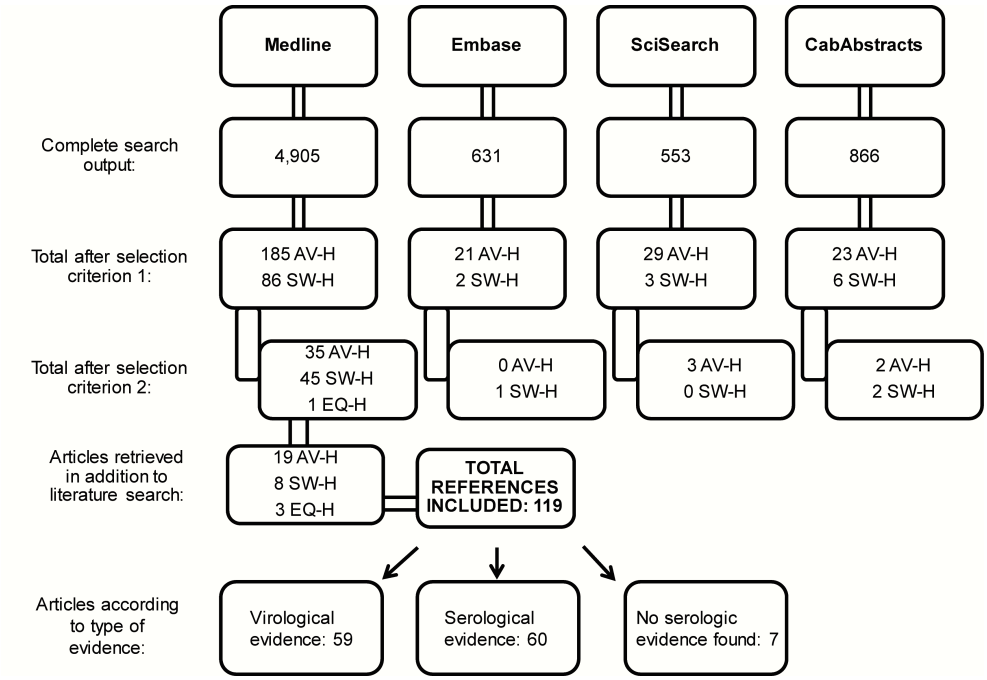
Search output and article selection

The initial search yielded 6,955 articles, with 4,905 articles resulting from the Medline search, and the others from additional searches (Figure 1). Search outputs from Embase, SciSearch and CabAbstracts yielded 631, 553 and 866 references, respectively. After screening of titles, abstracts and application of the second selection criterion, a total of 89 publications were selected. The majority of these would also have been identified solely through the Medline search.

Thirty additional studies were retrieved through scanning of reference lists of articles identified via the literature review, were retrieved from grey literature or came to our attention after February 2012. Of these 119, 59 publications and reports described virological evidence for infection of humans and met all other inclusion criteria; 60 papers provided some evidence for human infection, but only based on antibody testing and will therefore be described elsewhere. Seven publications containing both serological and virological evidence were counted once in the total reference count but were included twice in the subdivision according to type of evidence in Figure 1. Most studies, discussed in a review of case reports of SIV infections in humans by Myers et al. [26], were also identified in our literature search. For completeness of the human case count, virologically confirmed civilian (n=23) and military cases of Fort Dix (n=5), although discussed in detail in the review by Myers et al. [26], were also included in the current review [21,27-42] (Table 2). For two virus-confirmed cases from the review by Myers et al. [26], the reference could not be retrieved or did not provide full confirmation; these cases were therefore excluded from our listing in Table 2 [43,44].

Figure 1. Search strategy for the literature review on animal influenza A virus infections in humans. Selection step 1 extracted studies indicating information on human infection with animal influenza viruses from title and abstract. Selection step 2 excluded papers as specified in Inclusion– and exclusion criteria. Seven articles described virological as well as serologic evidence of infection. These references were counted once in the total count and were listed twice in row 'Articles according to type of evidence'. The virological evidence of human infection is presented

in this paper. The indirect serologic evidence will be described elsewhere. (AV, avian; SW, swine; EQ, Equine; H, Human)



RESULTS

The evidence for virologically confirmed infections of humans with avian or SIV is listed in Tables 1, 2 and 3. The exposure status of infected patients is summarised in Table 4. A total of 386 cases of human infection with non-A(H5N1) AIV were described, of which 375 were caused by natural infection and 11 were infected experimentally (Table1). Regarding human infections with SIV, a total of 401 naturally (n=396) and experimentally (n=5) infected cases were detected in the published and grey literature in English. This included, three virologically confirmed SIV A(H1N1) cases originally published in Russian by Chuvakova et al. [27] because they were listed in the review by Myers et al. [26]. The majority of cases (n=340) were naturally infected by a SIV variant A(H3N2)v [25]. Recognised in US swine in 2010, this variant combines seven genes from the contemporary North-American A(H3N2) SIV lineage and has acquired the M gene of the A(H1N1)pdm09 virus [25]. The remaining 56 naturally infected, virologically confirmed human cases were caused by different circulating SIV or SIV reassortants (Table 2). Five persons were experimentally infected with SIV [45]. The majority of AIV- and SIV-infected patients had been exposed to animals (Table 4).

Table 3. Humans naturally infected with avian influenza virus subtypes other than A(H5N1) and swine influenza virus subtypes, 1959–2014 (n=771)

Source virus	**Number of human cases infected with**										
AVIAN subtype	LP[a] H6N1	H7N2	HP[b] H7N3	LP H7N3	HP H7N7	LP H7N7	LP H7N9[c]	H9N2	H10N7	H10N8	**Total**
	1	4	3	2	89	4	251	15	4	2	**375**
SWINE subtype	H1N1	H1N2	H3N2	H3N2v[d]							**Total**
	47	2	7	340							**396**

[a] LP = low pathogenic, [b] HP = highly pathogenic, [c] as of January 27th, 2014 [23], [d] as of October 18th, 2013 [25]

Table 4. Exposure status of patients infected with avian influenza virus, excluding 251 A(H7N9) and including 11 experimentally infected cases, and with swine influenza virus, 1959–2014 (n=536)

Source	**Number of cases**					
	Exposed	Not exposed	Exposure status unknown	Likely exposed (H3N2v)	Other[a]	Total
Avian	114	3	5	n.a.[b]	13	**135**
Swine	46	17	4	328	6	**401**

[a]Experimental (avian n=11, swine n=5) or laboratory exposure (avian n=2), or human–to–human transmission (swine n=1), [b]not applicable

Human infections with avian influenza viruses

Infections with highly pathogenic avian influenza virus A(H5N1)

To date, highly pathogenic avian influenza (HPAI) A(H5N1) viruses are the most frequently diagnosed zoonotic influenza virus infections related to avian exposure [46], although this picture may change in the near future given the recent upsurge in low pathogenic avian influenza (LPAI) A(H7N9) cases. The HPAI A(H5N1) viruses first attracted major attention in the scientific community in 1996, when a large number of domestic waterfowl died in the course of an A(H5N1) outbreak in Guangdong province in southern China. In 1997, HPAI A(H5N1) resurfaced in Hong Kong SAR, China (in the following referred to as Hong Kong); it caused a massive die-off in poultry and crossed the species barrier for the first time, infecting 18 humans, of whom six died [47,48]. From mid-2003 to March 2004, HPAI A(H5N1) spread to seven south-east Asian countries with outbreaks in poultry and waterfowl, and the first confirmed human cases, were reported in Thailand and Vietnam in 2004 [49]. In 2005, HPAI A(H5N1) accounted for the death of a large number of migratory waterfowl at Qinghai lake, China. Shortly after this event, the virus rapidly spread to other Asian countries, Africa,

Europe, the Middle East, Mongolia and Russia [50]. Over time, the viruses evolved into multiple lineages, some of which persisted and have become endemic in China, Bangladesh, Egypt, India, Indonesia and Vietnam [51].

As of 10 December 2013, the WHO has listed 648 HPAI A(H5N1) infected cases from 15 countries, confirmed according to WHO criteria and covering a time span of 10 years [24]. In total, 59% of the reported cases died [24]. Indonesia, Egypt and Vietnam reported 195, 173 and 125 cases, respectively, accounting for about 75% of the total influenza A(H5N1) human case count. These three countries also reported the majority of fatalities [24].

Infections with H7 subtype avian influenza viruses

In total, we identified 353 human cases with virologically confirmed H7 infection (Table 1). The majority of these cases (n=251) were reported in China, followed by 95 cases in Europe, six in North America and one in Australia [17,18,23,52–59]. In China, all cases were caused by the recently emerged subtype A(H7N9) [23]. Of the remaining 102 cases, 93 cases had influenza A(H7N7), five had influenza A(H7N3) and four influenza A(H7N2) (Table 3). The first two human cases infected with influenza A(H7N7) were reported in 1959 and 1977. One of these patients had keratoconjunctivitis, thought to be caused by the AIV infection [52,53]. This predilection for the ocular mucosa was confirmed when a person involved in an experimental infection of a seal with an avian-like influenza A(H7N7) developed conjunctivitis, and virus was cultured from a conjunctival swab [54,60]. In the United Kingdom (UK) in 1996, LPAI A(H7N7) virus infection was associated with mild conjunctivitis in a woman who cleaned a duck house and mentioned getting a piece of straw in her eye [55].

Among European cases, 89 humans were infected in the course of a large outbreak with HPAI A(H7N7) in poultry in the Netherlands in 2003 [17]. In contrast to the severe consequences in poultry, only mild symptoms were seen in 88 of the infected people. There was one exception. a veterinarian who died of acute respiratory distress syndrome and multiple organ failure. This person had contracted a virus with several mutations, including a known virulence marker in PB2 [56]. Most of these mutations had accumulated during circulation of the virus in poultry, showing that the public health risk may change over the course of an outbreak [61]. In February 2004, a mixed LPAI and HPAI A(H7N3) virus outbreak was reported in poultry in British Columbia, Canada [18]. Enhanced surveillance for influenza-like illness (ILI) and conjunctivitis in the course of this outbreak led to the identification of two poultry workers showing symptoms of unilateral conjunctivitis. Neither had used the recommended goggles or taken prophylactic oseltamivir. Interestingly, both virus types led to human infection: the isolate cultured from the first worker had the LPAI phenotype, whereas the strain retrieved from the second worker was classified as HPAI [19,62,63]. In 2006 and 2012, LPAI A(H7N3) was associated with one patient in the UK and HPAI A(H7N3) with two patients in Mexico. In both instances, exposure to infected poultry was documented and all patients presented with conjunctivitis [57,59]. Finally, LPAI A(H7N2) was reported as the infectious

agent causing mild influenza-like symptoms and conjunctivitis in four cases in the UK in 2007 [58].

The assumption that LPAI influenza viruses were mostly associated with mild disease was challenged with the emergence of influenza A(H7N9) viruses in March 2013, when China notified the WHO of three cases infected with LPAI A(H7N9) who were severely ill and eventually died [64]. During the first wave of infections from February to May 2013, 133 human cases were reported and an additional two cases in July and August [23]. Phylogenetic studies concluded that all genes of this newly detected virus were of avian origin [64]. In October 2013, the second wave started and was still ongoing at the time of writing (31 January 2014) [23]. Between February 2012 and 27 January 2014, a total of 251 influenza A(H7N9) cases were reported, 56 of whom died [23]. Infections occurred in Anhui (n=4), Beijing (n=3), Fujian (n=15), Guangdong (n=32), Guizhou (n=1), Hebei (n=1), Henan (n=4), Hong Kong (n=3), Hunan (n=4), Jiangsu (n=31), Jiangxi (n=5), Shandong (n=2), Shanghai (n=42) and Zheijang (n=102). Two cases were imported from mainland China into Taiwan [23]. In response to these events, China culled thousands of birds and closed several poultry markets [65], although only 39 of 48,000 samples representing 1,000 poultry markets tested positive. Most human cases had a history of exposure to birds or live bird markets [23]. As of 31 January 2014, no conclusive evidence of human-to-human transmission has been reported and the ecology of the viruses remains to be resolved.

Infections with H9 subtype avian influenza viruses

In total, we detected 15 human cases infected with AIV A(H9N2) (Table 1, Table 3). Since the mid-1990s, influenza viruses of the H9 subtype have established stable lineages in poultry in Asia and have occasionally infected humans and swine (Table 1) [16,66-69]. As of 31 January 2014, human A(H9N2) cases have only been detected in Asia, particularly in China. Six cases were identified via the literature search [16,66-68]. Of those, three reported poultry exposure and all presented with mild ILI (Table 1). Reviews conducted by Peiris [46] and Cheng et al. [68] identified six additional human infections in China reported in the Chinese literature [70,71]. Three additional cases from Bangladesh, Hunan and Shenzhen, two with and one without poultry exposure, complete the total count of fifteen human cases caused by AIV A(H9N2) [69,72,73] (Table 3). Infections with AIV A(H9N2) viruses gained public health interest when researchers found that strains circulating in Asian poultry had a receptor specificity similar to human influenza A viruses, which is considered one of the essential features of a human-to-human transmissible virus [74]. So far, however, no sustained human-to-human transmission of A(H9N2) influenza viruses has been reported.

Infections with other avian influenza virus subtypes

Experimental inoculation of human volunteers with influenza strains A(H4N8), A(H6N1) or A(H10N7) resulted in mild clinical symptoms and virus shedding in eleven volunteers [75]. In 2004, the National Influenza Center in Egypt and the WHO Influenza Collaborating Centre in the UK announced the isolation of influenza A(H10N7) virus from two children presenting

with fever and cough in Egypt [76]. In Australia, virus of the same subtype could be detected by PCR in two abattoir workers with conjunctivitis who were exposed to infected poultry [20] (Table 3). In December 2013 and January 2014, human infection with A(H10N8) virus was reported for the first time in Jiangxi province, China [77,78]. Both patients were female and had visited a poultry and an agricultural market, respectively, before onset of illness. One of them was immunocompromised and had died whereas the other case was still in critical condition as of 31 January 2014. In 2013, CDC Taiwan reported a human case of AIV A(H6N1) infection causing mild pneumonia, although an avian source could not be identified [79,80] (Table 3).

Human infection with swine influenza viruses

An overview of all studies describing virologically confirmed human SIV cases is given in Table 2. In total, we identified 396 SIV-confirmed patients who were naturally infected (401 including experimental infections) (Table 2). Beare et al. [45] successfully recovered SIV from five of 20 human volunteers after experimental infection: of seven volunteers infected with SIV A(H3N2) related to A/Hong Kong/1/68, three tested virus-positive), and of 13 infected with a classical swine A(H1N1) virus strain, two tested positive. Of the naturally infected cases, 47 were infected with SIV A(H1N1), two with SIV A(H1N2), seven with SIV A(H3N2) and 340 with SIV A(H3N2)v (Table 2, Figure 2). The majority of these cases were reported in North America, 11 in Europe and six in Asia. One of the six Asian cases was infected with SIV A(H3N2) from the European lineage (Figure 2) [39]. SIV epidemiology differs between continents and was extensively reviewed for North America, Europe and Asia [81-84]. In addition to the studies discussed in the review by Myers et al. [26] we identified fourteen studies and reports describing a further 28 human SIV cases; 368 when taking into account 340 cases with SIV A(H3N2)v infection. Details on these studies are described in more detail in the following sections grouped by continent.

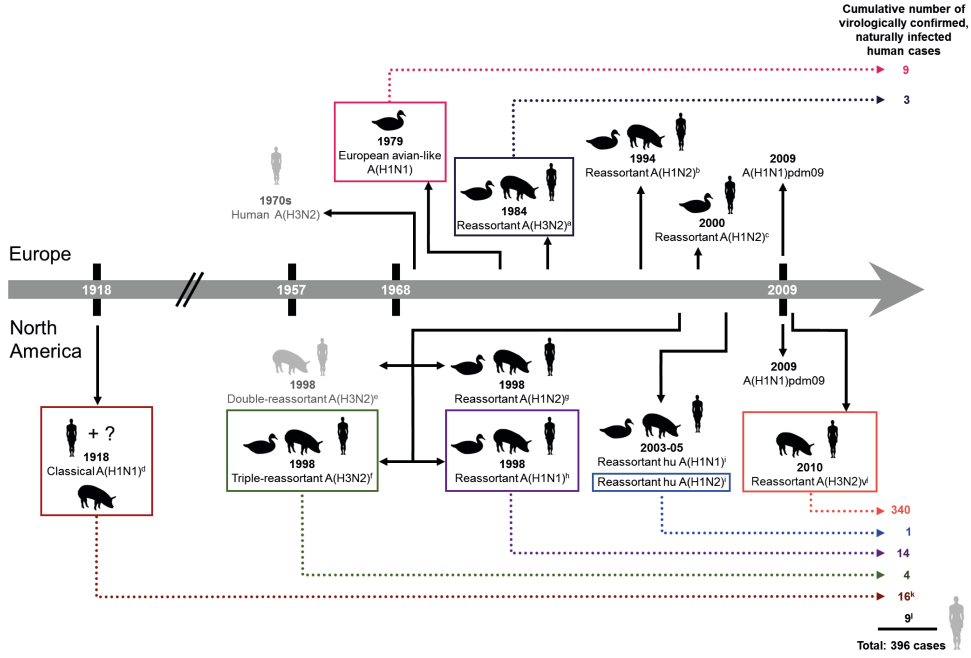
Infections with swine influenza A H1 subtype viruses

Asia: A 25 year-old male from the Philippines and a four year-old male from Thailand were infected with swine-like A(H1N2) and A(H1N1), respectively [85]. The isolated viruses carried HA genes most closely related to classical swine viruses circulating in Asia and North America. NA genes were most similar to circulating European SIV. Both cases showed mild ILI and neither of them had direct contact with swine, although occasional contact with backyard swine could not be ruled out.

Europe: In Spain, a 50 year-old woman developed ILI after having been closely exposed to swine on a family farm [22]. No symptoms in swine were observed and sequencing of the isolate revealed that it was closely related to avian-like SIV A(H1N1) circulating in swine in western Europe. Three cases from Switzerland were detected who had worked with influenza-confirmed swine [86].

North America: For the US, 19 confirmed cases of SIV A(H1N1) infection were described in the published literature. This number could possibly be higher because Vincent et al. [87] reported on 26 human cases presenting with ILI after exposure to ill swine on a county fair in Ohio. The authors described that isolation and sequencing was performed for at least two of the human cases. Since the exact number of virologically confirmed cases was not given, we only added the two confirmed patients to the overall SIV A(H1N1) count (Table 3). Sequences from swine and human isolates from this outbreak were identical and were similar to triple–reassortant (tr) viruses currently circulating in swine herds in the US [87,88]. Another triple–reassortant A(H1N1) SIV was detected in a 17 year-old male from Wisconsin who assisted in butchering healthy appearing swine [89]. The patient presented with acute, mild respiratory illness without fever. Similarly, Dawood et al. [90] reported infection with trSIV A(H1N1) in a 19 year-old asthmatic male who visited a swine show in South Dakota.

Figure 2. Timeline of emergence of swine influenza virus lineages circulating in Europe and North America indicating natural human infections from swine



Year numbers on main arrow denote human pandemics: A(H1N1) in 1918, A(H2N2) in 1957, A(H3N2) in 1968 and A(H1N1)pdm09 in 2009. Pictograms denote the origin of genes. Items in gray indicate no further circulation amongswine. Boxed viruses have infected humans.

Superscript letters refer to the genome segment constellation:
[a]HA and NA from human HongKong/68–like virus, remaining genes from European avian–like swine A(H1N1); [b]HA from human A(H1N1) (England/80–like), NA from European reassortant swine A(H3N2), remaining genes from European avian–like swine A(H1N1); [c]Classical SIV A(H1N1) constitutes a reassortant between human A/BM/1918 and unknown virus; [d]HA, NA of human A(H3N2)–origin, remaining genes from classical swine A(H1N1) and avian influenza origin; [e]HA, NA from classical SIV A(H1N1), remaining genes from triple reassortant OR swine A(H1N2); [f]HA, NA from seasonal human (hu) influenza viruses, remaining genes from triple reassortant swine A(H3N2); [g]A(H3N2)variant: M–gene from A(H1N1)pdm09, remaining genes from triple reassortant swine A(H3N2), N2 antigenically different compared with N2 from triple reassortant A(H3N2) from 1998; [h]Including 5 virus–confirmed human cases reported from Fort Dix outbreak among soldiers in New Jersey, US in 1976; [i]Nine human cases were not assignable, six of them were infected with reassortants that did not group with current SIV lineages, two cases from the Philippines and Thailand infected with A(H1N1) and A(H1N2) bearing HA from North American lineage from the 1990s and NA from European swine influenza lineages [83], three cases from Canada infected with an A(H1N1)–reassortant with HA and NA genes most similar to those of A/Brisbane/59/2007(H1N1)–like viruses and internal genes (NS, NP, M, PA, PB1, and PB2) similar to those of a contemporary North American SIV A(H3N2) triple reassortant [87], and one case infected with trH1N1 with HA, PA, PB1,PB2, NP, M, NS from North American trSIV A(H1N1)–lineage and NA from North American, classical swA(H1N1) [88,113]; and three additional cases infected with SIV A(H1N1) were described in Russian by Chuvakova et al. [24] for which no further isolate–characterization was given in the abstract of the paper.

Symptoms included fever, ILI, vomiting and diarrhoea. No respiratory illness was observed in swine at this event. A trSIV A(H1N1) was also detected in three infected swine workers in Sasketchewan, Canada [91]. Household members did not report any signs of disease. Mild respiratory illness was reported in less than 1% of the swine; however, no confirmatory test had been conducted in ill swine. Unlike trSIV identified earlier in North America, this isolate contained an HA and a NA belonging to the A/Brisbane/58/2007 A(H1N1) lineage, whereas the remaining genes were derived from trSIV A(H3N2) viruses circulating in North America since 1998. Gray et al. [92] found another trSIV A(H1N1) in the course of a prospective survey, which was isolated from an ill swine farmer exposed to swine showing respiratory symptoms. Routine national influenza surveillance reported another 10 human cases infected with trSIV A(H1N1), distinct from A(H1N1)pdm09, and one case caused by trSIV A(H1N2). The majority of those twelve patients stated exposure to swine prior to disease onset and all made a full recovery [93].

The CDC reported additional human infections with SIV-variant viruses of subtype A(H1N1)v and A(H1N2)v identified in the US since 2005 [94]. These figures have not been included in this review due to missing case history and in order to avoid double counting of cases described in the published literature.

Human infections with swine influenza A H3 subtype viruses

North America: In our search we detected two cases of trSIV A(H3N2) from Canada [95,96] and one from Kansas, US [97] infected before 2011. In Canada, a six year-old boy who lived on a swine farm presented with parotitis, nasal congestion, cough and pharyngitis, but had no fever. The swine appeared clinically healthy [95]. No swine exposure was reported in the second Canadian patient, a seven month-old child, who lived on a community farm and showed ILI symptoms [96]. Similarly, the third case from Kansas presented with ILI and is likely to have contracted trSIV A(H3N2) from swine he was exposed to at a county fair [97]. It is assumed that swine harboured the virus; PCR results performed on swine samples were negative but sera showed raised titres against SIV A(H3N2) indicating prior infection.

Between July and August 2011, a SIV variant, which accounted for the majority of reported human SIV A(H3N2) cases, appeared in the US, possibly reflecting enhanced surveillance activities in the country. This SIV A(H3N2) variant, A(H3N2)v, was first found in two children presenting with fever and respiratory signs [98,99]. Sequencing showed that this variant contained seven genes derived from the contemporary trSIV A(H3N2), circulating in the US swine population since 1998, as well as the M,gene from the A(H1N1)pdm09 virus. Since its first occurrence in 2011, A(H3N2)v has been detected in 340 humans according to data as of 18 October 2013 [25]. Since July 2012, 17 patients have been hospitalised and one patient has died due to SIV A(H3N2)v infection. Most cases have reported prolonged exposure to swine before getting ill.

Human infections following exposure to other animals

We have identified three studies describing human susceptibility to equine influenza virus, demonstrated by experimental infection with equine subtype A(H3N8) [100-102]. The literature search did not reveal evidence of humans naturally infected with equine influenza virus.

DISCUSSION

Here we present a review of the literature for studies presenting any evidence for human infection with animal influenza viruses. Virological techniques, e.g. virus culture, PCR and sequencing provide more solid evidence of infection, whereas serological methods can help reaching a diagnosis after the virus has been cleared from the body. Virus isolation is still the gold standard in detecting AIV infection. Human cases with virological evidence identified by PCR only should be interpreted with caution as detection of viral RNA without additional serological evidence (seroconversion, more than fourfold rise in the titre of paired samples) does not necessary imply infection, although current diagnostic methods heavily rely on case identification by PCR [103,104]. Serological results can be misleading because of the existence of cross–reactive antibodies and thus provide less solid evidence than direct detection of the infecting virus itself [105]. Therefore, we limited the current paper to studies providing virological evidence only.

Most evidence of human infection with AIV is associated with subtypes H5 and H7. Both subtypes can be linked to devastating outbreaks in poultry with high mortality, if transition from a low to a highly pathogenic state occurs [106]. Pathogenicity shown in poultry clearly does not reflect disease severity in humans: before the emergence in LPAI A(H7N9) as a cause of severe human illness, LPAI and HPAI viruses of subtype H7 had in the majority of cases been associated with mild eye infections or ILI [107]. The LPAI A(H7N9) infections diagnosed to date, however, have been unusually severe [23,64]. In addition to the severity of illness associated with these and also A(H5N1) viruses, the widespread circulation of different lineages, made possible by mutations and reassortments, justifies enhanced surveillance activities, given that few genetic changes may lead to a human-to-human transmissible virus [108,109].

Nevertheless, the evidence from experimental infections and anecdotal natural infections shows that other AIV may infect humans as well. There is insufficient systematic surveillance data to address the question whether the identified human cases reflect the level of virus circulation among wild or domestic birds, or whether certain subtypes infect humans preferentially.

Regarding SIV, there is ample evidence of human infection with A(H3N2), A(H1N1) and A(H1N2) subtypes, as well as reassortants derived from these endemic SIV lineages. Nevertheless, one has to be aware that the true number of human SIV cases is probably higher than reported since clinical symptoms of SIV are indistinguishable from seasonal influenza [86]. Whereas recent human cases in Europe were detected almost accidentally, the larger number of cases reported in the US since 2005, especially for influenza A(H3N2)v since 2011, may be the result of increased surveillance activities [86,110]. Swine were assumed to play an important role as intermediate hosts or 'mixing vessels' for strains of human, avian and swine origin, because they possess avian and human influenza-specific receptors in the tracheal epithelium [111]. However, recent research showed that the distribution of sialic acid receptors in the porcine respiratory tract is similar to that in humans, leading to the conclusion that humans are equally likely to constitute 'mixing vessels' [112,113]. The fact that influenza viruses can circulate unnoticed in swine populations [114] warrants close surveillance in this animal species as well. Co-circulation of different influenza virus strains in swine may facilitate the generation of new variants that could potentially pose a threat for public health [115]. For instance, it is assumed that influenza A(H1N1)pdm09 was present in swine herds for months before it emerged as a pandemic strain in humans [116]. Conversely, Nelson et al. [117] reported at least 49 transmission events of influenza A(H1N1)pdm09 from humans to swine between 2009 and 2011, as well as at least 23 separate introductions of human seasonal influenza into swine since 1990.

Although there is some evidence for infection in swine with non-H1 and non-H3 subtype viruses (H9N2, H4N6) [115,118], we found no case reports describing human infection with these influenza A virus subtypes after swine exposure. Since most human infection events

are associated with swine exposure, awareness of risk factors and personal protective equipment is paramount to limiting the chance of infection and preventing people working with or recreationally exposed to swine (including children) from becoming 'bridging links' between swine and community contacts and vice versa [92,119].

There are a few limitations to this review. The language restriction set to papers published in English only and the unsystematic search of the grey literature probably lead to the omission of additional documented human infections with animal influenza virus. Although this limitation may affect the total count of human cases, the aim of this review was to identify animal influenza subtypes, which crossed the species barrier to humans, and to our knowledge, all relevant subtypes were covered by this review.

Conclusions

There is evidence of infection of humans with animal influenza viruses belonging to various subtypes. All reported SIV cases have been exclusively associated with subtypes H1 and H3, and most AIV cases were caused by subtypes H5 and H7. Whether this reflects the prevalence of these viruses in birds kept or sold for consumption or a preferential ability to transmit to humans cannot be concluded from the available evidence. Given the often, mild illness associated with non-H5 and non-H7 animal influenza virus infections in humans, such cases likely are underreported [120]. Standardisation of diagnostic methods has significantly improved case ascertainment in recent years, but the monitoring of the evolution of these viruses is less advanced. Recent research pointed out that the majority of Asian and African countries have contributed only few sequences to surveillance networks and do not regularly sequence viruses as part of their surveillance programme [121]. Genetic sequencing is paramount to identifying changes with potential effect on the phenotype of circulating influenza viruses, and could thereby strengthen worldwide epidemic and pandemic preparedness [121]. To be prepared for a potentially emerging influenza virus of animal origin in humans, enhanced global surveillance in animal populations is therefore indicated to monitor evolution and circulation of viruses with yet unknown public health risks.

Note: Numbers on influenza H5N1 and H7N9 are as of 31 January 2014.

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Conflict of interest None declared.

Table 1. Virological evidence of human infection with avian influenza A viruses, excluding high pathogenicity A(H5N1)

Year	(Tested) [a]/ confirmed cases	Subtype	Symptoms	Method	Nature of exposure	Location	Reference
1959	1	H7N7	Unknown	Culture in embryonated chicken eggs; subtyping with specific antisera	Unknown; Isolated from patient suffering from hepatitis	USA	Campbell et al. 1970 [52]
1977	1	H7N7	Keratoconjunctivits	Culture in embryonated chicken eggs	Laboratory technician accidentally infected with fowl plague	Melbourne, Australia	Taylor & Turner 1977 [53]
1979	1	H7N7	Conjunctivitis	Virus typed with specific sera	Infected by experimentally infected harbour seal; from 4 possible cases who performed autopsies on infected harbour seals virus isolation was not attempted	Massachusetts, USA	Webster et al. 1981 [54,60]
1991	11/ 2 14/ 3 15/ 6	H6N1 H4N8 H10N7	Ranging from respiratory and some constitutional symptoms to no symptoms	Culture in embryonated chicken eggs	Experimental infection	USA	Beare & Webster 1991 [75]
1996	1	LP H7N7	Conjunctivitis	Culture on rhesus monkey kidney cells	Straw contaminated with duck feces	United Kingdom	Kurtz et al. 1996 [55]
1998	5	H9N2	Acute respiratory symptoms	Culture in embryonated chicken-eggs, HI- and NI-assay	Poultry contact	Shantou (n=3), Shaoguan (n=2), China	Guo et al. 1999 [70]
1999	2	H9N2	Mild ILI	Cultured by WHO-Reference laboratory	4- and 1-year-old girl; One patient probably had contact with live poultry	Hong Kong SAR, China	Peiris et al. 1999 [16]
1999	1	H9N2	Fever, cough, bronchitis	MDCK-cell culture, HI- and NI-assay	2-year-old female with probable poultry	Guangzhou, China	Guo et al. 2000 [71]

						contact		
2003	453/ 89 1 [including fatality]	HP H7N7	Conjunctivitis, ILI [fatality: fever, pneumonia, multi-organ failure, respiratory insufficiency]	Cell culture (used for the first 25 confirmed cases, afterwards RT-PCR used as screening method), typed and subtyped by HI assay [turkey RBC], RT-PCR	Poultry famer/family, cullers, veterinarians, medical personnel, others	Gelderland, North Brabant, Limburg; The Netherlands	Koopmans et al. 2004; Fouchier et al. 2004 [17,56]
2003	1	H9N2	Mild ILI	Culture in embryonated chicken eggs. HI- and NI assay, sequencing	No history of contact with poultry	Hong Kong SAR, China	Butt et al. 2005 [66]
2004	2	LP and HP H7N3	Conjunctivitis and mild ILI	RT-PCR, cell-culture, sequencing	Occupational exposure to infected poultry [Veterinarian, general worker]	British Columbia, Canada	Tweed et al. 2004 [18]
2004	2	H10N7	"Illness"	Virus isolation	Infants with indirect contact to poultry [father of one is poultry merchant]	Egypt	Pan American Health Organization [PAHO], 2004 [76]
2006	1	LP H7N3	Conjunctivitis	PCR (no serological confirmation reported)	Infected poultry	United Kingdom	Nguyen-Van-Tam et al. 2006 [57]
2007	4	LP H7N2	Conjunctivitis, ILI	Influenza A-confirmed[b]	Infected poultry	United Kingdom	Editorial team, 2007 [58]
2007	1	H9N2	Mild ILI	n.a.	Probably from bird market	Hong Kong SAR, China	United States Center for Disease Control and Precvention, 2008 [66]
2008 2009	2[c]	H9N2	ILI, vomiting, dyspnoa	Rapid test, RT-PCR; MDCK-cell culture; immunofluorescence assay; sequencing	Immunocompromised persons [with and without poultry contact]	Shenzhen and Hong Kong, China	Cheng et al., 2011 [68]
2010	7/ 2	H10N7	Conjunctivitis, rhinorrhea, sore	PCR, partial sequencing of haemagglutinin genes (no	Abattoir workers exposed to infected	New South Wales, Australia	Arzey et al., 2012 [20]

			throat	virus culture); no evidence of seroconversion	poultry		
2011	1	H9N2[d]	Fever, headache, runny nose, cough, sneezing	Partial sequencing	51-month-old female exposed to slaughtered chickens	Bangladesh	International Centre for Diarrhoeal Disease Research Bangladesh, 2011 [69]
2012	2	HP H7N3	Conjunctivitis; no fever or respiratory symptoms	rt-RT-PCR (n=2), Culture in embryonated chicken eggs (n=1), sequencing (n=1)	32- and 52-year-old female/ male poultry worker exposed to infected poultry	Jalisco, Mexico	United States Center for Disease Control and Precvention, 2012 [59]
2013	251 (including 56 fatalities)[e]	LP H7N9	Ranging from mild symptoms and recovery to severe respiratory symptoms and death	Virus isolation, PCR	Not specified	Shanghai, Beijing, Hong Kong; Anhui, Fujian, Jiangsu, Jiangxi, Guangdong, Guizhou, Henan, Hunan, Hebei, , Shandong,Zheijang, provinces, China; 2 cases were imported to Taiwan from mainland China	European Centre for Disease Prevention and Control updated rapid risk assessment [23]
2013	1	H6N1	ILI, mild pneumonia	Virus isolation, full genome sequencing	20-year-old female working in breakfast shop; no exposure to poultry	Taiwan	Centres for Disease Control, 2013 [79]
2013	1	H10N8	Severe pneumonia and death	Not specified	73-year-old immunocompromised female with underlying illness; exposed to live poultry	Jiangxi province, China	ProMED, 2013 [77]
2013	1	H9N2	Chest infection, low fever, chills and	Not specified	86-year-old male with underlying illness; no	Shenzhen/ Hong Kong SAR, China	Promed, 2013 [72]

			cough		recent poultry exposure			
2014	1	H9N2	Illness (not further specified); recovered	Not specified	7-year-old male with poultry exposure	Hunan, China	Promed, [73]	2014
2014	1	H10N8	Sore throat, dizziness, loss of strength, severe pneumonia[f]	Not specified	55-year-old female; visited agricultural market	Jiangxi, China	Promed, [78]	2014
Total	**784/386** (375 when experimentally infected human cases are subtracted)							29

Where more than one human case is stated, diagnostic tests were performed for all cases as stated in the Methods section, unless otherwise specified.

HI, hemagglutination inhibition assay; HP, high pathogenic; ILI, influenza-like illness, LP, low pathogenic; MDCK, Madin Darby canine kidney; MN, microneutralization; n.a., not available; NI, neuraminidase inhibition; n.a., not applicable; PCR, polymerase chain reaction; RBC, red blood cells; RT-PCR, reverse transcription polymerase chain reaction; rtRT-PCR, real time reverse transcription polymerase chain reaction; WHO, World Health Organization.

a In most cases the number tested reflects only the confirmed cases reported in the reviewed paper where the authors did not report on the true number of suspected cases tested

b Authors assume that influenza A-positive test points towards A(H7N2)-infection due to close temporal-spatial links with A(H7N2)-infected poultry and low seasonal influenza activity at that time.

c One patient and her asymptomatic husband showed a MN-titer of 80, three and two weeks after onset of illness respectively.

d Belongs to G1-lineage

e At the time of writing (31 January 2014) the number of cases was still on the increase

f At the time of writing the patient was hospitalized and in critical condition

Table 2. Virological evidence of human infection with swine influenza A viruses

Year	(Tested)[a]/ confirmed cases	Subtype	Symptoms	Method	Nature of exposure	Location	Reference
Not specified	7/3[b] 13/2	swH3N2[c] clH1N1	Mild: coryza	Virus isolation (not further specified)	Experimentally infected humans (intranasal, $10^{5.5}$ egg infective doses)	Not specified (according to author affiliations: England)	Beare et al., 1972 [45]
1974	1 (fatal)	swH1N1[d]	Pneumonia	Culture on WI-38-, HeLa- and rhesus-monkey kidney-cells, inoculation of mice; HI-assay (guinea pig RBC)	16 year-old Hodgkin's disease-patient living on swine farm	Minnesota	Smith et al. 1976 [42]
1976	20/5 (including 1 fatality)	swH1N1	Acute respiratory illness, pneumonia	Embryonated-chicken-egg-culture, HA	Previously healthy soldiers without known swine exposure	New Jersey, USA	Gaydos et al., 1977 [28]
1976	1	swH1N1[e]	Mild ILI	Virus isolation; HI-assay	22-year old swine worker exposed to ill, influenza-positive swine	Wisconsin, USA	United States Center for Disease Control and Prevention, 1976 [29]
1976	1	swH1N1[f]	ILI	Virus isolation; HI-assay	13-year-old boy living on swine farm	Wisconsin, USA	United States Center for Disease Control and Prevention, 1976 [30]
1979/ 80	2	swH1N1[f]	ILI	Rhesus-monkey-kidney cell-culture; embryonated chicken egg-culture; HI-assay	20-year-old having had close contact with swine; 6-year old livestock show visitor without direct swine contact	Texas, USA	Dacso et al., 1984 [31]
1982	1 (fatal)	swH1N1[f]	Pneumonia	Cynomolgus-monkey-kidney-cells; Embryonated-chicken-	4-year old female leukemia-patient; no	Nevada, USA	Patriarca et al, 1984 [32]

				egg-culture; RNA-oligonucleotide mapping	known swine exposure		
1983	3	swH1N1	NA	Isolation	65-year old male with occupational exposure to swine; 10-year old female and 27-year old male with unknown swine exposure	Russia	Chuvakova et al., 1985 [27]
1986	a) 1 b) 2	swH1N1[g,h]	a) Pneumonia, b) Mild ILI	a) Various cell-cultures; Embryonated chicken-egg-culture; HI- and NI-assay b) Various cell-cultures; HI-assay, complement fixation assay	a) 29-year old farmer exposed to ill, influenza-infected pigs b) 50-year-old employee exposed to ill, influenza-infected pigs and 3-year-old no known swine contact	a) Netherlands b) Switzerland	De Jong et al, 1988 [33]
1988	1 (fatal)[i]	SwLH1N1	Pneumonia	RNA fingerprinting; partial sequencing;	32-year-old pregnant woman exposed to influenza infected pigs in county fair	Wisconsin, USA	McKinney et al 1990 [34]
1991	1 (fatal)	SwLH1N1[j]	Pneumonia	Rhesus-monkey-kidney-cell-culture; Embryonated-chicken-egg-culture; rtRT-PCR; Oligonucleotide mapping; HI- and NI-assay; sequencing; Experimental infection of swine	27-year old animal caretaker exposed to swine showing respiratory symptoms	Maryland, USA	Wentworth et al, 1994 [35]
1992/ 93	2	swH3N2[k]	Mild respiratory symptoms	Virus isolation; HI-assay; Sequencing	1- and 2-year old with no known swine exposure	Netherlands	Claas et al, 1994 [41]
1993	1	swH1N1[h]	Pneumonia	Tertiary-monkey-kidney-cell-culture; RT-PCR; Immunofluorescence assay on MDCK-cells; Sequencing; HI-assay	5-year-old living on swine farm [health status of swine not known]	Netherlands	Rimmelzwaan et al., 2001 [36]
1994	2	swH1N1[l]	Mild ILI	Embryonated-chicken-egg-culture; MDCK-cell culture; HI-	39- and 30-year old BSL3-laboratory	Wisconsin, USA	Wentworth et al, 1997 [37]

				and NI-assay; Sequencing; RT-PCR and other PCR-types; Experimental infection of swine	workers exposed to influenza-infected pigs		
1995	1 (fatal)	swH1N1	Severe pneumonia	Virus isolation and subtyping	37-year-old healthy woman working on pig farm (health status of pigs unknown)	Minnesota, USA	Kimura et al., 1998 [38]
1999	1[m]	H3N2[n]	Mild ILI	MDCK-cell culture; HI- and NI-assay; RT-PCR; sequencing	10-months-old girl (neither her nor her family had recent contact with pigs)	Hong Kong SAR, China	Gregory et al., 2001 [39]
2002	1	H1N1[o]	ILI	MDCK-cell culture; HI-assay [also serologically confirmed by HI]	50-year old farmer [possibly from pigs showing respiratory symptoms]	Switzerland	Gregory et al., 2003 [21]
2004 **2005**	1 1	H1N2[p] H1N1[p]	Both mild ILI	MDCK-cell culture; hemagglutination-assay (turkey RBC); HI-assay[q]; rapid tests[r]; RT-PCR; sequencing	25-year-old male and 4 year-old male; neither had direct contact to pigs (incidental contact with backyard pigs could not be excluded)	Philippines Thailand	Komadina et al., 2007 [85]
2005	1	trH1N1[s]	Sore throat, runny/ stuffed nose, cough, fever	Cell culture, RT-PCR, sequencing	50-year old swine farm resident exposed to ill swine (not influenza confirmed)	Iowa, USA	Gray et al., 2007 [92]
2005	1[t]	trH1N1[u]	Acute mild respiratory illness; no fever	MDCK-cell-culture; RT-PCR; sequencing	Patient assisted in butchering swine; vaccinated one month before illness	Wisconsin, USA	Newman et al., 2008 [89]
2005	1	trH3N2[v]	ILI	Culture on primary rhesus monkey kidney cells; sequencing	Previously healthy swine farmer exposed to influenza-positive pigs	Canada	Olsen et al., 2006 [40]
2006	1[w]	sw trH3N2[v]	ILI	HI-assay (guinea pig RBC) RT-PCR, sequencing	7-months old child living on communal farm; no direct exposure	Canada	Robinson et al., 2007 [96]

					to animals, seropositive swine found on farm		
2007	1[x]	trH3N2[v]	Parotitis, nasal congestion; no fever, cough or pharyngitis	Virus isolation; HI-assay (turkey RBC); sequencing	6-year-old boy living on swine farm [no illness in swine observed]	Canada	Bastien et al., 2009 [95]
2007	26/2[y]	swH1N1[z]	ILI	Virus isolation; sequencing	People having been exposed to ill swine on county fair	Ohio, USA	Vincent et al., 2009 [87]; Yassine et al., 2009 [88]
2005-2009	a) 10 b) 1	a) sw trH1N1[aa] b) trH1N2[bb]	Ranging from mild ILI to pneumonia	Rapid point of care test (n=8); Virus culture (n=7); rt RT-PCR (n=6); HI-assay; complete genome pyrosequencing	7 males [age: 16months- 36 years] 4 females (age: 4 – 48 years) exposure ranging from unknown contact, close proximity and direct contact with swine (partly with ill pigs)	Wisconsin, Missouri, Iowa (n=3), Ohio (n=2), Illinois, Michigan, Minnesota and Texas, USA	Shinde et al., 2009 [93]
2008	1[cc]	SwL trH1N1[dd]	ILI, vomiting, diarrhea	Rapid test; culture; rRT-PCR; sequencing	19 year-old male exposed to healthy appearing pigs in course of judging event (no physical contact)	South Dakota, USA	Dawood et al., 2011 [90]
2008	1	swH1N1[ee]	ILI	MDCK-cell culture; immunofluorescence using MAb; PCR; partial sequencing	50 year-old-female with direct contact to asymptomatic pigs	Spain	Adiego Sancho et al., 2009 [22]
2009	1[ff]	sw trH3N2	Fever, cough, sore throat	Rapid test; rtRT-PCR; sequencing	12 year-old boy (touched healthy appearing swine while on a county fair-> were seropositive)	Kansas, USA	Cox et al., 2011 [97]
2009	3[gg]	sw trH1N1[hh]	ILI	MDCK-cell culture; rtRT-PCR; HI-assay (turkey RBC)[ii] sequencing	Workers involved in hog operation[jj]	Saskatchewan, Canada	Bastien et al., 2010 [91]
2009-2011	3	swH1N1[kk]	n.s.	n.s.	Three adult, male swine workers exposed to ill, influenza- confirmed pigs	Switzerland	European Centre for Disease Prevention and Control, 2012 [86]

Aug- Dec 2011	12[ll]	H3N2v[mm]	ILI, vomiting, diarrhea	Rapid tests; rRT-PCR; sequencing	50% exposed and 50% unexposed to swine; (11 children, 1 adult male)	Hawaii, Illinois, Indiana, Iowa, Maine, Maryland,	United States Centers for Disease Control and Prevention 2012 [99], 2013 [25]
2012	309				Most attending agriculture fair	Michigan, Minnesota, Ohio,	
2013	19[nn]				Most attending agriculture fair	Pennsylvania, Utah, West Virginia, Wisconsin; USA	
Total	455/ 401						33

Where more than one human case is stated, diagnostic tests were performed for all cases as stated under "Methods" unless otherwise specified.

No. tested, only if available; cl, classical swine lineage; HA, haemagglutination assay; HeLa, Henriette Lacks cervical cancer; HI, haemagglutination inhibition; ILI, Influenza-Like Illness; MAb, monoclonal antibodies; MDCK, Madin Darby Canine Kidney; NA, information not available; NI, neuraminidase inhibition; n.a., not applicable; n.s., not specified; RBC, red blood cells; RT-PCR, reverse transcriptase polymerase chain reaction; rtRT-PCR, real-time reverse transcriptase polymerase chain reaction; sw, swine; SwL, swine-like; tr, triple reassortant; WI-38, human embryo lung fibroblast.

a In most cases the number tested reflects only the confirmed cases reported in the reviewed paper where the authors did not report on the true number of suspected cases tested
b Number of humans in which virus could be reisolated.
c Swine/Taiwan/7310/70 related to A/Hong Kong/1/68.
d Named A/Mayo Clinic/103/74 – inhibited by antisera against A/swine/1976/31 and A/swine/Wisconsin/67.
e A/New Jersey/8/76.
f A/New Jersey/8/76-like influenza virus.
g A/Netherlands/386/86, A/Geneva/5521/86, A/Geneva/5200/86.
h Highest homology with European swine-influenza viruses.
i Patient's husband developed ILI-symptoms one day before the patient (virus isolation not done): people exposed to the patient and/or diseased.
j A/Maryland/12/91.
k Human-avian reassortants: A/Netherlands/5/93, A/Netherlands/35/93.
l Strain was similar to strain used in swine experiment and is closely related to Sw/IN and A/WI/3523/88.
m Patient showed a titer of 160 by HI-assay, mother a titer of 20, father, brother and grandparents less than 10.
n Virus was closely related to viruses circulating in European pigs.
o A/Switzerland/8808/02 (European avian-like lineage).
p Swine-like viruses: HA genetically similar to swine viruses circulating in swine in Asia at the time and viruses that circulated in North America in the 1990s wheras NA and internal genes were similar to European swine viruses.

q	Both isolates tested negative against human strains.
r	To determine whether isolates belong to influenza A or B.
s	HA, PB1, PB2, PA, NP, NS, M descended from North American trA(H1N1), NA descended from North American classical A(H1N1) [113].
t	Patient showed a 2-fold titer increase in MN-assay (not in HI-assay) and 4 family members and the patient's brother-in-law were sero-negative by MN- and HI-assay.
u	Predominant genotype of subtype H1N1 in North American pigs.
v	Same genotype as human/ classical swine/ avian reassortant that emerged in 1998 in North America.
w	Four of 7 household members of the patient and 4 of 46 other residents of the farm showed serological evidence of infection.
x	Household members were only serologically screened.
y	Virus from at least two individuals was isolated and sequenced and turned out to be nearly identical to the swH1N1 isolated from the ill swine; not done for all human cases.
z	HA related to H1γ cluster (H1N2-like) of contemporary H1-SIV, NA-gene related to swine N1 phylogenetic cluster and internal genes were from tr reassortant SIV lineage and group with cluster IV of A(H3N2) viruses.
aa	H1N1: HA, NA, NP, NS, M (classical swine, North American lineage), PB2, PA (Avian, North American lineage), PB1 (human seasonal H3N2).
bb	H1N2: HA (human seasonal H1N1), NP, M, NS (classical swine, North American lineage), PB2, PA (Avian, North American lineage), PB1, NA (human seasonal H3N2).
cc	Contacts of patient and people exposed to swine in course of judging event were serologically screened.
dd	Distinct from A(H1N1)pdm09 and similar to recently circulating triple-reassortant swine viruses in the US
ee	Phylogenetically close to A/Switzerland/8808/02 (European avian-like lineage) [19].
ff	In addition 27 of 34 county fair visitors participated in a survey: none reported ILI.
gg	No household members were ill at the time.
hh	Distinct from A(H1N1)pdm09: NS, NP, M, PA, PB1 and PB2 were similar to a North American Swine triple reassortant and HA and NA were most similar to A/Brisbane/59/2007 (H1N1)-like viruses.
ii	For antigenic characterization.
jj	Mild respiratory illness was present in <1% of the swine herd the workers were exposed to (no confirmation whether due to influenza A infection).
kk	Infected with European SIV similar to viruses identified in ill pigs the workers were exposed to.
ll	Eleven children (including 2 children attending the same daycare) and one adult male.
mm	Influenza A H3N2 variant (comprises genes from avian, swine and human origin) with M-gene derived from A(H1N1)pdm09.
nn	Case count from January 2013 until October 18, 2013 (most recent update as of January 31st, 2014)

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CHAPTER 3.1

GETTING MORE OUT OF LESS – A QUANTITATIVE SEROLOGICAL SCREENING TOOL FOR SIMULTANEOUS DETECTION OF MULTIPLE INFLUENZA A HEMAGGLUTININ-TYPES IN CHICKENS

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ABSTRACT

Current avian influenza surveillance in poultry primarily targets subtypes of interest for the veterinary sector (H5, H7). However, as virological and serological evidence suggest, surveillance of additional subtypes is important for public health as well as for the poultry industry. Therefore, we developed a protein microarray enabling simultaneous identification of antibodies directed against different HA-types of influenza A viruses in chickens. The assay successfully discriminated negative from experimentally and naturally infected, seropositive chickens. Sensitivity and specificity depended on the cut-off level used but ranged from 84.4% to 100% and 100%, respectively, for a cut off level of ≥1:40, showing minimal cross reactivity. As this testing platform is also validated for the use in humans, it constitutes a surveillance tool that can be applied in human-animal interface studies.

INTRODUCTION

Avian influenza A viruses (AIV) belong to the family *Orthomyxoviridae* and comprise eight gene segments consisting of negative sense single-stranded RNA. The classification of AIV into different subtypes is based on two surface structures, hemagglutinin (HA) and neuraminidase (NA). To date, 18 distinct HA-types and 11 NA-types are known of [1–3]. With the exception of subtypes H17N10 and H18N11 of which RNA was recently detected in bats, aquatic birds constitute reservoirs for AIV, usually without showing signs of disease [1,2]. To date, influenza A viruses have crossed the species barrier to humans, swine, aquatic mammals, domestic poultry, birds of prey, horses, mustelids, civets, felines and canines [4–6]. Several avian and swine influenza viruses have zoonotic potential. While AIV subtype A (H5N1) virus infections have had the largest economic and public health impact so far, AIV with HA types 6, 7, 9 and 10 have also caused virologically confirmed human infection with varying severity [4,6]. Until recently, human H7-infections have been associated with mild symptoms in humans. However, since early 2013, a newly emerging H7-subtype, A(H7N9), has formed an exception by causing a more severe clinical picture and death in about 36% of the recorded patients, possibly related to specific host susceptibility factors [7,8]. Although the symptoms shown by patients largely resembled infection with highly pathogenic (HP) A(H5N1), the manifestation in poultry – the putative source of direct human infection – is different [9,10]. Unlike HP A(H5N1) viruses that cause severe illness and death in poultry, this novel influenza A(H7N9) strain causes subclinical infection in poultry, which allowed the virus to spread unnoticed over a large geographic region in China [10]. Consequently, the general population can be exposed to animals shedding this virus without warning signs. Indeed, serological investigations in poultry workers suggest more widespread infections in humans, possibly reflecting mild or unapparent illness [11].

This example and additional serological evidence for human infection with influenza viruses *other than* H5, H7, H9 and H10 – including H4, H6 and H11 [12–14] – highlight the importance of influenza monitoring at the human-animal interface, where humans are currently sentinels for circulation of zoonotic viruses [15,16]. Therefore, ideally, future serological studies evaluating influenza viruses at the human-animal interface would include these "neglected" subtypes.

Given the ability of AIV H5 and H7 to mutate into HP forms and the economic consequences associated with such infections, a compulsory European Union-wide surveillance system was implemented in 2005 [17]. In the Netherlands, serological monitoring is more intensive than required by EU-regulations [18] and includes screening of all poultry flocks at least once a year and high risk-groups, e.g. free-range flocks every three months. In practice, a representative number of farms and individuals per country are pre-screened with an indirect or competitive enzyme-linked immunosorbent assay (ELISA), identifying antibodies against conserved regions (matrix or nucleoprotein) that all influenza virus subtypes have in common [19]. Upon a positive pre-screening result, the presence of H5- or H7-antibodies is confirmed or ruled out by means of a hemagglutination inhibition (HI)-assay, and flocks are

tested for active virus circulation. While this screening regimen meets the requirements for veterinary surveillance, the characterization of non-H5 and -H7 but ELISA-positive samples may be relevant for the poultry industry and for public health.

Here, we describe the development and use of a protein microarray (PA) that enables simultaneous screening for antibodies to multiple influenza HA-types in poultry, using minute quantities of serum (10µl) that can be collected through routine veterinary surveillance.

MATERIALS AND METHODS

Sera

Three different serum sets (hereafter referred to as group 1-3) were used to evaluate the performance of the PA for the use in chicken:

Negative sera

Negative sera were obtained from different sources. In total 38 chicken sera which tested negative by ELISA (Idexx FlockChek AI, MultiS-Screen Ab Test Kit, Hoofddorp, the Netherlands) were used:

1a) One serum pool of 52-week-old, specific pathogen free (SPF) white layers (flock from GD AHS)
1b) Ten sera from 3-week-old, non-infected, non-vaccinated, conventional Lohman Brown layers
1c) 27 sera from a commercial 6-week-old Ross broiler flock (hereafter named "negative field chickens")

Consecutive serum samples from SPF chickens experimentally infected with live field strains.

Four groups of 15 white SPF laying hens (GD AHS) were intratracheally infected with live field strains (0.5 ml; ~10^6 EID_{50}) belonging to the subtypes H5N2, H6N2, H7N1 or H9N2 (Table 1) at 12 weeks of age. For the duration of the experiment infected chickens were kept in isolators with twelve hours light per day, 20-25°C and were given ad libitum access to food and water. Serum was collected from the wing vein at day 7, 14 and 22 post infection (p.i.) and seropositivity was confirmed by testing sera at one dilution (1:8) by standard HI-assay, as is done routinely in the animal health service. Therefore, data were available as positive/ negative results only.

Sera from outbreaks of avian influenza detected during routine surveillance in the Netherlands

To evaluate applicability of the test in the field, we analyzed samples from four different laying hen flocks having undergone past infection with low pathogenic (LP) AIV subtypes, hereafter named "naturally infected field chickens". All flocks were identified as AIV exposed by ELISA-testing (Idexx FlockChek AI, MultiS-Screen Ab Test Kit) of samples collected during routine surveillance performed by the AHS. HI typing of sera, and/ or virus isolation and virus typing (CVI, Lelystad) confirmed initial diagnosis. Samples were derived from two outbreaks caused by subtype H6, both in flocks of 16-month-old, free-range brown laying hens (outbreak 1: n=10; outbreak 2: n=7). In addition, ten sera seropositive for LP H7N3 were obtained from 16-month-old, free-range brown layers, and eight sera from an H9N2-outbreak in 19-month-old, brown laying hens housed in cages were screened. Individual HI-titers were available for the H9- and one H6-outbreak (outbreak 2). Sera of the remaining two outbreaks were screened qualitatively at one dilution only (1:8).

Ethics statement

All experiments were approved by the Animal Experimental Committee of the Faculty of Veterinary Medicine of the Utrecht University or the Animal Welfare Committee (DEC) of the GD Animal Health Service, Deventer, the Netherlands, in accordance with the Dutch regulations on experimental animals.

Production of protein microarray-slides and sample analysis

We used a modification of the technique that has been described elsewhere [20]. In our study, 22 recombinant HA1-proteins comprising representatives of 13 different subtypes (Table 1) were printed onto 16-pad nitrocellulose slides as described before [20]. Antigens were produced in human embryonic kidney (HEK) cells, were purified by HIS-tag and were delivered at a protein concentration of 1mg/ml (see manufacturer for details, Table 2). To determine the optimal working concentration for the recombinant HA1-proteins used in the PA, checkerboard titrations were performed for each protein using four different dilutions (2x, 4x, 8x, 16x). When necessary, proteins were concentrated using Amicon Ultra-0.5 mL Centrifugal Filters for Protein Purification and Concentration according to manufacturer's instructions (Merck Millipore, Massachusetts, USA) and checkerboard titrations were repeated thereafter.

Prior to testing, all sera were inactivated in a water bath at 56^{o}C for one hour. For serum analysis, four slides fixed in a FAST frame slide holder (Whatman, Kent, UK) could be used simultaneously. Each holder accommodated up to seven sera and one in house-standard. Serum was titrated in two fold dilution series ranging from 1:20 (10µl of serum) to1:2560. Known negative sera were tested in two-fold dilutions ranging from 1:20 to 1:160. An in house-standard, comprising of a serum-pool of hyperimmunized chickens infected with strains of subtypes H5, H6, H7 and H9 was included in each test run. After serum incubation, bound antibodies were visualized using a Cy5 AffiniPure rabbit anti-chicken IgY Fc-

fragment-specific conjugate (Jackson ImmunoResearch, West Grove, USA) diluted in Blotto Blocking Buffer (Thermo Fisher Scientific Inc., Rockford, MA, USA) and 0.1% Surfact-Amps (Thermo Fisher Scientific Inc.) at a concentration of 1:1300. IgY represents the avian equivalent of mammalian IgG [21].

Data analysis and statistics

Fluorescent signals were quantified and converted into titers as described before [20]. The PA spanned a detection range of titers from 1:20 to 1:2560. We calculated geometric mean titers (GMTs) including 95% confidence intervals (CI) as well as homologous versus heterologous GMT ratios of the validation data using GraphPad Prism for Windows (Version 6.03, GraphPad Software Inc., California, USA). Log2-transformed median antibody titer ratios of field chickens were plotted in R (R Foundation for Statistical Computing, version 2.15). For consecutively collected samples, seroconversion or a significant rise was defined as a ≥4 fold increase in antibody titer [22]. Correlations between the PA and HI-test were calculated using a two-sided Spearman's rank correlation coefficient (ρ). A p-value of less than 0.05 was considered statistically significant.

The overall antibody reactivity for all seropositive individuals was visualized by means of a heat map, generated by applying hierarchical clustering (pairwise correlation distance and Ward's method) to log-transformed titers. No cut off titer was applied to the data. Bright red color indicates high titers whereas faint red and white corresponds to low titers and no reactivity, respectively. Amino acid (AA) sequence similarity of HA1s was determined using a fast algorithm with pairwise alignment in Bionumerics (version 6.6, Applied Maths).

Antigen stability and batch control

Antigen quality and stability between different batches was tested using an in-house serum pool comprising HA-specific polyclonal rabbit-antisera (Immune Technology Corp., New York, USA) raised against all antigens included on the PA. Testing the last slide from each batch of 25 slides showed that all antigens were stable over time (data not shown). Prior experiments showed that spotted PA slides containing recombinant influenza HA1-proteins are stable for at least one year (unpublished data). Day-to-day variation was controlled for by correcting all titers according to the reactivity of the reference antigen H6.07 against the in house-standard, as previously described [20].

Table 1. Recombinant HA1-proteins used on the PA and viruses used for infection of chickens of group 2.

	Subtype	*Strain*	*Source*
H1.18	H1N1	A/South Carolina/1/18	IT
H1.33	H1N1	A/WS/33	IT
H1.99	H1N1	A/New Caledonia/20/99	IT
H1.07	H1N1	A/Brisbane/59/2007	IT
H1.09	H1N1	A/California/6/2009	IT
H2.05	H2N2	A/Canada/720/05	IT
H3.68	H3N2	A/Aichi/2/1968	SB
H3.03	H3N2	A/Wyoming/3/03	IT
H4.02	H4N6	A/mallard/Ohio/657/2002	E
H5.97	H5N1	A/Hong Kong/156/97 (clade 0)	IT
H5.06	H5N1	A/Turkey/15/2006 (clade 2.2)	G
H5.02	H5N8	A/duck/NY/191255-59/2002 (LP)	SB
H5.07	H5N3	A/duck/Hokkaido/167/2007 (LP)	SB
H6.07	H6N1	A/northern shoveler/California/HKWF115/2007	SB
H7.03	H7N7	A/Chicken/Netherlands/1/03	IT
H8.79	H8N4	A/pintail duck/Alberta/114/1979	E
H9.99	H9N2	A/Guinea fowl/Hong Kong/WF10/99	IT
H9.07	H9N2	A/Chicken/Yunnan/YA114/2007	G
H11.02	H11N2	A/duck/Yangzhou/906/2002	IT
H12.91	H12N5	A/green-winged teal/ALB/199/1991	IT
H13.00	H13N8	A/black-headed gull/Netherlands/1/00	IT
H16.99	H16N3	A/black-headed gull/Sweden/5/99	IT
Infection	*Subtype*	*Strain*	*GISAID accession number*
	H5N2	A/chicken/Belgium/150/1999	EPI238402
	H6N2	A/turkey/Massachusetts/3740/1965	EPI3187
	H7N1	A/parrot/Northern Ireland/VF-73-67/73	EPI6514
	H9N2	A/chicken/Saudi Arabia/SP02525/3AAV/2000	AHS

LP, low pathogenic; IT, Immune Technology Corp.; SB, Sino Biological Inc.; E, e-enzyme; G, Genscript; AHS, from Animal Health Service, Deventer, the Netherlands

RESULTS

Four out of 38 negative sera (1.5%) – all four belonging to group 1c (negative field chickens) – showed minor low-level reactivity with titers ranging between 21 and 30 against antigens H2.05 and H12.91, respectively. All other samples tested negative for all antigens (data not shown). These findings result in a specificity of the PA of 94.6% to 100% at a cutoff titer of >1:20 across all antigens, and of 100% when the cutoff was raised to ≥1:40 or higher.

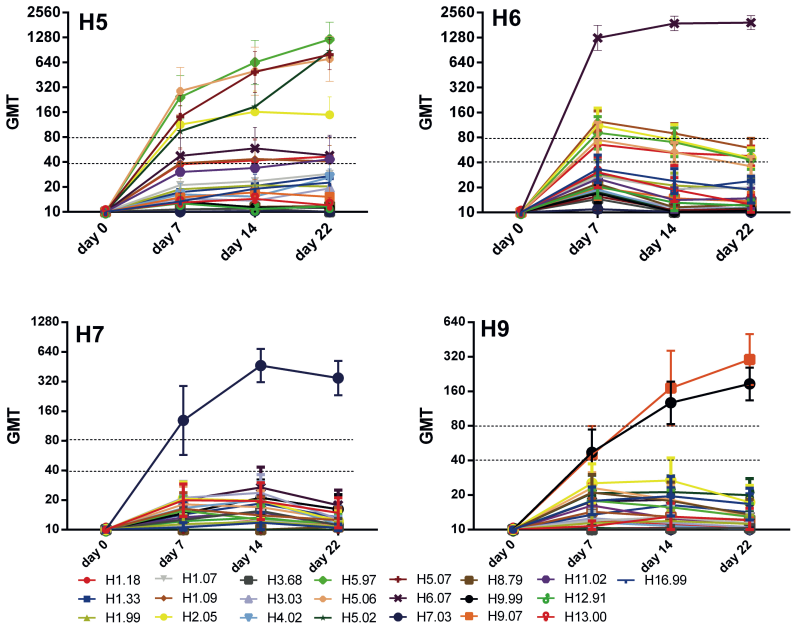
In contrast, all experimentally infected chickens (group 2) seroconverted to the homologous antigens, although the kinetics of response differed slightly. H5- and H6-infected animals were the fastest to show 100% seroconversion at day 7 p.i. at a cut off of ≥1:40 for at least one PA-antigen used, whereas for H7- and H9-exposed animals complete seroconversion (100% of animals) occurred at a later time point (Figure 1, Table 2). At day 22 p.i. all animals showed a significant (≥ 4-fold) titer increase. With advancing antibody rise (at days 14 and 22 p.i.), sensitivities further increased for antigens matching the infecting subtype. In addition, we combined all serum collection time points to investigate the ability of the PA to identify positive individuals in different stages of antibody development and sensitivity remained high (Table 2).

Interestingly, although H5-infected SPF chickens were inoculated with a low-pathogenic H5-strain (Table 1), we observed the strongest antibody response against H5.97, an antigen representing HP AIV clade 0 (Table 1, Figure 1). For the H9-infection cohort, chickens showed mixed antibody reactivity against the two H9-antigens, with half the individuals reacting stronger against H9.99 and the other half displaying a higher titer against H9.07 at day 7 p.i. One individual had an equally high titer for both antigens at that time point. At day 14 and 22 p.i., reactivity profiles shifted towards H9.07 in the majority of chickens, ten and nine out of 15, respectively, displaying a higher titer against H9.07 compared to H9.99 (data not shown).

Cross-reactivity against heterologous antigens of experimentally infected chickens (group 2)

In general, we observed some degree of heterogeneity in kinetics and cross reactivity of antibody responses within all infection groups (Figure 1). The ratio of homologous versus heterologous GMTs of all sampling days combined ranged from 1.8 to 57.9 in H5-, 19.1 to 161.1 in H6-, versus 12.8 to 27.4 for H7- and 4.6 to 13.3 in H9-infected individuals (Figures 1 and 2). The highest level of cross reactivity was observed in H5-infected animals reacting with the H2-antigen (GMT-ratio 1.8-4.2). Nevertheless, a clear distinction between homologous and heterologous reactivity was observed for the remaining antigens, with GMT ratios of >4 for all other antigen combinations (Figures 1 and 2). Therefore, the infecting

Figure 1. Kinetics of serological responses of SPF chickens after intratracheal infection with live virus (group 2). Titles of each graph indicate infection group. X-axes depict the day of serum collection post infection. Y-axes indicate geometric mean titers (GMT). Error bars represent 95% confidence intervals of the measurements. Note differences in log-scale. Heterologous reactions above the dotted line represent cross-reactive responses with a titer higher than 1:40 or 1:80, respectively.



strain could clearly be identified independent of the cutoff level chosen (Figure 1). To minimize or dismiss the "noise" caused by cross-reacting antibodies, the application of a cutoff level of ≥1:80 seems appropriate (Table 2, Figure 1).

Serological profiles of naturally infected laying hens (group 3)

Serum samples from naturally infected field chickens showed similar discriminatory serological profiles compared with the data from the validation experiments (Figure 3). In the analysis, we combined the data of both H6-outbreaks. The PA correctly identified 100% of the tested field chickens as positive up to a cut-off titer of ≥1:80 (data not shown). Cross-reactivity was negligible for H6- and H7-infected individuals and generally matched the patterns observed in group 2 (Figure 3, light red, light green). Among the field chickens naturally infected with H9, we observed somewhat more cross reactivity (Figure 3 and 4).

Nevertheless, the infecting subtype was still evident by resulting in the highest median log2-titer ratio (Figure 4).

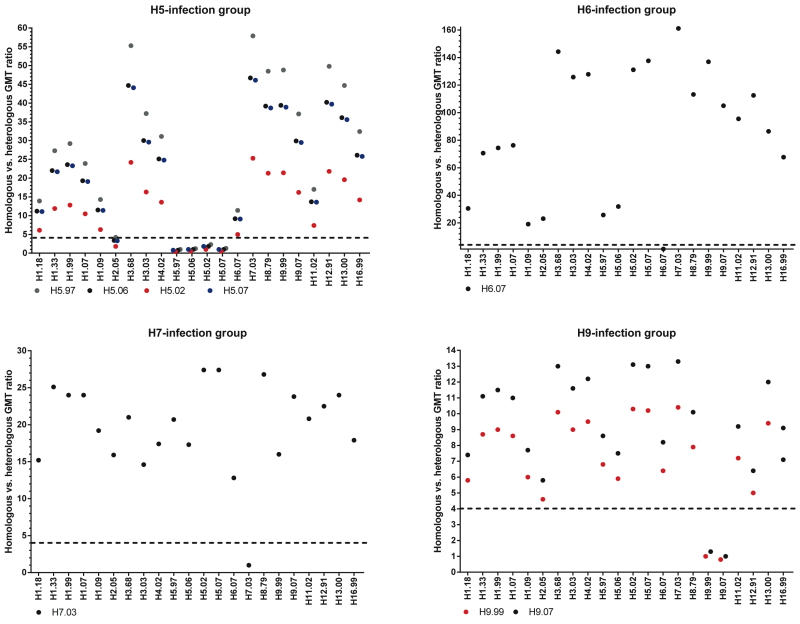
Overall, the PA results showed good correlation with the HI-assay. Spearman's rank correlation coefficients showed strong, significant associations between the HI-assay and PA-antigens H9.07 (ρ=0.804, p=0.021) and H6.07 (ρ=0.850, p=0.029), whereas a relatively strong but not statistically significant association could be demonstrated between HI-data and PA-antigen H9.99 (ρ=0.600, p=0.121).

DISCUSSION

Here we present a highly sensitive and specific multiplex-screening tool to detect antibodies against different HA-types of AIV in chickens. We show that the PA discriminates between negative and experimentally infected, seropositive chickens. We further demonstrate that our test can serve as a surveillance tool in commercial field chicken flocks, by reliably identifying the infecting subtypes in laying hens from free-range- and indoor husbandry. An asset of the technique is that it requires a minute quantity of serum (5-10µl) to simultaneously screen for *multiple* subtypes, whereas the HI-assay usually requires about the same amount to detect antibodies against only *one* subtype [23]. This characteristic is particularly advantageous when screening small animal species of which only small volumes of sera are available. Lee et al. [28] speculated that immunogenicity, and therefore antibody titer heights, can depend on the protein itself and can vary between strains of different subtypes in chickens immunized with different DNA-vaccines. Failure to regularly update antigens in HI-assay can result in a reduced ability to detect antibodies against more recent field isolates [27] and it is unclear if this also can be observed in our assay system. The strains used for the infections of group 2 animals were closely related to the strains from which antigens were produced, with the lowest level of AA-identity for antigen H9.07 (94.4%) (Table 3). Analysis of consecutive sera of SPF chickens infected with live field strains of different AIV subtypes showed that the PA was able to quantify varying titer heights per sampling time point and infection group. Such variation could either be due to differences in immunogenicity of strains used for infection [24], infectious dose [24,25], different chicken breeds or genetic lineages [24,26]. From a technical aspect, differing quality of antigens used on the PA and distant relatedness of strains used for infection and the assay antigen [27] could account for the differences in titer heights between infection groups. As infectious dose and breed were the same for all experimental infection cohorts and antigen quality was checked prior to testing and monitored throughout the experiment, these factors can be disregarded as a possible source of variability.

This lower AA-identity in combination with individual variation could be a possible explanation why H9.07 did not yet react at day 7 p.i. for some experimentally infected chickens of group 2 (Figure 3, square with black solid line). On the other hand, the lower AA-

Figure 2. Homologous versus heterologous geometric mean titer (GMT) ratios for different groups of experimentally infected chickens (H5, H6, H7, H9) for all sampling days combined. A high homologous versus heterologous ratio in GMT indicates low cross-reactivity and vice versa. For instance, as for the H6-infection group the GMT against the homologous antigen H6.07 is 1668.8 and the GMT against the heterologous antigen H7.03 is 10.4, the homologous vs. heterologous ratio is the highest (~161), implying that the level of cross-reactivity is lowest for the H7-antigen in the H6-infection group. The dotted line demarkates a ratio of 4. Note differences in scale.



It is not known how AA differences in HA1 translate to antigenic reactivity in the PA system. Cattoli et al. [29] examined serological responses of drift variants of H5 strains in chickens using HI- and microneutralization assay. Of the 11 AA substitutions found in the HA1, the researchers demonstrated that only five substitutions sufficed to cause antigenic drift. These findings stress that a high AA sequence similarity in the HA1 of two strains does not necessarily translate into similar serological reactivity, if critical substitutions occur in epitopes influencing antigenicity. Hence, AA sequence similarity is not a good indicator for antigenicity and cross reactivity, so no inferences about the compatibility between the viruses used for infection and PA-antigens can be made.

Overall, the observed cross-reactivities were negligible in comparison to the titer height of the antigens matching the subtype of infection. Interestingly, we noted that heterologous patterns largely reflected phylogenic relationships. The 16 currently known HA-types derived from birds divide into two phylogenetic groups which further segregate into 5 clades.

Table 2. Sensitivities (%) for microarray antigens corresponding to subtype of virus strains used for infection of SPF chickens (group 2) according to time point of serum collection and different cut of levels. Bold font indicates 100% sensitivity. (p.i., post infection)

	Virus subtype	H5				H6	H7	H9	
p.i.	Cut off ≥	H5.97	H5.06	H5.02	H5.07	H6.07	H7.03	H9.99	H9.07
Day 7	1:20	100.0	100.0	85.7	100.0	100.0	80.0	86.7	73.3
	1:40	92.9	92.9	78.6	100.0	100.0	73.3	73.3	66.7
	1:80	85.7	85.7	57.1	85.7	100.0	66.7	20.0	26.7
	1:160	78.6	78.6	28.6	57.1	100.0	53.3	6.7	13.3
	1:320	50.0	57.1	21.4	0.0	93.3	40.0	0.0	0.0
	1:640	14.3	28.6	0.0	0.0	86.7	6.7	0.0	0.0
	1:1280	0.0	0.0	0.0	0.0	53.3	0.0	0.0	0.0
	1:2560	0.0	0.0	0.0	0.0	13.3	0.0	0.0	0.0
Day 14	1:20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.3
	1:40	100.0	100.0	85.7	100.0	100.0	100.0	93.3	86.7
	1:80	92.9	92.9	78.6	100.0	100.0	100.0	80.0	86.7
	1:160	85.7	78.6	57.1	100.0	100.0	93.3	20.0	46.7
	1:320	78.6	71.4	42.9	71.4	100.0	66.7	13.3	26.7
	1:640	71.4	50.0	14.3	28.6	100.0	60.0	6.7	20.0
	1:1280	21.4	14.3	0.0	14.3	80.0	0.0	0.0	6.7
	1:2560	7.1	7.1	0.0	0.0	40.0	0.0	0.0	0.0
Day 22	1:20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	1:40	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	1:80	100.0	93.3	100.0	100.0	100.0	100.0	100.0	93.3
	1:160	100.0	86.7	100.0	100.0	100.0	78.6	46.7	73.3
	1:320	86.7	73.3	86.7	80.0	100.0	64.3	20.0	46.7
	1:640	80.0	53.3	66.7	53.3	100.0	21.4	6.7	20.0
	1:1280	73.3	40.0	26.7	26.7	73.3	0.0	0.0	6.7
	1:2560	13.3	0.0	6.7	13.3	40.0	0.0	0.0	0.0
Days combined	1:20	100.0	100.0	95.3	100.0	100.0	93.2	95.6	88.9
	1:40	97.7	97.7	88.4	100.0	100.0	90.9	88.9	84.4
	1:80	93.0	90.7	79.1	96.6	100.0	88.6	66.7	68.9
	1:160	88.4	81.4	62.8	89.7	100.0	75.0	24.4	44.4
	1:320	72.1	67.4	51.2	58.6	97.8	56.8	11.1	24.4
	1:640	55.8	44.2	27.9	34.5	95.6	29.5	4.4	13.3
	1:1280	32.6	18.6	9.3	17.2	68.9	0.0	0.0	4.4
	1:2560	7.0	2.3	2.3	6.9	31.1	0.0	0.0	0.0

Figure 3. Heat map depicting serological patterns of naturally (n.i.) and experimentally infected (e.i.) chickens (H5, H6, H7, H9) spanning all samplings days. Dendrograms reflect clustering based on similarity of serological profiles. Microarray antigens are depicted on the X- axis. Different infection groups are color coded on the Y-axis according to the avian influenza virus subtype causing the infection. Rows represent reaction profiles of individual chickens across the entire antigen panel. Columns represent the reactivity of all individuals against a specific antigen as stated on the X-axis. Intensity of the red color is proportional to the log-titer height. Black dotted squares indicate missing antigens H5.07 (n=6) and H11.02 (n=7) due to spotting failure. Black square with solid line indicates no biological reactivity against the H9.07 antigen. The clustering algorithm automatically excluded negative sera.

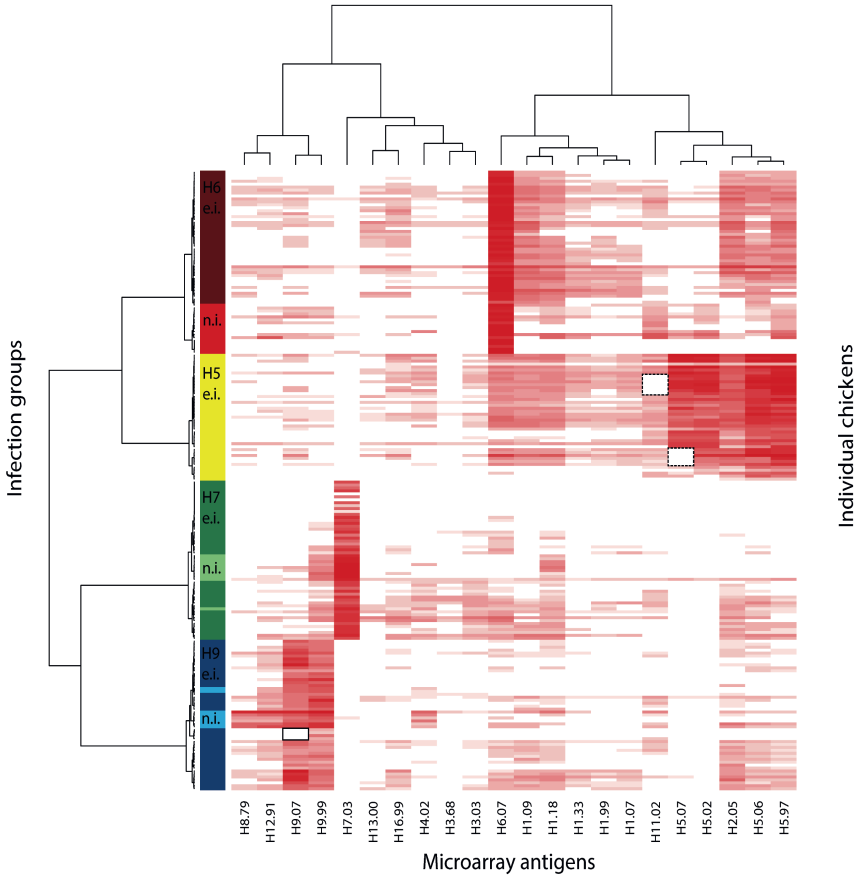
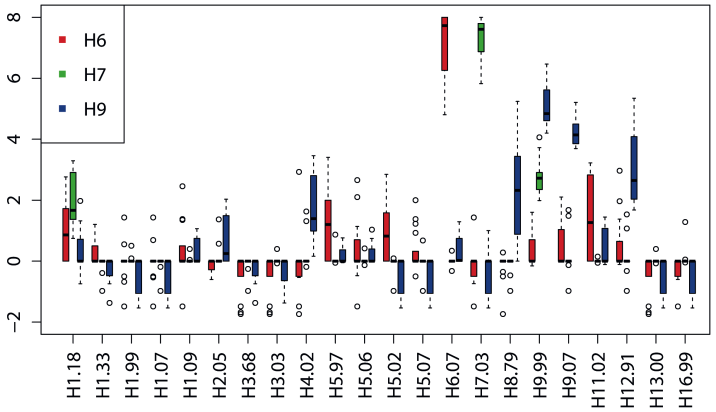


Table 3. Amino acid (AA) similarity matrix for strains of a particular subtype used to infect SPF chickens of group 2 (in bold) versus PA-antigens per HA-type (table 1). Similarity was calculated based on the HA1 part of the hemagglutinin (sequence length 318 AA). Percentages in bold and italics denote similarity between strains used for infection versus corresponding PA-antigen.

H5N2	100.00				
H5.07	97.37	100.00			
H5.02	96.15	96.42	100.00		
H5.97	95.75	95.39	93.52	100.00	
H5.06	95.29	95.45	94.21	97.48	100.00
H6N2	100.00				
H6.07	96.01	100.00			
H7N1	100				
H7.03	97.11	100.00			
H9N2	100.00				
H9.99	97.49	100.00			
H9.07	94.39	93.77	100.00		

Figure 4. Antibody profiles of field chickens expressed as log2-transformed median antibody titer ratios plotted per outbreak. Antibody titer ratios were derived by log transforming the data, calculating the median antibody reactivity across all antigens included on the PA and subtracting it from the antibody reactivity against individual antigens. This was calculated for every chicken. By doing that, every individual's values are normalized according to its own background reactivity. Individual ratios were summarized in boxplots. Horizontal bars within each box represent log2-transformed median antibody titer ratios per antigen and outbreak. Chickens naturally infected with H6 are depicted in red (n=17), H7 in green (n=10) and H9 in blue (n=8). The two H6-outbreaks were combined in this analysis.



identity of H9.07 did not seem to have a major influence, as this antigen showed a higher GMT at day 14 and 22 p.i. in H9-infected chickens of group 2, compared to H9.99, which was 97.5% similar to the infecting strain (Table 3). Group 1 consists of 3 clades (H1, H2, H5 and H6; H8, H9 and H12; H11, H13 and H16) whereas group 2 comprises 2 clades (H3, H4 and H14; H7, H10 and H15) [1,30].

Heterosubtypic immunity has mainly been attributed to cytotoxic T-cells specific for internal proteins [31], but neutralizing antibodies also play an important role in protection [32,33]. To date, a number of broad reacting intra-subtype-, intra-clade-, intra-group- and inter-group-specific neutralizing monoclonal antibodies have been identified [34–37]. Of all vaccination cohorts, H5-vaccinated chickens displayed the highest level of cross-reactivity with antigen H2.05 (Figures 1, 2 and 3). This finding is not surprising due to the high sequence similarity of these two subtypes [28,38]. Likewise, H9-positive serum cross-reacted somewhat with members of the same clade, H8 and H12. Together with the calculation of the median-log2-titer ratios – as was performed for the field chickens in this study –, the knowledge of these patterns can be useful in distinguishing cross-reactivity from potential dual infections involving subtypes of different clades. Although we only tested one serum of a chicken simultaneously immunized with influenza virus strains belonging to two different subtypes (H7 and H9), the PA showed clear antibody titers against both HA-types (median log2-titer ratio for H7.03=8, H9.99=6 and H9.07=5.8, respectively) with no cross-reactivity to other antigens (median log2-titer ratio=0). This capacity can be especially interesting for regions where AI surveillance is not implemented in poultry and where animals might experience multiple consecutive- or co-infections with different subtypes. To further investigate this potential, serum of experimentally infected chickens consecutively or simultaneously immunized with different subtypes would need to be analyzed, which were not available in this study.

Heterologous reaction was lowest in chickens experimentally and naturally infected with subtype H7 compared to other serum cohorts. This can possibly be explained by the fact that, apart from H3- and H4-antigens, no other representatives of phylogenetic group 2 (H10, H14, H15) were included in the PA setup. Similarly, Latorre-Margaleff et al. [39] found that after infection with a certain subtype, infection with the homologous- or subtypes within the same clade and group were uncommon, suggesting heterosubtypic immunity.

In this project, we showed that the PA can discriminate between different HA-types. Strain-discrimination was not possible yet with the PA, when more than one antigen per subtype was included, e.g., H5. This intra-subtype reactivity is not unexpected since a study found an intra-subtype similarity (based on AA-sequences of the HA1) of >92%, whereas inter-subtype identity based on AA-similarity was much lower (38.5%) [40]. Broad intra-subtype reactivity is exploited in diagnostics. Ducatez et al. [41] discovered that ancestral strain A of HP H5N1 as well as strains belonging to clade 2.2 (represented by H5.06 in our study) proved to be the most suitable antigen as they correctly identified most HP H5N1 antigens/-sera of other clades [41]. On the other hand, as genetic changes can lead to escape mutants eliciting

different serological responses, it is important to monitor and regularly update the PA-antigen setup, as is done for other serological assays [27]. The extent to which strain discrimination can be achieved by means of the PA is currently focus of a follow up project.

It is important to stress that the PA does not give information on the presence or absence of neutralizing antibodies and can therefore not be used to determine the immune status, i.e. protection. In serological avian influenza surveillance the HI assay is currently the gold standard with a sensitivity and specificity of 98.8% and 99.5%, respectively [42]. Overall, the PA showed a good correlation with the HI test. Other currently known serological multiplex techniques for the use in poultry, e.g. bead-based Luminex assays, either target conserved regions of influenza virus (nucleoprotein, matrix protein, non-structural protein 1) [43], screen for antibodies against HA-types relevant for the poultry sector (H5 and H7) [44] or combine the two approaches, eg. nucleoprotein with H5 [45]. In addition, simultaneous serological screening for influenza virus in combination with other poultry diseases of economic importance (e.g., Newcastle Disease Virus, Infectious Bronchitis Virus, Infectious Bursal Disease Virus) are described in the literature [46,47]. To our knowledge, the PA technique is the first to allow simultaneous detection of influenza virus antibodies against more than two HA-types in chickens.

In this study, we aimed at including the full range of HA-types known to be present in birds at the time. The dependence on commercial availability lead to the random assembly of antigens of Eurasian as well as North American lineages and failure to cover all AIV subtypes. It is known that strains descending from Eurasian and North American lineages of H5 and H7 differ antigenically, as is reflected in differing titer heights in serological assays [28]. Therefore, to achieve optimal results, the PA should ideally comprise antigens relevant and topical for the region in which the test is to be deployed. A limitation that should be acknowledged is that the PA has only been tested with sera of subtypes H5, H6, H7 and H9. To evaluate the performance against other subtypes, additional serum cohorts would need to be analyzed. Furthermore, the PA is limited to the detection of HA-type specific antibodies and cannot identify antibodies against the neuraminidase. It is not known as to what extent NA-specific antibodies influence reactivities against the HA-proteins (due to steric hindrance) in this testing platform [28].

In conclusion, we present a sensitive and specific test for the simultaneous detection of HA-type specific antibodies against different AIVs in chicken that requires very low amounts of serum. In combination with a screening-ELISA targeting antibodies against a conserved region of AIV, the PA can provide a valuable epidemiological surveillance tool to monitor dispersal of different subtypes. As this testing platform is also validated for the use in humans [20,48] it lends itself for conducting exposure studies at the human-animal interface. Current research centers on the development of the PA for the use in swine.

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CHAPTER 3.2

EXPLORING NOVEL SEROEPIDEMIOLOGICAL TOOLS – EFFECT OF DIFFERENT STORAGE CONDITIONS ON LONGITUDINAL STABILITY OF MICROARRAY SLIDES COMPRISING INFLUENZA A-, MEASLES- AND *STREPTOCOCCUS PNEUMONIAE* ANTIGENS

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ABSTRACT

In this study we evaluated the long-term stability of a microarray-based serological screening platform, containing antigens to influenza A, measles and Streptococcus pneumoniae, as part of a preparedness research programme aiming to develop assays for syndromic disease detection. Spotted microarray slides were kept at four different storage regimes with varying temperature and humidity conditions. We showed that under the standard storage condition in a temperature-controlled (21 °C) and desiccated environment (0% relative humidity), microarray slides remained stable for at least 22 months without loss of antigen quality, whereas the other three conditions (37°C, desiccated; room temperature, non-desiccated; frozen, desiccated) produced acceptable results for some antigens (influenza A, S.pneumoniae), but not for others (measles). We conclude that these arrays for multiplex antibody testing can be prepared and stored for prolonged periods of time, which aids lab-preparedness and facilitates seroepidemiological studies.

HIGHLIGHTS

- Monitoring long-term stability of spotted MA slides is important for quality assurance
- Under temperature-controlled and dry conditions, spotted slides were stable for at least 22 months
- Our findings allow stockpiling of spotted MA slides which facilitates lab preparedness

INTRODUCTION

Laboratory methods for infectious diseases detection and diagnosis either directly target infectious organisms by means of virus detection methods, like polymerase chain reaction (PCR), or indirectly confirm past infections via serological techniques, such as hemagglutination inhibition assay, microneutralization assay, ELISA or Western Blotting. Molecular methods, like PCR, are fast and useful tools, given appropriate types of sample are collected within an appropriate time window. However, for some acute infections, for instance those caused by Dengue- [1] or Chikungunya virus [2], the relatively short period of pathogen excretion limits the applicability of virus detection methods as they are usually rapidly cleared in an immunocompetent host. Serological methods, on the other hand, detect antibody responses against pathogens and can thereby provide information on exposure when the infectious agent is no longer present. Antibody detecting techniques are not only useful to retrospectively confirm infections in individuals when paired sera are available; they also provide important information during outbreak settings. The ability to detect mild or asymptomatic infections allows estimation of attack rates, transmissibility and geographic distribution of a pathogen on a population level, as well as unbiased case fatality rates. In combination with epidemiological and clinical data, these measures are important to guide effective control strategies to contain infectious disease outbreaks [3–5].

For influenza virus infections, for example, patients with influenza-like illness or acute respiratory infection are identified when referred by physicians, and this forms the basis for the global sentinel surveillance systems [6]. Whereas such a symptom-dependent system is useful for virological surveillance, it tends to predominantly capture the most severe cases, as patients with mild- or asymptomatic infection are less likely to seek health care [7]. Hence, during infectious disease outbreaks there is a risk that morbidity and mortality rates can be biased when basing the denominator only on severe cases [7,8].

With the occurrence of the most recent influenza pandemic caused by a novel H1N1 subtype in 2009 [A(H1N1)pdm09], the importance of including serological studies into pandemic preparedness planning and the use of standardized serological assays for improved comparability between studies, became apparent [5]. Serological methods, such as the hemagglutination inhibition- and microneutralization assay were widely used during the different stages of the pandemic. However, despite the availability of an international antibody standard, limited awareness thereof precluded its wide use. Another challenge was that laboratory capacity and storage for conducting extensive and high-volume serological studies was insufficient [5].

We previously reported on the development and use of a standardized serological assay termed protein microarray, which is a platform able to simultaneously screen for antibodies against multiple influenza hemagglutinin types in humans [9–12], chickens [13] and bats [14]. This antibody detection assay was developed as part of an emerging disease preparedness program, and was piloted to monitor the evolution of the A(H1N1)pdm09 in13 different countries [12]. In another study, this technique was used in real-time to assess pre-

existing antibody levels to H7 subtypes during the emergence of a novel zoonotic A(H7N9) avian influenza virus subtype in rural and urban locations of Vietnam [10]. For such large-scale seroepidemiological studies, a high number of spotted microarray slides are required and are ideally stockpiled within the framework of lab preparedness for rapid deployment during outbreak situations. This potential use of the technology is conditional to validation of storage conditions. In the current study, we evaluated the long-term stability of viral proteins and bacterial polysaccharides printed onto microarray slides and investigated the influence of four different storage conditions on antigen quality over a period of 22 months.

MATERIALS AND METHODS

Antigen selection and production of microarray slides

We evaluated the stability of recombinant proteins of the HA1 part of different influenza virus hemagglutinins, whole inactivated measles virus and capsular polysaccharides of *Streptococcus pneumoniae* spotted onto microarray slides (Table 1). Stability of antigens was evaluated for four different temperature- and humidity conditions as further specified below. Recombinant HA1 proteins were produced in HEK293 cells and purified using HIS-tag purification as described by the manufacturers (Table 1). Antigen H1.09 was previously extensively validated in serosurveillance studies in humans during the H1N1 influenza virus pandemic of 2009 [9,11,12]. Antigens H5.05 and H5.07 were validated for antibody screening of chicken serum samples [13]. Antigens representing measles virus and *Streptococcus pneumoniae* (used as a surrogate for *Streptococcus suis*), are currently being validated for the use in diagnostics and were included to evaluate antigen stability in the microarray platform for future purposes.

Optimal concentration per antigen was determined by checkerboard titration using suitable anti-sera (Table 1&2). Proteins (influenza and measles virus antigens) were diluted in working strength protein arraying buffer (Maine Manufacturing, ME, USA) containing proteinase inhibitor cocktail (BioVision, Mountain View, CA, USA). Bacterial polysaccharides were printed in working strength protein arraying buffer only. All antigens (Table 1) were spotted onto nitrocellulose-coated glass slides of the same lot number (16-pad, Oncyte Avid, Grace Biolabs, Bend, OR, USA) using a non-contact spotter (Piezorray, Perkin Elmer, Mass., USA). Two microarray batches were produced on the same day using the same reagents and antigens. Each batch consisted of 25 slides, which constituted the maximum capacity per spot run. Immediately after spotting, slides were transferred to a dark plastic box and were stored in a drying chamber to allow optimal protein linkage to the nitrocellulose. All slides were kept in the drying chamber with an average temperature of 21°C under dark conditions until further use (~3 weeks later). For quality control prior to the study, we tested one slide per batch at the onset of the study and demonstrated that results with slides from different batches were comparable as overall antibody titers did not differ significantly at baseline (Time point 0, Figure 2; Wilcoxon rank sum test, p-value=0.69).

Table 1. Selection of antigens used for spotting of microarray slides. Optimal antigen concentrations were determined by checkerboard titration using antisera presented below the respective antigen. Two anti-serum pools (rabbit and human) were prepared for periodic testing of spotted microarray slides. Serum pools were filled out in twelve aliquots and were stored at -80°C until further use.

	Code	Nature of antigen/ antiserum	Strain/ Serotype	Concentration	Source
Influenza A virus	H1.09	Recombinant protein (HA1[1])	A/California/6/2009 (subtype H1N1)	~0.125 mg/ml	Immune Technology Corp., USA
	Anti-H1.09	Polyclonal rabbit serum	A/California/06/2009		Immune Technology Corp., USA
	H5.05	Recombinant protein (HA1[1])	A/Indonesia/5/2005 (subtype H5N1)	~0.250 mg/ml	GenScript USA Inc., USA
	Anti-H5.05	Polyclonal rabbit serum	A/Indonesia/5/05		Immune Technology Corp., USA
	H5.07	Recombinant protein (HA1[1])	A/duck/Hokkaido/167/2007 (subtype H5N1)	~0.5 mg/ml	Sino Biological Inc., China
	Anti-H5.97	Polyclonal rabbit serum	A/Hong Kong/438/97		Immune Technology Corp., USA
Measles virus	Measles	Inactivated whole virus	Edmonston strain	Baseline concentration unknown; 2x dilution used	In-house vaccine formulation, RIVM, the Netherlands
	Anti-measles	Pool of measles-seropositive humans			RIVM, surplus of anonymized diagnostic samples
Streptococcus pneumoniae	S. pneumoniae	Purified polysaccharides	Serotype 14	0.5 mg/ml	Statens Serum Institute, Denmark
	Anti-S. pneumoniae	Polyclonal rabbit antiserum (Serotype 14)			Statens Serum Institute, Denmark

[1]Head domain of the hemagglutinin receptor

Similarly, batch 1 and 2 did not differ over the entire study period (Wilcoxon rank sum test, p-value= 0.92). Calculation of geometric coefficients of variation (GCV) showed comparable variations in titers for both batches (GCV batch 1: 126%, GCV batch 2: 130%).

Microarray protocol

Microarray slides were essentially tested as described before (Koopmans et al., 2012). Briefly, we first incubated microarray slides with Blotto blocking buffer containing 0.1% Surfact-Amps (both Thermo Fisher Scientific, Rockford, MA, USA), followed by incubation of serum pools and finally used specific conjugates to visualize bound antibodies. All incubation steps lasted an hour. Conjugates used were AlexaFluor647 AffiniPure labeled goat-anti-rabbit IgG, and Alexa647 AffiniPure labeled goat-anti-human IgG (both Fc-fragment specific and polyclonal, Jackson Immuno Research, West Grove, USA), at dilutions 1: 1300 as determined using checkerboard titration. Following the manufacturer's instructions, we updated both conjugates once during the study period. Before replacing the conjugates (same product from same manufacturer), we tested and verified that old- and new conjugates yielded comparable fluorescence signals at the same dilution (data not shown). After slide analysis, fluorescent signals were quantified using a ScanArray Gx Plus microarray scanner (Perkin Elmer) and sigmoidal fluorescence curves were converted into titers as described previously [9].

Antisera used for checkerboard titration were used to prepare specific rabbit- and human serum pools (Table 1). After pooling, we filled out twelve aliquots per serum pool which were subsequently stored at -80°C until further use. From each aliquot we prepared two-fold dilution series in Blotto blocking buffer containing 0.1% Surfact-Amps, starting at a dilution of 1:80 for the rabbit- (anti-influenza A and anti-*S.pneumoniae*), and 1:320 for the human serum pool (anti-measles). For periodic testing, four slides – one per storage condition – were tested simultaneously.

Storage conditions

All slides were stored under dark conditions. The stability of spotted microarray slides was evaluated under the following four storage conditions:

- Temperature-controlled, desiccated (hereafter abbreviated 'TC_D'): Slides were stored in a desiccation chamber placed in a temperature-controlled room (average temperature 21°C).
- Frozen at -20°C, desiccated (abbreviated 'Frozen'): Slides were stored in individual plastic boxes containing silica sachets and were sealed in plastic bags prior to freezing.
- 37°C, desiccated (abbreviated '37C'): Slides were stored in a dark plastic box containing silica sachets and were stored in an incubator set to 37°C.

- Room temperature, non-desiccated (abbreviated RT_ND). As this was the only storage condition for which temperature and humidity were not controlled, we daily recorded temperature in degrees Celsius and relative humidity expressed in percentages daily (Lascar Electronics, Easy Log USB, version 7.2.0.0) over a period of ~16 months (data was logged between October 24th, 2012 and January 16th, 2013, as well as between March 19th, 2013 and February 21st, 2014, respectively).

The above conditions were chosen to evaluate the suitability of the currently employed standard storage condition 'TC_D' and to compare it to potentially alternative long-term storage options ('Frozen'). We furthermore examined the influence of a temperate climate on spotted slides kept at room-temperature without regulation of temperature and humidity (RT_ND), and aimed to study the effect of warm temperature in combination with controlled humidity (37°C) to mimic conditions in countries with warmer climates in which collaborations are ongoing.

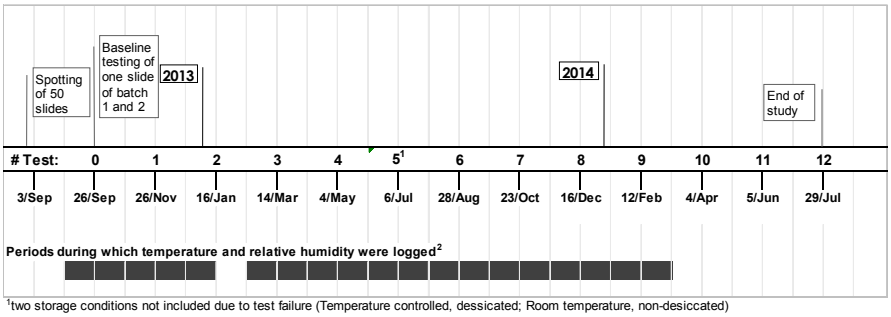
Study set up

As microarray slides were printed in two separate spot runs (=two batches) we checked for equal quality of batches by simultaneously testing one slide of each batch. This initial test served as a baseline for subsequent measurements established during twelve time points spaced at two-month intervals, covering a total study period of 22 months (Figure 1). At each testing time point four slides – one slide per storage condition – were analyzed simultaneously. Spotted microarray slides were randomly allocated to the four storage conditions, in a way that slides from the two batches alternated at every test point.

Statistical analysis

All statistical analyses were performed in R, a language for statistical computing (version 3.1.0, Vienna, Austria). Descriptive analyses were performed using the 'psych' package [15]. Line graphs were created using package 'ggplot2' [16]. To compare the variation between the two microarray slide batches we calculated *geometric* coefficients of variation (GVC, expressed as percentages) on natural log-transformed data, due to the underlying log-normal distribution of antibody titers [17]. We furthermore examined comparability of the two microarray batches by means of a non-parametric Wilcoxon rank sum test. To account for day-to-day variation and determine whether titer measurements remain within an acceptable range over the duration of the study, we calculated mean titers on log2 transformed data (=equivalent to geometric mean titer, GMT) per antigen and storage condition, and calculated how many measurements transgressed the range of plus/ minus one dilution step around the mean, as variation between antibody titer measurements is considered acceptable if it does not exceed a range of plus/ minus one dilution step from a central tendency measure, e.g. GMT or median [18–20].

Figure 1. Experiment timeline. The study period spanned a total of 22 months. Periodic testing of spotted microarray slides was performed at a 2-month interval. Per testing time point, four slides – one slide per storage condition – were tested simultaneously. Temperature and relative humidity were logged (indicated by grey blocks) during part of the study for condition 'Room temperature, non-desiccated'.



[1]two storage conditions not included due to test failure (Temperature controlled, dessicated; Room temperature, non-desiccated)

[2] for storage condition 'Room temperature, non-desiccated'

To quantify the effect of long term storage under different storage conditions on titers per respective antigen over time, we used a linear mixed effects model using package 'lme4' [21]. We chose this approach, as this type of analysis is frequently applied in longitudinal studies, and accounts for dependence (repeated measures of the same entity) and potentially unbalanced nature of the data (different number of measurements per storage condition, Figure 1) [22–24]. The package 'multcomp' was used to retrieve 95% confidence intervals (95% CI) for the estimates [25]. To meet the assumption of a normal distribution of residuals, titers were similarly log2-transformed for this analysis. We entered the predictors 'storage condition' and 'testing time point' as fixed effects combined in an interaction term into the linear mixed effects model to allow for different slopes per storage condition. To account for an observed – possibly seasonal – trend (Figure 2), we added trigonometric (sine & cosine) functions. To account for the data possibly being clustered by batch and by antigen, we included random intercepts for 'batch' and 'antigen', and moreover, random effects for the interaction of time and storing condition per antigen. This allows per antigen, that the slopes of time differ for each storing condition. We checked model assumptions by inspecting residual plots, which did not reveal overt deviations from homoscedasticity and normality. P-values were obtained by means of the likelihood ratio test. A p-value of less than 0.05 was considered statistically significant.

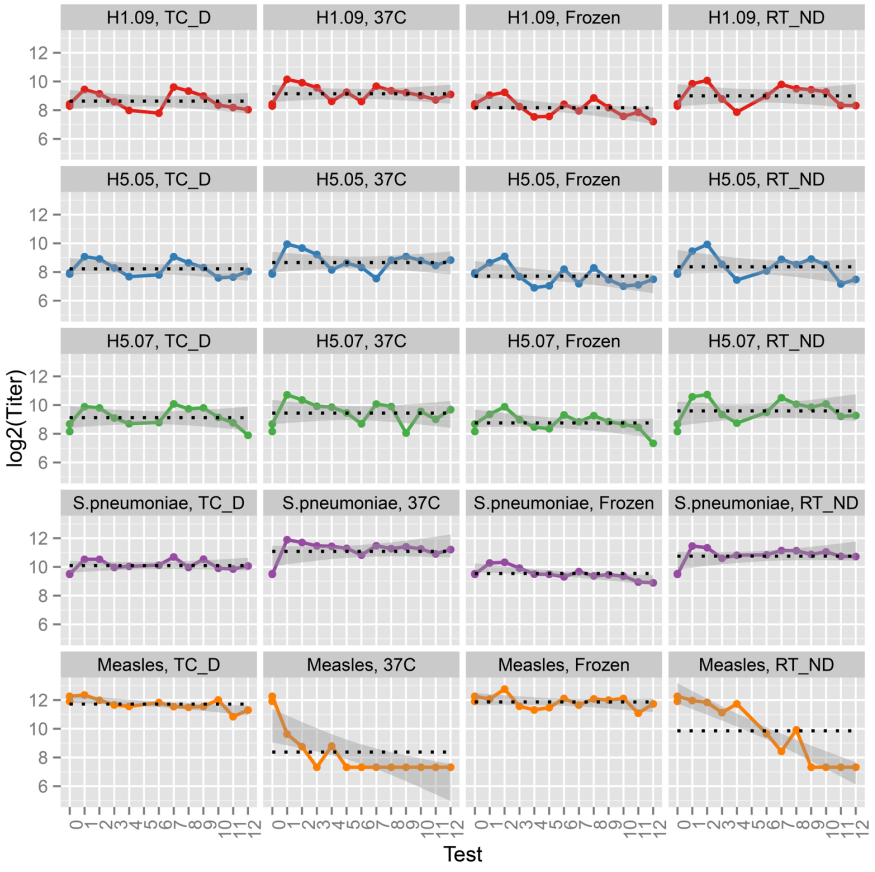
RESULTS

3.1 Effect of storage conditions on different antigen classes and longitudinal trends

Antibody titer measurements per storage condition and antigen for the entire study period are presented in Figure 2. Spotted microarray slides stored at the current standard storage

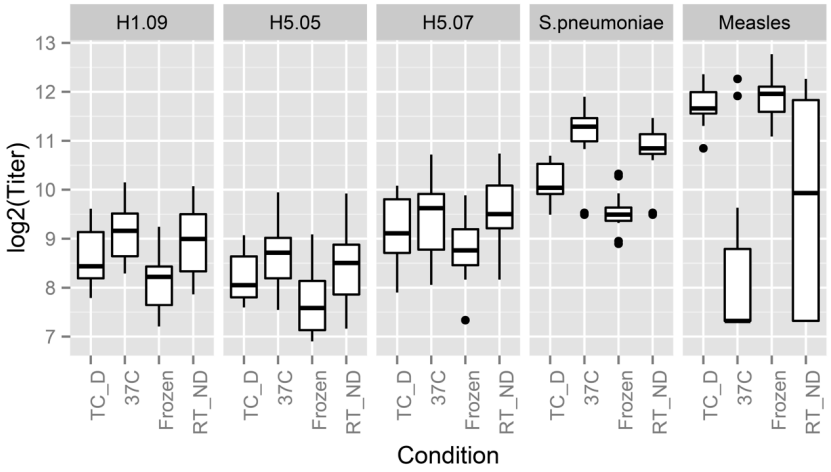
condition 'TC_D' proved the most stable for all antigen classes and showed the least variability over time, compared to other conditions (Figure 2&3). Freezing spotted microarray slides at -20°C yielded the second best results (Figure 2&3).

Figure 2. Stability of viral (influenza A- and measles virus)- and bacterial antigens (S. pneumoniae) printed onto microarray slides stored at four different temperature- and humidity conditions. Antigen stability was assessed by periodic testing using specific serum pool aliquots at two-month-intervals (x-axis) covering a total study period of 22 months. Log2-titers are shown on the y-axis. Horizontal panels represent different storage conditions per antigen. Colored lines show titer measurements per antigen and storage condition. Horizontal, dotted black lines correspond to respective geometric mean titers. Measurements were acceptable if they remained within a range of plus/minus one dilution step from the mean log2-titer. Grey ribbons show a 95% confidence interval around an invisible linear regression line to indicate trends in antigen stability over time. Baseline measurements (Test 0) are combined under one tick.



TC_D: temperature-controlled and desiccated; 37C: 37°C and desiccated; Frozen: frozen and desiccated; RT_ND: room temperature and non-desiccated

Figure 3. Boxplots showing distributions of log2-titers (y-axis) against different storage conditions (x-axis) presented per antigen (panel) over the entire study period. Titers to S. pneumoniae antigens showed the least variation for all storage conditions (log2-titer ranges: 1.2-2.41). Within the three influenza A antigens (H1.09, H5.05, H5.07) we found comparable titer distributions across the four storage conditions, with the lowest variation observed for condition 'TC_D' (ranges: 1.47-2.18) and the highest for 'RT_ND' (log2-titer ranges for H1.09: 2.2; H5.05: 2.75) and 37°C (H5.07: 2.66), respectively. With exception of conditions '37°C' and 'RT_ND' which had a detrimental effect on antigen stability, variation in log2-titers against the measles antigen was low.



TC_D: temperature-controlled and desiccated; 37C: 37°C and desiccated; Frozen: frozen and desiccated; RT_ND: room temperature and non-desiccated

In contrast, slides stored under conditions 'RT_ND' and '37°C' resulted in higher variation in titers for different antigens (Figure 2&3). While results were reasonable for influenza and streptococcus antigens, the measles antigen was no longer detectable after test time point 5 for 'RT_ND', and test time point 9 for '37°C', respectively. A further observation was that storage conditions affected the titer estimates, again in an antigen dependent manner: microarray slides stored under condition '37°C' generally showed higher mean/ median log2-titers to influenza A- and *S.pneumoniae* antigens, compared to other conditions (Figure 2&3). Conversely, mean/ median titers against these antigens were lowest for frozen slides, whereas this was not observed for the measles antigen (Figure 2&3).

To quantify the titer trends across different storage conditions, we fitted a linear mixed effects model on log2-titers in which we accounted for the observed seasonal fluctuations (Figure 2) and used predictors as described in the methods section. To rule out more subtle

changes between batches over time we included 'batch' in the model which confirmed results of our initial comparison that no variation between the two microarray batches was observed over time (data not shown). Given the different nature of the antigens, as expected, the model output showed some variability between the different antigen classes (variance: 1.65). Overall, we found a slight decreasing trend on antigen stability for all storage conditions with estimates ranging from -0.012 to -0.065. The smallest signal decrease over time for all antigens combined was observed for condition 'TC_D' (estimate: -0.012, 95%CI [-0.049, 0.024]). Back-transformed to titers, the estimate can be interpreted as follows: every two months (interval between testing time points) for condition 'TC_D', the overall titer for all antigens combined decreased by 0.86%. This further corroborates our observation that spotted microarray slides stored at condition 'TC_D' remain the most stable, irrespective of antigen type. Titer decreases for other conditions were slightly higher but still relatively low, with overall decreases across different antigens of 2.73% for 'RT_ND' [estimate: -0.040; 95% CI (-0.253, 0.173)], 3.18% for '37C' [estimate: -0.047; 95% CI; (-0.338, 0.245)] and 4.37% for 'Frozen' [estimate: -0.065; 95% CI (-0.134, 0.005)], respectively. When comparing a model including 'storage condition' versus a model without this predictor, 'storage condition' did significantly improve the model (Likelihood Ratio χ^2-test: 10.4, p-value=0.0158), confirming that storage conditions have a significant influence on antigen stability. Titer changes over time for individual antigens, expressed as percentage increase or decrease, differed markedly between the different storage conditions (Table 3).

Table 3. Percent titer change (increase or decrease) per antigen and storage condition for one unit change in testing time points, i.e. between two testing time points (2 months apart).

	Storage conditions			
Antigens	TC_D	37C	Frozen	RT_ND
H1.09	-1.30%	3.66%	-6.37%	1.74%
H5.05	-1.70%	2.22%	-7.27%	0.16%
H5.07	-0.91%	3.00%	-5.27%	1.81%
S. pneumoniae	0.02%	9.19%	-3.40%	7.37%
Measles	-0.41%	-28.62%	0.64%	-21.81%

TC_D, temperature-controlled and desiccated; 37C, 37 Celsius and desiccated; Frozen, frozen and desiccated; RT_ND, room-temperature and non-desiccated

3.2 Titer fluctuations due to seasonal and day-to-day variation

Temperature and relative humidity measured at condition 'RT_ND' showed fluctuations concordant with changing seasons (data not shown), but did not seem to have a large influence on antigen stability, as relative to each other, titers against different antigens were largely clustered across the different storage conditions and showed a comparable trend over the entire study period (Figure 2). When applying the conservative quality score of less than one log2-dilution step deviation from the mean for acceptance of the results, likewise, storage condition 'TC_D' yielded the best result for all antigens. For this condition, only one measurement of one antigen, influenza H5.07, was rejected (time point 12; Table 3). For

condition 'Frozen', all measurements against *S. pneumoniae*- and measles virus antigens remained within the acceptable titer range throughout the study period, but some (n=4) of the influenza antibody titer estimates fluctuated more than the accepted norm. For storage conditions '37C 'and 'RT_ND', the number of measurements deviating one dilution step from the mean was higher for all antigen classes (Table 3). For these two conditions, means for the measles antigen and deviations of one dilution step from it were calculated using only titer measurements of testing time points 0-4 and 0-8, respectively. Time points thereafter were excluded due to antigen degradation (37°C: test 5-12, RT_D: test 9-12, respectively).

Table 3. Number of data points not passing the quality score of deviating less than one dilution step above (>1) or below (<1) the geometric mean titer per storage condition and antigen over time.

Storage condition	Antigen	<1	>1	Total n
TC_D	H1.09	0	0	1
	H5.05	0	0	
	H5.07	1	0	
	S.pneumoniae	0	0	
	Measles	0	0	
RT_ND	H1.09	1	1	13
	H5.05	1	2	
	H5.07	1	1	
	S.pneumoniae	2	0	
	Measles	3	1	
Frozen	H1.09	0	1	4
	H5.05	0	1	
	H5.07	1	1	
	S.pneumoniae	0	0	
	Measles	0	0	
37C	H1.09	0	1	13
	H5.05	1	2	
	H5.07	2	1	
	S.pneumoniae	2	0	
	Measles	2	2	

Discussion

In this study, we assessed the stability of spotted microarray slides containing different antigen classes under four storage conditions with varying temperature- and humidity settings over a total period of 22 months. A broad range of antigens, varying from crude lysates from infected cells or microbial lysates to highly purified microbial components (e.g. expressed proteins and bacterial polysaccharides) are used for serological assays, e.g. ELISA, hemagglutination inhibition assay, Western Blot, as well as multiplexed techniques (protein microarrays and bead-based platforms) [26,27]. Antigen stability can be influenced by the composition of the antigen, and by various chemical and physical factors, such as temperature, pH, oxidation, salt concentration, freeze-thaw cycles, proteases, mechanical destruction as well as contact with solvents or contamination with bacteria.

Proteins linked to nitrocellulose slides were previously reported to remain stable for at least 3 years, when kept at -20°C under dry conditions [28]. The manufacturer of the microarray slides used in this study recommends storage of slides at room temperature before and after spotting, but storing slides at -20°C has also been reported without quality loss. However, the use of a desiccant during the storage period was not recommended as it supposedly could negatively influence results [29]. This recommendation is in contrast with our findings, as slides kept in a temperature-controlled and desiccated environment showed less variation than slides stored at room temperature and uncontrolled humidity. Our data also shows that it is important to validate these findings for different antigens types. In this study, the most detrimental effects on antigen quality were observed for the measles antigen, which consisted of crude cell lysate, whereas the recombinant proteins used gave more consistent results throughout the study period. The instability of the measles antigen under the conditions '37C' and 'RT_ND' show that this particular antigen would be less suitable for studies in warmer climates, unless stored appropriately. These findings highlight that it is paramount to evaluate the stability and performance of a diagnostic assay under extreme temperature and humidity conditions, if the test is to be deployed in such settings; for instance in tropical countries [30].

We took care to keep variation due to external factors, e.g. operator-induced, different lots of reagents etc., to a minimum. To avoid batch effects, ideally the same batches of assays are used throughout the entire study period. However, particularly in large-scale studies, that is not always feasible and batch effects can have a significant impact on the outcome [31]14/12/15 21:24 and therefore we compared results for different batches priori to their use. We performed this work as part of a project for improved avian influenza surveillance. There is a strong push for enhanced standardization of serological testing, after comparing reproducibility of serological assays for diagnosis of the pandemic A(H1N1)pdm09 which – without using a standard – revealed high inter-laboratory variation; median GCVs ranged from 95-345% for hemagglutination inhibition-, and 204-383% for virus neutralization assay, i.e. a 80- and 109-fold difference, respectively [32].

In conclusion, this study showed that spotted microarray slides containing influenza, measles and streptococcus antigens can be stored up to 22 months without quality loss when kept in a dark, desiccated and temperature-controlled environment. We also find good results at conditions compatible with situation in developing countries, although this needs to be assessed for each antigen separately. Our findings are assuring for large-scale studies, as this property allows production and stockpiling of multiple batches within a short period of time using the same reagents, thereby keeping variation between batches to a minimum.

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Conflict of interest

none

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CHAPTER 4

WEIGHING SEROLOGICAL EVIDENCE OF HUMAN EXPOSURE TO ANIMAL INFLUENZA VIRUSES - A LITERATURE REVIEW

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Abstract

In this literature review, we assess studies describing *serological* evidence of human infection with animal influenza viruses and grade them based on a newly developed scoring system in order to weigh their evidence for such infections. Comparing serological data is challenging due to a lack of standardization in the collection of epidemiological data and the laboratory methods used in published zoonotic influenza studies. Many studies report reliable evidence of antibodies to swine influenza viruses A(H1N1), A(H1N2), and A(H3N2) among persons occupationally exposed to pigs compared to those not exposed. Most studies on human exposure to avian influenza target the H5, H7 and H9 serotypes and, concordant with that, most serological evidence is reported for these subtypes. Interestingly, the studies receiving a low grade in this review were generally those reporting higher seroprevalences to avian influenza viruses in humans. Currently, most surveillance efforts are targeted towards avian influenza subtypes H5 and H7. However, efforts should be taken to increase surveillance for swine influenza and avian influenza subtypes other than H5 and H7. Moreover, very little information is available on zoonotic influenza in humans in understudied areas Africa and Southern America. Efforts should also be directed towards increasing influenza surveillance and research efforts in those areas.

INTRODUCTION

The family *Orthomyxoviridae* contains three distinct genera of influenza: A, B and C. The viruses have a segmented, negative-strand RNA genome which requires an RNA-dependent RNA polymerase of viral origin for replication. The error-prone nature of the polymerase causes mutations which contribute to the diversity and evolution of influenza viruses. When mutations change antigenic properties of the surface proteins, facilitating immune evasion, this is called 'antigenic drift.' The segmented RNA genome allows reassortment: the mixing of the RNA segments from diverse influenza viruses, resulting in virus particles with genetic material from diverse influenza subtypes. Reassortment has not been described at the genus level [1].

Influenza A and B viruses are known to cause high human morbidity and mortality during the yearly seasonal epidemics. In contrast to influenza B viruses, influenza A viruses circulate in many animal species and are able to cross *the species barrier*. Moreover, they are the only type of influenza virus to have caused pandemics, because of a mechanism called 'antigenic shift', a unique type of reassortment that results from the replacement of HA (and less frequently NA) subtypes with novel ones[2]. Viruses resulting from 'antigenic shift' have caused four influenza pandemics in the last 100 years: the 'Spanish flu' A(H1N1) in 1918, the 'Asian flu' A(H2N2) in 1957, the 'Hong Kong flu' A(H3N2) in 1968, and most recently the A(H1N1)pdm09 pandemic in 2009.

Animal influenza viruses are of concern because of the small but real risk of their adaptation to humans, possibly leading to efficient human-to-human transmission and sustainable circulation in the human population. It has been suggested that rising global trade and travel and changes in human demographics, consumption patterns and behaviors have caused an increase of emerging infectious diseases in general [3-5] and zoonotic influenza in particular [6]. Well-known examples of animal influenza viruses that have recently infected humans include A(H5N1), A(H7N9), A(H6N1), and A(H10N8) [7].

When an influenza virus crosses the species barrier, the risk ranges from mild to severe disease and death in individuals to the emergence of a pandemic. To improve human influenza surveillance and preparedness, it is important to be able to assess influenza A virus strains circulating in the animal population as to their potential to cross the species barrier and cause human infections. The first step is to collect and review existing scientific studies that assess the prevalence of zoonotic influenza in human populations. Recently, a comprehensive literature review listed all published virological evidence for human infection with swine and avian influenza viruses other than A(H5N1)[8].

While surveillance based on virologically confirmed human influenza cases has a high positive predictive value, the approach has some downsides. Virus shedding in infected persons typically lasts only a week [9]and has often diminished or ended by the time of sampling. In addition, infections may cause only mild illness, leading to underreporting of cases. Therefore, studies investigating serological evidence of infection thus have a wider

window of detection and have been used to study exposure in human-animal interface settings. A pitfall is that serological data need to be interpreted with caution due to cross-reactivity of antibodies among and within virus subtypes [10, 11] and the problems of sensitivity and reliability with standard serological tests when used to detect antibodies against novel influenza subtypes [12, 13].

Another difficulty is that seroepidemiological studies of zoonotic influenza are difficult to compare due to a lack of standardisation in design and laboratory methods used [14, 15]. The World Health Organization (WHO) published two guidelines with recommendations for the detection of human antibodies against animal influenza. One targets the detection of avian influenza virus A(H5N1) or antibodies against this subtype in humans (2007), and the other advises on detection of animal influenza viruses in general in animals and humans (2002). Despite these guidelines, methods used in serological screening continue to vary among studies. Moreover, there are no specific tools for quality assessment of influenza prevalence studies available [16].

In this review, we assess studies describing *serological* evidence of human infection with animal influenza viruses and grade the studies based on a newly developed scoring system in order to weigh their evidence. This review can serve as input for an evidence-based risk assessment framework to evaluate novel influenza viruses or variants in light of their pandemic potential.

MATERIAL AND METHODS

Search strategy

We performed a comprehensive literature search for serological studies dealing with zoonotic influenza, using the same search strategy as described in Freidl et al., 2014, but expanding the search period from February 2012 to February 2014 [7]. We conducted a more cursory search to include studies published between February and December 2014.

Inclusion and exclusion criteria

Two investigators first screened all papers by title and, when necessary, by abstract. They selected papers containing serological evidence from observational studies describing human infection with animal influenza viruses . Studies of influenza A(H5N1) were excluded, as serological evidence of H5N1 in humans has been extensively studied previously [18, 19].

The selected studies excluded those describing influenza antibody findings only in animals and those reporting only human-to-human transmission of animal influenza viruses. We also disregarded reviews, commentaries, and articles describing data that were described in previous publications.

Scoring the quality of the evidence

To be able to assess the value of the outcomes of the selected studies, we developed a scoring system (table 1) , taking a number of variables into account, i.e. study design, laboratory methods used, as well as availability of essential background information such as age group and vaccination status (see rationale below). The maximum score was 18. A detailed breakdown of the scoring system is shown in table 1. Based on their overall score, we assigned all studies into four arbitrary categories (A, B, C, D), ranging from best to worst. Category A spanned studies with scores ranging from 15-18 points, category B from 10-14 points, category C from 5-9 points and category D from 0-4 points.

Rationale of the scoring system

In our scoring system (table 2), studies including a **control group** matched for age (less than 10 years difference in average age), gender (less than 10% difference in the percentage of women and men) and region (same country) received a higher score (Table 1: 6 out of 18), as age, gender and location are possible confounding factors for influenza serology [17-20]. The inclusion of an age-stratified control group is also recommended by the Consortium for the Standardisation of Influenza Seroepidemiology (CONCISE) [21]. A control group is of particular interest for zoonotic influenza serology, because influenza infections occur repeatedly over a human lifetime, boosting pre-existing antibodies against human influenza viruses which might cross-react with animal influenza virus subtypes [22-26] [27]. Comparison of an animal-exposed study population with a well-defined non-animal-exposed control group is important in avoiding over-estimation of the importance of the serological findings. We also assigned a higher score to studies that did not include an age-matched control group but did report the age of study subjects and corrected their results for age differences, or to studies that stratified their findings in separate age groups.

An **antibody titer rise** between two samples from the same individual was considered a more reliable measure of infection than obtained by a single serum sample, as individuals served as their own control.

A higher score was assigned to studies which addressed the possibility that antibodies may result from **cross-reactivity** among influenza subtypes. These studies included vaccination rates and/or tested for human influenza types, both variables known to have an effect on the generation of cross–reactive antibodies [17, 28]. In order to score all studies in an objective manner, we did not evaluate their analysis of cross-reactivity but assigned scores based only on their inclusion of vaccination rate and/or testing for human influenza types.

A higher score was also assigned to studies that added non-serological evidence of exposure of humans, particularly when they provided virological evidence for infection with animal influenza in their human subjects or in the animal population to which the subjects had been exposed.

Table 1. Scoring system for evaluation of published papers describing seroprevalence studies of zoonotic influenza virus infections.

Individual scores / Attribute	Maximum score	0	1	2	3
Control group	6	No	Unmatched	Age-matched (2) Gender-matched (2) Region-matched (2)	
Repeated sampling[a]	2	No	/	Yes	/
Correction for age or reporting in age groups [a]	1	No	Yes	/	/
Vaccination status reported	1	No	Yes	/	/
Seasonal flu included in testing	1	No	Yes	/	/
Other evidence	3	No	Serological evidence in animals to which humans were exposed	Virological evidence[b] in animals to which humans were exposed	Virological evidence[b] in human study subjects
Diagnostic method	5 (see below)	/	/	/	/
Total	18				

[a]Score applied only if there was no age-matched control group; [b]virus detection by culture or (real-time) reverse transcription polymerase chain reaction (rtRT-PCR) and sequencing is listed as virological evidence.

The rationale for our scoring of the **diagnostic methods** used in the studies we reviewed is based on the official WHO case definitions for human infections with influenza A(H5N1) virus[29]. A confirmed case, according to WHO, has "a fourfold or greater rise in neutralisation antibody titer" or "a microneutralisation antibody titer of 1:80 or greater and a positive result using a different serological assay, for example, a horse red-blood cell haemagglutination inhibition titer (of 1:160 or greater) or an H5-specific Western blot-positive result".

Data analysis

All statistical analyses were performed in STATA (StataSE 13.0). For all analyses, a p-value of less than 0.05 was considered statistically significant.

RESULTS

Search output

The final output of the literature search was 95 articles (Table 3). Included were 12 prospective cohort studies, 13 cross-sectional studies in general population or rural populations, 57 cross-sectional studies in populations with routine exposure to animals, nine cross- sectional studies in hospital populations, and 11 animal influenza outbreak investigations. We found one paper investigating human serological evidence for canine influenza, four for equine influenza, 39 for swine influenza, and 57 for avian influenza A. The majority of the studies investigated serological evidence for antibodies to avian subtypes with HA-type H9 (45 articles), H7 (39 articles), H5 (excluding H5N1, 27 articles), and swine subtype H1N1 (36 articles) (Table 3).

Table 2. Scoring criteria for the diagnostic method used in published literature on zoonotic influenza viruses.

Confirmation \ Screening	NT[a]	HI[b]	ELISA	None[d]
NT		5	5	3
HI	5		4	2
ELISA	5	4		2
Western blot	5	4	4	0
NI[c]	3	3	3	0
None	3	2	2	

a. Neutralisation Test (NT): microneutralisation (MN) assay or virus neutralisation (VN) assay; b. Hemagglutination Inhibition assay; c. Neuraminidase Inhibition assay; d. no description of method provided.

Study populations were from Asia (n = 38), North America (n = 28), Europe (n = 19), and the Middle East (n = 8, of which 6 were from Iran). For Africa and South America, the search yielded only one publication. In North America, most studies focussed on human infections with swine influenza, whereas in other parts of the world, such as Asia, more emphasis was placed on avian influenza viruses (Figure 1).

Scoring the studies

An overview of the scoring of all studies investigating serological evidence of swine and avian influenza viruses in humans is presented in Figures 2 and 3, respectively. Assuming an arbitrary quality threshold at 9 points (half the maximum score), only 24% of the studies (n=24) were graded A or B, of which only one met the requirements for grade A. In total, 56% (n=53) and 20% (n=19) of the studies fell into category C and/or D, respectively. All but four grade A and B studies had a control group that was matched for at least two out of the three desired characteristics (age, gender and region). Such controls were missing or insufficiently matched for the grade D studies. Grade A and B studies significantly more often included a serological confirmation test compared to C and D studies ($p<0.05$). Of the 23 studies graded A or B, 9 investigated serological evidence for swine influenza (swH1N1, swH1N2, swH2N3, and swH3N2), 14 pertained to avian influenza viruses (H4-H16), and one study investigated human antibodies against a canine influenza virus (H3N8).

Prevalence and incidence of human antibodies against animal influenza A viruses

The reported seroprevalence rates of both the exposed group and the control group of the cross- sectional studies investigating swine influenza and avian influenza are plotted by animal source, virus subtype, and grade of the study in figures 4 and 5. For cohort studies, the seroprevalence at enrollment was included.

Swine influenza

Cross-sectional studies (figure 4)

The vast majority of the swine influenza sero-epidemiology studies detected antibodies in a proportion of the population under investigation (94% of studies looking for swH1N1 antibodies, 100% for swH1N2, and 90% for swH3N2). When single serum samples were analyzed, cut-off values of serological assays (HI-assays and neutralisation assays) ranged from 1:10 to 1:100. For paired sera, a four-fold titer rise was considered proof of infection, but in cohort studies any titer increase during the study period was reported. The reported seroprevalences differed greatly among studies. In populations occupationally exposed to swine, the prevalence of antibodies to swH1N1 ranged from 0% [30] to almost 80% [31]; to swH1N2, from 4% [32] to 67% [31], and to swH3N2, from 9% [32] to almost 80% [33]. Looking only at the high quality studies (grades A or B), the reported prevalences are similar to those for C and D studies, with the exception of swH3N2, for which the highest reported seroprevalence was 28% [34]. In the unexposed control groups the antibody prevalence to swH1N1 ranged from 0 [35] to 18.7 [36]. One study, published in 1968, found a seroprevalence to swH1N1 of 67.4% in the general population, but this could be explained by cross-reacting antibodies against the 1918 pandemic influenza virus in the older population. [37].

Table 3. Output of literature search.(aStudies in which a significant difference was explicitly mentioned or for which it could be calculated based on the data provided; bother than H5N1)

Influenza virus	Influenza subtype	#studies included	#studies detecting antibodies in study group (% of included studies)	#studies detecting significant difference with control group[a] (%studies with control group)
Canine Influenza Virus	H3N8	1	1 (100%)	0 (0/1)
Equine Influenza virus	H3N8	4	4(100%)	0 (0/2)
	H7N7	2	0(0%)	0 (0/0)
Swine Influenza virus	H1N1	35	32 (91%)	12 (12/20: 60%)
	H1N2	6	6 (100%)	5 (5/6: 83%)
	H2N3	1	1 (100%)	0 (0/1)
	H3N2	11	9 (81%)	3 (3/7: 43%)
Avian Influenza virus	H1	4	1 (25%)	0 (0/1)
	H2	5	2 (40%)	0 (0/1)
	H3	5	3 (60%)	0 (0/1)
	H4	22	6 (27%)	1 (1/14: 7%)
	H5[b]	27	12 (44%)	4 (4/16: 25%)
	H6	21	12 (57%)	2 (2/13: 15%)
	H7	39	16 (41%)	5 (5/23: 22%)
	H8	16	2 (13%)	0 (0/11)
	H9	45	37 (82%)	12 (12/30: 40%)
	H10	19	6 (35%)	1 (1/12: 9%)
	H11	19	9 (47%)	0 (0/11)
	H12	14	5 (36%)	0 (0/7)
	H13	4	1 (25%)	0 (0/1)
	H14	1	0 (0%)	0 (0/1)
	H15	1	0 (0%)	0 (0/1)
	H16	1	0 (0%)	0 (0/1)

Figure 1. Avian and swine influenza serological studies in humans in the period 1946-2014.

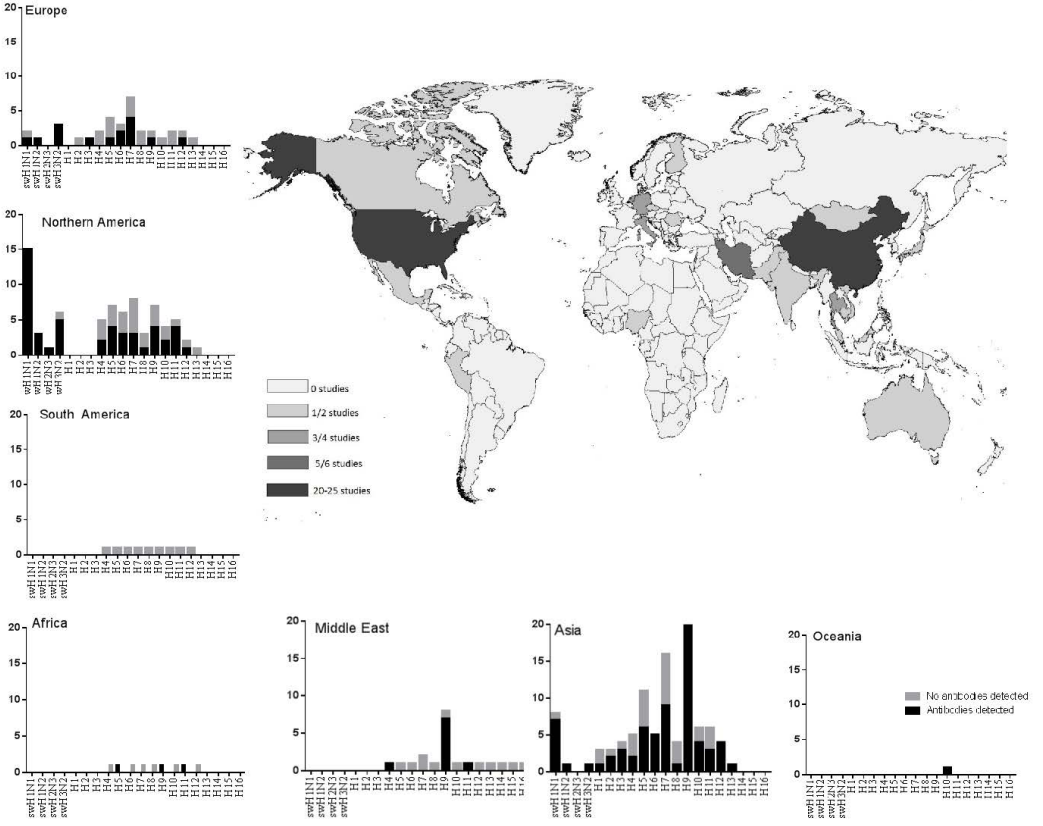
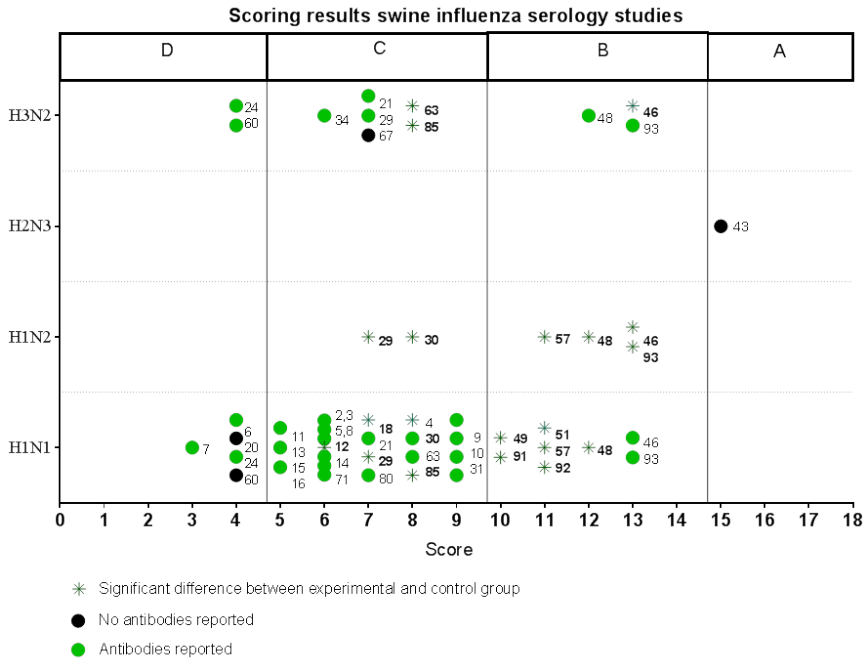


Figure 2. Scoring results of the included swine influenza serological studies in humans. The numbering refers to supplementary table 4.



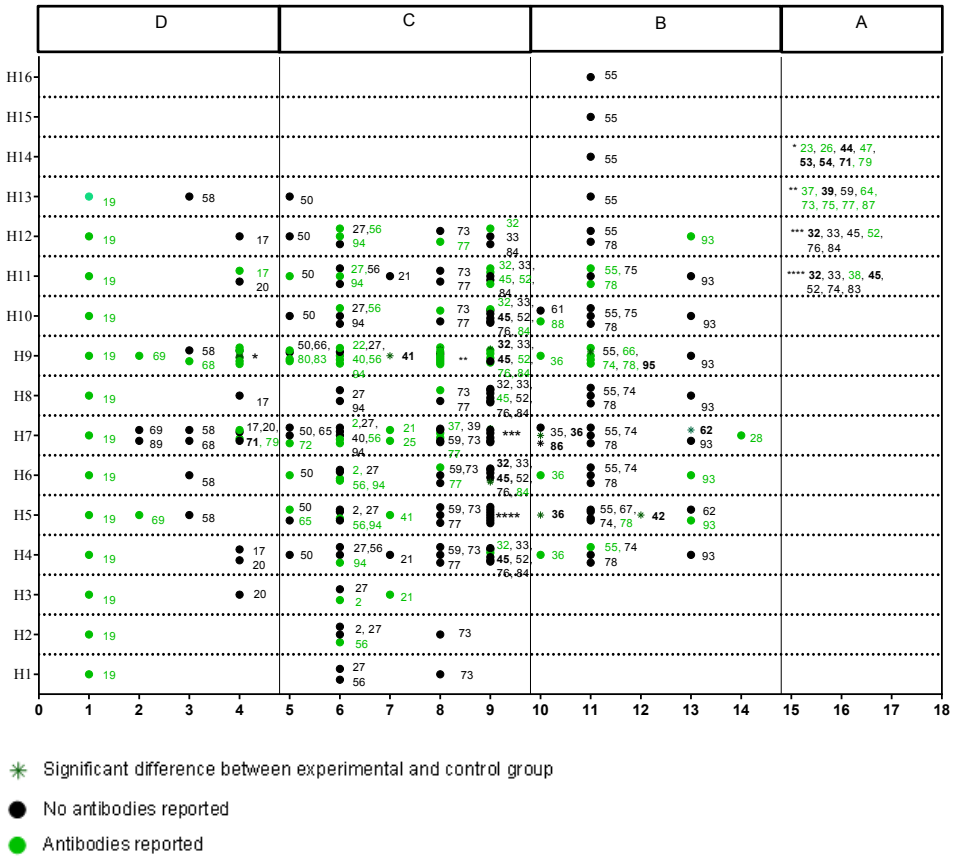
For swH1N2 and swH3N2 the prevalence ranged between 1.0 [32]and 11.4[34] and between 0[32] and 85.0[33], respectively.

In studies that investigated the difference between an unexposed control group and the study population groups (i.e. subjects exposed to animals or subjects from a hospital population), a significantly higher number of seropositive people was found by 60% (12/18) of the swH1N1 studies, 83% (5/6) of the H1N2 studies, and 43% (3/7) of the H3N2 studies.

Cohort studies

Woods et al. [38] and Terebuh et al. [34] found titer increases in antibodies against swH1N1 of 0% to 8.5% per year in serum of farm workers and abattoir workers exposed to swine. Gray et al. [39] found that 25% of rural residents showed a four-fold increase in antibodies to swH1N1 over a time- period of two years. Slightly lower rates were found for swH1N2 [34, 39], for which 5% and 8% had evidence of exposure over two years [34, 39]. In the period 2008-2011, both Coman et al. and Gray et al. found a high percentage of seroconversions for swineH1N1 and H1N2, which were most likely due to cross-reactions with pandemic H1N1[40, 41]. Both Coman et al. and Terebuh et al. investigated serological evidence of swH3N2 exposure during a time-period of two years, concluding that the number of titer increases for different types of swH3N2 in the swine-exposed group were not significantly higher than in the control group [34, 41].

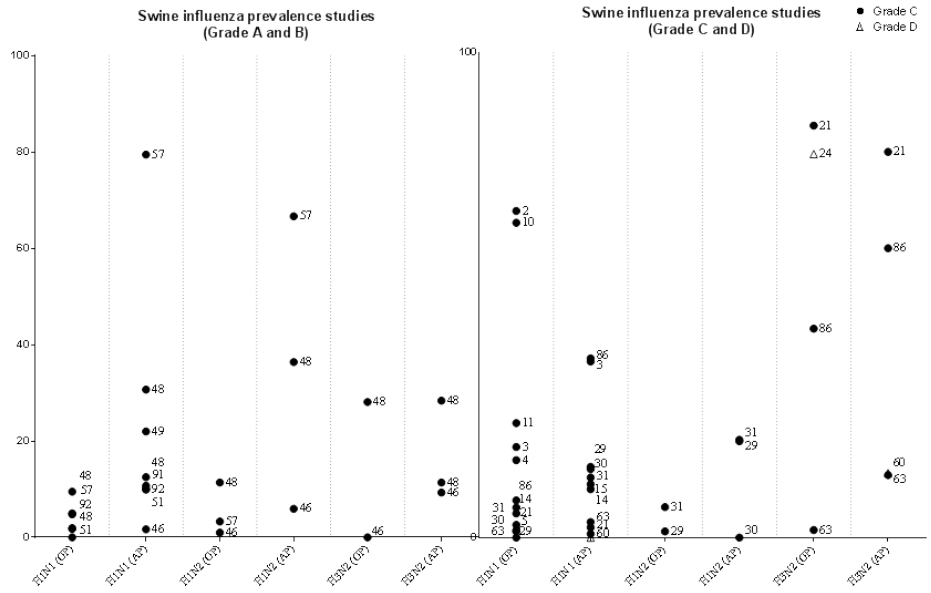
Figure 3 Scoring results of the included avian influenza serological studies in humans. The numbering refers to references listed in supplementary table 4.



Outbreak studies

The five outbreak studies included in this review targeted people who were exposed to swine infected with H1N1 (n=4) or H2N3 (n=1). Those that investigated people exposed to H1N1 [42-45] reported seroprevalences ranging from 15% [44] to 40% [45] (using various cut-offs), and three out of four reported a significant difference between the exposed individuals and a control group. All four H1N1 studies were graded C, except the study by DaWood et al., which received grade B. It reported that 40% of pig-exposed students had a MN titer ≥ 80 and HI titer ≥ 20 [45]. The one study of H2N3, graded A, investigated workers that were exposed to H2N3- infected swine and found, remarkably, a higher seroprevalence in the unexposed control group [19].

Figure 4. Seroprevalence of antibodies to different swine influenza viruses (x-axis) in exposed and control humans. The numbering of references, diagnostic method, and cut-offs can be found in supplementary table 4. (GP: general population or control population; AP: animal-exposed population)



Avian influenza

Cross-sectional studies (figure 5)

Most studies screening for antibodies against avian influenza viruses (>60%) failed to detect antibodies in any proportion of the population under investigation, except for subtype H9 to which 82% of the studies detected antibodies. The cut-off values used in the studies ranged from 1:2 to 1:160 for HI and 1:10-1:80 for neutralisation tests. In the cross-sectional studies over all, antibodies were detected against avian influenza A virus hemagglutinin subtypes H1 through H13. However, in studies graded A or B, fewer subtypes were detected: H5, H6, H7, H9, H10, H11 and H12.

When reviewing only studies that compared prevalence of antibodies in risk groups (subjects in contact with animals or from a hospital population) with those from a control group, we found significant differences in seroprevalence between both groups for avian influenza subtypes H4 (only 1 study of 14), H5 (4 studies of 16), H6 (2 of 13), H7 (5 of 23), H9 (9 of 30), and H10 (1 of 12). Insufficient standardisation or description of methods and cut-offs did not allow a direct comparison of the data. *Cross-sectional studies: grade A and B studies*

Most grade A and grade B studies reported serological evidence of H5, H7 and H9 exposure, but with considerable variation. Gray et al. found a significant difference in the

seroprevalence of H5N2 antibodies in a swine- and poultry-exposed rural population from Iowa, U.S.A., versus unexposed controls from the same region (8.8 vs. 0%). Okoye et al. found H5N2 antibodies in swine-exposed and -unexposed groups from Nigeria [46, 47]. However, two studies executed in Romania and Vietnam found no antibodies in either group [18, 48]. Gray et al. also found neutralizing antibodies to H6N2 and H7N2 influenza virus in the same study, but the prevalences were not significantly different between the exposed group and non-exposed controls. Five other studies failed to find serological evidence of H6 or H7 exposure [18, 41, 46, 47, 49]. Only one-cross sectional study looked at H7N9 exposure, finding a seroprevalence of 6.3% in poultry workers in Guangdong, China, and a significantly lower percentage in non-exposed controls [50]. Antibodies to H9N2 avian influenza virus were found in four of seven studies, with seroprevalences ranging from 1.3 to 12.3% [48, 51]. Only Wang et al. found a significantly higher prevalence in exposed versus control persons in Shanghai, China.

Uyeki et al. included other H9 antigens and found a low level of antibodies to H9N3 and H9N7 virus in Vietnamese poultry workers, although the prevalences of 0.5% and 2.5%, respectively, were not significantly different from the control group [48]. In 2013, Qi et al. found 0.4% prevalence of antibodies to H10N8 in animal workers in Guangdong province, China, which did not differ significantly from the non-exposed controls (0%) [52].

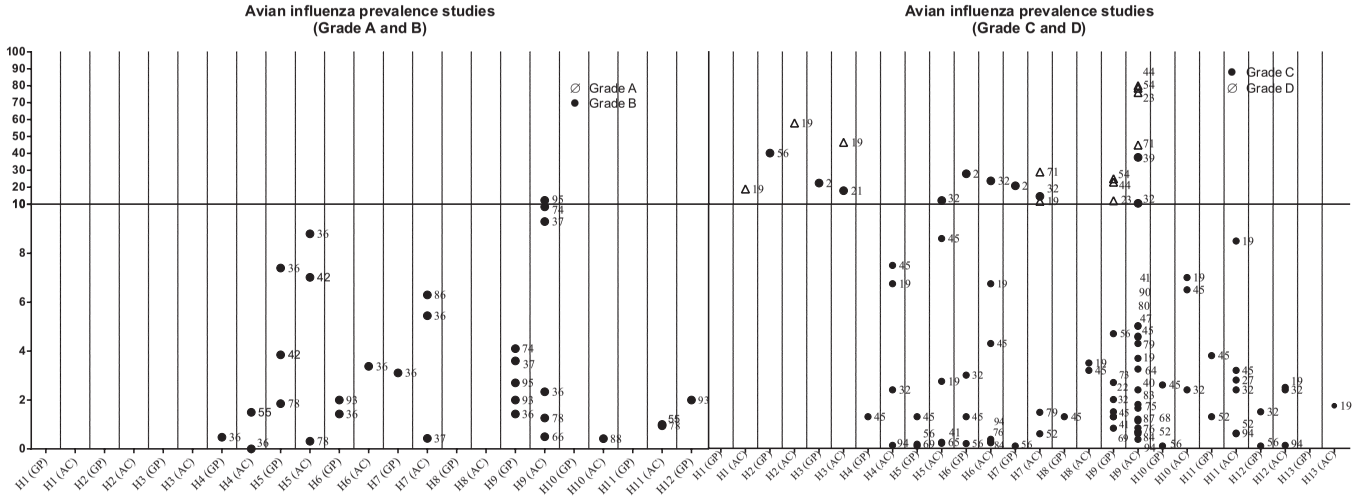
Cohort studies

Grey et al. found that 0.8% of Iowa agricultural workers experienced an antibody increase for H5N2 during the study period of two years [46]. Four cohort studies found antibodies to H6N1 during their two-year study periods. The percentage of the study population that experienced an increase in antibody titers ranged between 0.1% and 2% [40, 41, 53, 54]. Two out of the four studies could not find an association with animal exposure. A very low percentage (<0.3%) of three of the study populations experienced a slight increase in antibodies against H7N7[54], H10N4[53], or H12N5 [54].

Increases in H9N2 antibodies were detected in three two-year cohort studies studying poultry workers. In Thailand, between 2008 and 2010, 2% of the study population seroconverted in the first year of the study, and 2.5% seroconverted in the second year [54]. A similar number of antibody titer increases was found in Mongolia between 2009 and 2011: 2.2% of the adults experienced an increase in antibodies after either year 1 or year 2 of the study [53]. In both studies there were individuals that showed a four-fold antibody titer increase (0.25% and 0.8%) but did not report influenza like illness, suggesting subclinical infections [53, 54]. Gray et al. found that 0.3% of agricultural workers from Iowa experienced a titer increase during the two-year study period [46].

Besides information about the number of seroconversions, cohort studies provide information on antibody longevity. Lu et al. show that all individuals previously seropositive for H7N7 and H9N2 serums became seronegative after one year [55]. Krueger et al. likewise found that antibody titers against H6N1 and H7N7 were undetectable after one year [54]

Figure 5. Seroprevalence of antibodies to different avian influenza viruses (x-axis) in exposed and control humans. The numbering, diagnostic method, and cut-offs can be found in supplementary table 4. GP: general population or control population; AP: animal-exposed population



Studies with a lower score (C or D) appeared to report higher prevalences for avian influenza antibodies than did A or B studies, but the difference was not significant ($p>0.05$). Our conclusions were similar when we compared different cut-offs.

Outbreak investigations

The outbreak studies included in this review investigated people who had been exposed to poultry infected with H5, H7, H9 or H10. A study of H5N2 outbreaks at Japanese chicken farms found a positive H5N2 neutralising titer (MN≥1:40) in 25% of the workers, of whom 7.8% showed a four-fold antibody increase. [56] Di Trani et al. found that 2.1% of Italian poultry workers exposed to H5N2- or H5N7-infected poultry showed an antibody titer (HI≥1:10), but results were not confirmed by MN, nor was there a significant difference between subjects and unexposed controls [57].

The outbreak studies that found H7 antibodies reported seroprevalences from 0.4 to 2.7% in exposed poultry workers [57, 58].The outbreak study by Di Trani et al. found significantly more antibodies to H7N1 and H7N3 in H7-exposed poultry workers compared to unexposed controls [57]. Another outbreak investigation conducted in Italian poultry workers likewise found H7N1 and H7N3 antibodies, but included no controls. Moreover, 6 of the 983 workers in that study reported conjunctivitis but showed no H7 antibody response [58]. Using an MN assay with cut-off of 1:80, Skowronski et al. found no evidence for human antibody responses in Canadian workers involved in an H7N3 outbreak in poultry; but they reported that close contact with the infected poultry correlated with red or watery eyes [59]. In a study of H7N7 by Meijer et al. in the Netherlands, the results from the HI assay (≥1:10) indicated prevalence of 49%, but none of the titer rises could be confirmed by MN; however, ocular symptoms of infection appeared more frequently in subjects with HI-detected antibodies compared to subjects without antibodies [12].

One outbreak study investigating H9-exposed poultry farmers found antibodies in 11 of 34 subjects, but did not include a control group or describe a cut-off for the HI assay [60]. Arzey et al. investigated abattoir workers exposed to H10N7-infected poultry and found that 2 of 7 reporting conjunctivitis were PCR-positive for influenza A; partial sequence analysis of the HA confirmed the presence of H10 subtype, but the findings could not be serologically confirmed [61].

Equine and canine influenza

Two studies executed before 1970 in Europe investigated human exposure to equine influenza viruses and found prevalences from 4.2% to 20.9% for H3N8, using HI and neutralisation assays, but no non-exposed control group was included [37, 62]. Khurelbaatar et al. also investigated exposure to equine influenza virus in a Mongolian rural population during 2009-2011 and found a seroprevalence of 1.1% at enrollment. During the two-year follow-up period, 2.5% of the study population experienced a four-fold titer increase against equine influenza virus H3N8, but exposure to camels or horses was not associated with titers to H3N8 [53, 63]. Antibody responses have been detected against canine influenza A H3N8 in

dog-exposed subjects, but comparison with an unexposed control group yielded no significant difference [64]

DISCUSSION

There is currently no methodology or tool available for the quality assessment and comparison of influenza serology population studies [16]. In this review we therefore tried to develop a grading system to weigh the evidence for human infection with animal influenza viruses from the included studies. Each attribute of the grading system is either a known confounding factor, and should therefore be included in the analysis (e.g. age, influenza vaccination status, and cross-reactions with human influenza), or is an accepted method to improve the specificity of the outcome of serological influenza studies (e.g. comparison with a control group, use of paired sera, additional virological evidence, diagnostic tests with high specificity, and confirmation testing). Although the weights of variables of the scoring system were divided in an arbitrary manner, the scoring system comprises important factors that should be incorporated in future studies at the human-animal interface to improve reliability of serological evidence of animal influenza viruses.

It was often not possible to score all aspects of the execution of a study, as details were lacking from its methods section. Therefore, although we scored for the presence of information on confounding factors, we did not take into account how they were incorporated in the analysis of the data. Nor did we assess the quality and execution of the diagnostic tests, because descriptions often omitted details that can very much influence test outcome, e.g. the origin and quality of red blood cells (RBC) used in HI assays [65].

Moreover, it was difficult to interpret and compare the diverse antibody titers reported in the literature. Several studies addressing the inter-laboratory variability of influenza HI and MN assays have found differences in geometric mean titers up to six- (HI) or seven-fold (MN) [10, 65-68]. The interpretation of the test results is even more difficult because little is known about the agreement between HI and MN assays. It has been hypothesized that there is actually no linear correlation between VN and HI titers and that the ratio varies across serotypes [66, 68]. In addition, pre-existing antibodies against human influenza viruses may cross-react with animal influenza virus subtypes, resulting in titers that are unrelated to exposure or infection with an animal influenza virus [22-24]. Using RBCs of diverse animal origins in HI assays can also result in titer differences, and the RBC type that allows the best binding, and therefore the highest titers, can differ among and within influenza subtypes. [69-71] Moreover, for many zoonotic influenza A subtypes the optimal detection method is unknown.

In this review, the focus was on assessing the specificity of the reported findings. However, it is possible that clinical or subclinical infections are being missed and that the actual rate of infection is higher than the serological data suggest. For example, individuals exposed to H7 (other than H7N9) have developed a virologically confirmed conjunctivitis when no seroconversion could be detected [12, 59]. Also, infections with avian influenza causing fever

and/or respiratory symptoms can sometimes be confirmed virologically but not serologically [12, 50]. Moreover, serological responses to zoonotic influenza can wane rapidly, which can lead to underestimation of the frequency of spill-over of animal influenza viruses to humans [55, 72].

Looking at the studies collected for this review, it becomes clear there is no agreement on the diagnostic methods, cut-offs or study design that should be used to investigate the prevalence of zoonotic influenza in humans. This limits both the interpretation and the comparability of the available data. Following the H1N1 pandemic in 2009, the WHO reached the same conclusions in a review on the pandemic and requested standardised methods to improve the comparability of the serological data [73]. Although the Consortium for the Standardization of Influenza Seroepidemiology (CONCISE) published recommendations and protocols to standardise serological studies on zoonotic influenza virus outbreaks, human influenza virus epidemics, and seasonal influenza, there are no guidelines for the design and execution of population studies for influenza on the human-animal interface [14, 15].

An interesting finding is that studies graded C or D in this review generally reported higher seroprevalences to avian influenzaviruses in humans ($p>0.05$) than A or B studies. It is possible that using a less stringent study protocol leads to an overestimation of serological findings of animal influenza in humans. Therefore, to harden the reliability of the evidence and reduce the occurrence of false positive outcomes, inclusion of confounding factors either in the study design or the data analysis is important .

In this review we see that antibodies to swine influenza viruses A(H1N1), A(H1N2), and A(H3N2) are more prevalent amongst persons occupationally exposed to pigs compared to those not exposed. However, given the potential for cross-reactivity within subtype, serological studies that investigate human infection with swine influenza should be interpreted with great caution. Unlike avian influenza viruses, endemic swine influenza viruses have common origins with seasonal human influenza viruses. Novel pandemic influenza human viruses have originated from swine viruses or have been introduced in swine, and play an important role in the evolution of genetic diversity of swine influenza viruses. For instance, with the recent emergence of pandemic (H1N1) 2009 virus and subsequent reintroductions in the swine population, serological population studies investigating human infection with swine influenza viruses should be designed and interpreted with extreme caution to differentiate true exposures from cross-reactions [74, 75]. Nevertheless, the number of studies finding a significant difference in seroprevalence between swine-exposed study groups and unexposed control groups is strong evidence of frequent spill-over events from swine to humans. The high number of reported swine-to-human transmissions and evidence for subsequent human-to-human transmission, in combination with the recent emergence of pandemic (H1N1) 2009 virus, warrants increased serological and virological surveillance of swine and people that are exposed to swine [7, 76]. Unfortunately swine influenza surveillance is less prevalent than avian influenza surveillance, and there is relatively little knowledge on prevalence and circulation of swine influenza [77].

According to our assessment, the most reliable serological evidence (grade A and B) was found for human exposure to avian influenza virus HA-types H5, H7, and H9. The risk of infection with H5 and H7 subtypes is illustrated by the serious recent outbreaks of subtypes (H5N1) virus and A(H7N9) virus in humans, which, to date, have resulted in 429 [78] and 212 [79] deaths, respectively. Few cases of humans infected with avian H9 viruses have been reported: Freidl et al. described in their literature review the virological evidence of 15 cases of humans infected with H9N2[7]. However, avian H9N2 viruses are a growing concern, and the mild disease associated with H9 infection potentially leads to considerable underestimation of incidence [80-82]. H9 avian influenza can be found in poultry all over the world and is also described in multiple other avian species [83], pigs [84, 85], and dogs[86]. Moreover, internal genes of A(H9N2)v were found in A(H10N8)v [87], A(H7N9)v [88] and A(H5N1)v [89], showing that A(H9N2)v can reassort with other influenza subtypes, potentially resulting in the generation of new zoonotic influenza types. Recently, human cases of infection with H10N8 and H6N1 have been reported [87, 90], as well as presence of these serotypes in environmental samples from animal markets [91, 92], showing that H6 and H10 serotypes can likewise pose a risk to human health. Serological cohort studies of persons exposed to poultry provide information on the incidence and longevity of antibodies to zoonotic influenza viruses. For most avian influenza subtypes, this information is currently unknown and will greatly contribute to the risk analysis of zoonotic avian influenza.

Although human antibodies have been found against equine and canine influenza, these infections seem to be a minor public health risk. However, the very limited number of studies could lead to substantial underreporting.

CONCLUSIONS

Comparing serological data is difficult due to a lack of standardisation in the collection of epidemiological data and the laboratory methods used in the published zoonotic influenza studies. Researchers should take into account WHO guidelines, known confounding factors, and the need for a control group in order to produce research articles that can be used, compared, and appreciated by policy makers and other researchers to assess the true risks and prevalence of animal influenza exposure in humans.

Swine-to-human transmission is prevalent, but surveillance systems and standard serological surveillance of swine and human risk groups is scarce. Surveillance for avian influenza is more common, but most studies target H5, H7 and H9 serotypes and, accordingly, most serological evidence is reported for these subtypes. Given the zoonotic potential of avian influenza viruses, which can potentially reassort with circulating seasonal human influenza virus subtypes, systematic surveillance in poultry populations should be expanded beyond H5 and H7, the primary focus for the veterinary sector [93, 94]. Subtypes H6, H9 and H10 are known to be able to infect humans and should therefore be included. Finally, we found that the majority of studies conducted at the human-animal interface represent `Asia, Europe, and North America. Efforts should be made to shed light on understudied areas, such as South America and Africa.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Table 4. Total score per reference

Reference number	Author Country	Publication year	Study design	Study population	Screening test	Confirmatory assay	SCORE							
							Control group	Vaccination	Diagnostic method	Age	Human influenza	Additional evidence	Repeated sampling	TOTAL SCORE
1	N. Masurel et al. The Nether- lands	1966	Serial cross-sectional	General population	eqH3N8 (HI) 1958: 4.2 (74/1750) 1963:5.8 (52/900) eqH7N7 (HI) 0 (0/2650)	MN Unknown	0	0	5	1	1	0	0	7
2	B. Tumova et al. Czech republic	1968	Cross-sectional	General population	swH1N1 (HI≥1:32) 67.4 (126/187) H3N8 (HI≥1:32) 22.5 (42/187) eqH3N8 (HI≥1:32) 20.9 (39/187) H6N2 (HI≥1:32) 28.0 (21/75) H7N6 (HI≥1:32) 20.9 (39/187) H2N2 H5N3, eqH7N7 (HI≥1:32) 0 (0/187)	NT Unknown	0	0	5	1	0	0	0	6

3	P. R. Schnur-renberger et al. USA	1970	Cross-sectional	Occupationally swine-exposed population Control: general population	swH1N1 (HI≥1:20) 36.3 (360/1004)[b] 18.7 (157/840)	No	2	1	2	1	0	0	0	6
4	P. Cuneo-Crovari et al. Italy	1976	Cross-sectional	General population	swH1N1 (HI≥1:64) 16 (112/700)	swH1N1 (NI) Unknown	0	1	5	1	1	0	0	8
5	N. Masurel The Nether-lands	1976	Serial cross-sectional	General population Hospital population (influenza+)	swH1N1 (HI≥1:100) 1967: 16 (112/700) 1972: Not specified, 80% in age 49-75 1973: Not specified, 60% age 49-75 swH1N1 (HI 4fold rise) 2.7 (53/2000)	No	0	0	2	1	1	0	2	6
6	R. Pyhala Finland	1976	Cross-sectional	Hospital population (influenza A+) Hospital population (inf. disease+)	swH1N1 (HI≥1:12) 55.9 (19/34) “about 25% in Finnish population (before 1920: 91%, 1920-1930:55%, after 1930: 2%)”	No	0	0	2	1	1	0	0	4
7	J. W. Smith USA	1976	Case contact study	Family Co-workers in slaughterhouse	swH1N1 (HI≥1:20) 7 (2/28) 33(6/18)	No	0	0	2	0	1	0	0	3

8	R. L. Thomp-son et al. USA	1976	Cross-sectional and case contact study	Hospital population (pneumonia) Case contacts	swH1N1 (HI 4fold rise) 2 (2/100) 16 (4/25)	swH1N1 (NI) 50% (1/2) HI-positive could be confirmed	0	0	3	0	1	0	2	6
9	J. C. Gaydos et al. USA	1977	Cross-sectional and case study	Hospital population (acute respiratory disease+)	swH1N1 (HI 4fold rise) 8.3 (9/74)	No	0	1	2	0	1	3	2	9
10	R. A. Hodder et al. USA	1977	Cross-sectional	Basic combat trainees (BCT) Persons from military not in BCT Control: ambulatory care patients	swH1N1 (HI≥1:20) 22.6 (134/592) Cohorts with most swh1N1 cases: 9%-19%, cohorts without cases: 0-5% 15.3 (21/137) 27.8 (119/428) <5% in age <30	No	2	1	2	1	1	0	2	9
11	R. J. O'Brien et al. USA	1977	Cross-sectional and case-contact study	Persons living in the area and children from nearby schools Family members Contacts family	swH1N1 (HI≥1:20) 23.6 (61/258) >age 50: 80 75 (6/8) 4 (2/55) positive >age 50: approaching 100%	No	0	0	2	1	1	1	0	5

12	J. G. Olson Taiwan	1977	Outbreak investigation	Exposed and unexposed swine workers, one year after epizootic in swine Children of swine workers and classmates Control: outpatients without swine exposure and children	swH1N1 (HI≥1:10) Exposed: 24.6 (15/61)[b*] Unexposed: 19.6 (11/56) 0 (0/25) Outpatients: 35.9 (60/167) Children: 3.3 (1/30) * only a significant difference between unexposed workers in lower age groups vs. unexposed control group	No	2	0	2	1	1	2	0	6
13	E. Magurea-nu et al. Romania	1978	Cross-sectional	Hospital population (influenza+) Control: patients without respiratory complaints and healthy visitors of the clinic	swH1N1 (HI≥1:20) Paired sera: no 4fold increases or titers above 1/16 (0/116) 17.8 (288/1620)	No	4	0	2	1	0	0	2	9

14	D. S. Tan et al. Malaysia	1979	Cross-sectional	Pig slaughterers Control: unexposed veterinary laboratorium workers Swine	swH1N1 (HI) 10 (6/60) 6.2 (4/65) Serum (HI): 13.3 (23/173)	No	2	0	2	0	1	1	0	6
15	G. T. Woods et al. USA	1981	Cohort study and cross-sectional	Pre-employment and 1 year after samples of abattoir workers Veterinarians, veterinary students and pork producers Youth in touch with swine	swH1N1 (HI≥1:20) 6.3 (92/1466) After one year, 8.5% (26/305) experienced a titer increase. 22.9 (138/602) 0 (0/23)	No	0	0	2	1	0	0	2	5
16	H. Sinnecker et al. Germany	1983	Outbreak investigation	Exposed pig breeders Swine	swH1N1 (HI≥1:10) A/swine/potsdam/1/81: 14 (8/57) A/New Jersey/8/76: 17 (10/57) Serum convalescent pigs: 100 (120/120) against isolated strains Healthy control pigs: 0 (0/281) against isolated strains	NI (HI≥1:10)A/swine/Potsdam/1/81: 30 (17/57) A/New Jersey/8/76: 33 (19/57)	0	0	3	0	0	2	0	5

17	M. L. Profeta et al. Italy	1986	Cross-sectional	General population	H4N6, H7N7, H8N, H11N9, H12N5 (HI): 0 (0/294)	H4N6 (NI≥1:20) 3.4 (10/294) H7N7 (NI≥1:20) 6.4 (19/294) H8N4(NI≥1:20) 23.4 (69/294) H11N9 (NI≥1:20) 5.1 (15/294) H12N5 (NI≥1:20) 5.1 (15/294)	0	0	3	1	0	0	0	4
18	D. L. Wells et al. USA	1991	Outbreak investigation and case-contact study	Exposed junior pig exhibitors Family, direct contacts and exposed health care personnel Control: unexposed health care personnel and age matched junior swine exhibitors from other county	swH1N1 (HI≥1:20) 76.0 (19/25)[b] 30.6 (22/72) 0 (0/81)	No	4	1	2	0	0	0	0	7

19	K.F. Shortrid-ge China	1992	Cross-sectional	Rural dwellers	H1 (HI) 19 (57/300) H2 (HI) 58 174/300) H3 (HI) 46.5 (442/950) H4 (HI) 6.75 (64/950) H5 (HI) 2.75 (26/950) H6 (HI) 6.75 (64/950) H7 (HI) 11.75 (112/950) H8 (HI) 3.5 (33/950) H9 (HI) 3.25 (31/950) H10 (HI) 7 (67/950) H11 (HI) 8.5 (81/950) H12 (HI) 2.5 (24/950) H13 (HI) 1.25 (17/950)	No	0	0	1	0	0	0	0	1
20	L. L. Shu et al. China	1996	Serial cross-sectional	Farm workers and family Hospital population (respiratory complaints)	swH1N1,H3N8, H4N4, H7N4, H11N2(HI≥1:20) 0 (0/156) H3N8, H4N4, H7N4, H11N2(HI≥1:20) 0 (0/51)	swH1N1 (NI≥1:20) 3.8 (6/156) H3N8 (NI≥1:20) 0 (0/156) H4N4 (NI≥1:20) 1.2 (2/156) H7N4 (NI≥1:20) 5.1 (8/156) H11N2 (NI≥1:20) 24.4 (38/156) H3N8, H4N4, H7N4 (NI≥1:20) 0 (0/51) H11N2 (NI≥1:20) 11.8 (6/51)	0	0	3	0	1	0	0	4

21	N. Zhou et al. China	1996	Cross-sectional	Occupationally exposed population	swH1N1 (HI≥1:20) Sw/BJ/47/91: 1.7 (8/459) Sw/Ita/786/88: 0.7 (3/459) swH3N2 (HI≥1:20) 79.6 (300/459) H3N8 (HI≥1:20) 7 (18/459) H4N4, H7N4, H11N2(HI≥1:20) 0 (0/459) Purified H7 (ELISA ≥400) 8.1 (37/459)[b*]	swH1N1 (NI≥1:20) Sw/BJ/47/91: 0 (0/459) Sw/Ita/786/88: 6 (11/459) swH3N2 (NI≥1:20) 53.6 (246/459) H3N8 (NI≥1:20) 0.7 (2/268) H4N4 (NI≥1:20) 0 (0/268) H7N4 (NI≥1:20) 0.7 (2/268) H11N2(NI≥1:20) 26.1 (70/268)	2	1	3	0	1	0	0	7
				Control: local university students /US citizens without recent influenza vaccinations	swH1N1 (HI≥1:20) Sw/BJ/47/91: 1.5 (3/205)/ 16 (5/32) Sw/Ita/786/88: 5 (10/205)/ 9 (3/32) swH3N2 (HI≥1:20) 85 (175/205)/ 84 (27/32) H3N8 (HI≥1:20) 19 (7/32) H4N4, H7N4, H11N2(HI≥1:20) 0 (0/32) Purified H7 (ELISA ≥400) 3.3 (1/32)									
				Slaughtered pigs from same region	swH1N1 (HI≥1:20) 0 (0/200) huH1N1 (HI≥1:20) 10.0 (20/200) swH3N2 (HI≥1:20) 0.5 (1/200) huH3N2 (HI≥1:20) 5.0 (10/200) Purified H7 (ELISA ≥400) 0 (0/200)	* significant difference between "women raising pigs " vs. unexposed control group								

22	M. Peiris et al. Hongkong	1999	Cross-sectional	General population Control: UK general population	H9N2 (NT≥1:20) 2 (3/150) 0 (0/100) (G1-like) 3 (3/100) (G9-like)	H9N2 (HI≥1:40) 2.7 (4/150)	1	0	5	0	0	0	0	6
23	M. Mayahi Iran	2003	Cross-sectional	Poultry exposed population Control: unexposed population	H9N2 (HI≥2) 76 (76/100)[b] 12 (12/100)	No	2	0	2	0	0	0	0	4
24	G. Ayora-Talavera et al. Mexico	2005	Cross-sectional	Hospital population (any medical condition)	swH1N1 (HI≥1:40) 1.7 (2/115) swH3N2 (HI≥1:40) 79.1 (91/115)	No	0	0	2	1	1	0	0	4
25	S. Puzelli et al. Italy	2005	Outbreak investigation	Exposed poultry workers	H7N1 (MN≥1:20) 0.7 (7/983) H7N3(MN≥1:20) 3.8 (7/185)	H7N1 (HI≥1:10) 0.4 (4/983) H7N3(HI≥1:10) 2.2 (4/185)	0	0	5	0	0	2	0	7
26	E. K. Barbour et al. Lebanon	2006	Outbreak investigation	Exposed poultry farmers	H9N2 (HI) 32.3 (11/34)	No	0	0	2	0	0	2	0	4
27	J. S. Gill et al. USA	2006	Cross-sectional	Duck hunters and nature reserve employees	H1-H10 and H12 (MN ≥1:10) 0 (0/107) H11 (MN ≥1:10) 2.8 (3/107)	H1-H10 and H12 (HI ≥1:10) 0 (0/107) H11 (HI ≥1:10) 2.8 (3/107)	0	1	5	0	0	0	0	6

28	A. Meijer et al. The Netherlands	2006	Outbreak investigation and case-contact study	Exposed poultry workers Direct contacts Control: general population (vaccine trial)	H7N7 (HI≥1:40) 7.7 (36/469) 6.3 (4/63) 0 (0/100) HI titers correlated with occurrence of eye symptoms	H7N7 (MN) 0	4	1	5	0	1	3	0	14
29	K. P. Myers et al. USA	2006	Cross-sectional	Occupationally exposed population (farmers and veterinarians) Meat processing workers Control: unexposed population	swH1N1 (HI≥1:40)[b] 14.2 (25/176) swH1N2 (HI≥1:40)[b] 19.9 (35/176) swH3N2 (HI≥1:40) No significant difference with control population swH1N1, swH1N2, swH3N2 (HI≥1:40) Indication of exposure to swine H1N1 virus infection (OR, 6.5; 95% CI, 1.4–29.5) and swine H1N2 virus (OR, 2.7; 95% CI, 1.1–6.7) swH1N1 (HI≥1:40) 0 (0/79) swH1N2 (HI≥1:40) 1.3 (1/79) swH3N2 (HI≥1:40) Unknown	No	2	1	2	1	1	0	0	7

30	A. Ramirez et al. USA	2006	Cross-sectional	Swine confinement workers Control: unexposed population	swH1N1 (HI≥1:20) 14.6 (7/49)[b*] 1.3 (1/79) * significant difference between swine workers that wear gloves vs. unexposed control group	No	4	1	2	0	1	0	0	8
31	G. C. Gray et al. USA	2007	Cohort study and cross-sectional	Rural resident with /without swine contact Control: US unexposed population	swH1N1 (HI≥1:40) 12.4 (88/707)[b] / 5 (4/80)[b] swH1N2 (HI≥1:40) 20.2 (143/707)[b] / 6.3 (5/80)[b] swH1N1 (HI≥1:40) 0 (0/79) swH1N2 (HI≥1:40) 1.3 (1/79) In 2 years 25% experienced a titer increase for swH1N1 and 5% for H1N2	No	2	1	2	1	1	0	2	9

32	K. P. Myers et al. USA	2007	Cross-sectional	Veterinarians	H4N8 (MN≥1:10) 2.4 (1/42) H5N2(MN≥1:10) 12.2 (5/42)[b] H6N2(MN≥1:10) 23.8(10/42)[b] H7N2(MN≥1:10) 14.3 (6/42)[b] H8N5(MN≥1:10) 0 (0/42) H9N2(MN≥1:10) 9.5 (4/42) H10N7(MN≥1:10) 2.4 (1/42) H11N9(MN≥1:10) 2.4 (1/42) H12N5(MN≥1:10) 2.4 (1/42)	No	4	1	3	0	1	0	0	9
				Control: volunteers from the university	H4N8, H5N2, H7N2, H8N5, H10N7, H11N9 (MN≥1:10) 0 (0/66) H6N2(MN≥1:10) 3 (2/66) H9N2(MN≥1:10) 1.5 (1/66) H12N5(MN≥1:10) 1.5 (1/66)										

33	E. J. Ortiz et al. Peru	2007	Cross-sectional	Poultry workers from one farm Control: workers without poultry contact	H4N8, H5N2, H6N2 H7N2, H8N5, H9N2, H10N7, H11N9, H12N5(MN≥1:80) 0 (0/133) H4N8, H5N2, H6N2 H7N2, H8N5, H9N2, H10N7, H11N9, H12N5(MN≥1:80) 0 (0/17)	No	4	1	3	0	1	0	0	9
34	J. L. Robinson et al. Canada	2007	Case contact study	Family members Residents of communal farm with or without exposure to swine Swine on the farm	swH3N2 (HI≥1:32) 57.1 (4/7) 8.2 (4/46) Serum (ELISA swH3N2): 40 (4/10) 1 positive for the strain that was isolated from patient (HI)	No	0	0	2	0	1	3	0	6
35	D. M. Skowronski et al. Canada	2007	Outbreak investigation	People involved the outbreak	H7N3 (MN≥1:80) 0 (0/167) 19 subjects experienced ILI and 21 red or watery eyes. 2 cases were virus positive, but serologically negative. No association between symptoms and serologic response	H7N3 (HI and Westernblot) 0	0	1	5	0	0	2	2	10

36	G. C. Gray et al. USA	2008	Cohort study and cross-sectional	Swine or poultry exposed /unexposed rural agricultural workers Control: US unexposed university workers and students	H4N8 (MN≥1:10) 0 (0/385)/ 0.5 (2/418) H5N2 (MN≥1:10) 8.8 (34/385)[b] / 7.4 (31/418)[b] H6N2 (MN≥1:10) 1.4 (6/385) / 0.5 (2/418) H7N2 (MN≥1:10) 5.5 (21/385) / 3.1 (13/418) H9N2 (MN≥1:10) 2.3 (9/385) / 1.4 (6/418) H4N8, H5N2, H7N2 (MN≥1:10) 0 (0/66) H6N2 (MN≥1:10) 3.0 (2/66) H9N2 (MN≥1:10) 1.5 (1/66) H4N8, H5N2, H7N2 (MN≥1:10) 0 (0/66) H6N2 (MN≥1:10) 3.0 (2/66) H9N2(MN≥1:10) 1.5 (1/66) In a 2 year time period the swine or poultry exposed agricultural workers showed titer increases for H5N2 (y1:5.8%, y2:11.3%), H6N2 (y1:1.4%, y2: 0.3%) and H9N2 (y1:0.3). The swine or poultry unexposed agricultural workers showed titer increases for H4N8 (y1:0.6%), H5N2 (y1:7.7%, y2:13.9%), H6N2 (y1:1.2%) and H9N2 (y1:0.3)	No	2	1	3	1	1	0	2	10

37	C. Y. Lu et al. China	2008	Cohort study and cross-sectional	Occupationally poultry-exposed population Control: citizens living in H5N1-outbreak areas	H7N7 (HI≥1:20) 0.4 (1/231)[b] H9N2 (HI≥1:20) 9.5 (22/231) H7N7 (HI≥1:20) 0 (0/983) H9N2 (HI≥1:20) 3.7 (37/983) After one year, all positive cases had become negative.	Good correlation: all positives confirmed	2	0	5	0	1	0	2	10
38	T. Ogata et al. Japan	2008	Outbreak investigation	Exposed poultry farm workers	H5N2 (MN≥1:40) 16.4 (45/275) H5N2 (4fold titer rise) 7.3 (20/275)	No	0	1	3	1	0	2	2	9
39	H. S. Alizadeh E et al. Iran	2009	Cross-sectional	Poultry workers Control: minimally poultry exposed population	H7N7 (HI≥1:20) 0 (0/127) H9N2 (HI≥1:20) 37.7 (48/127)[b] H7N7 (HI≥1:20) 0 (0/25) H9N2 (HI≥1:20) 0 (0/25)	No	4	1	2	0	1	0	0	8
40	N. Jia et al. China	2009	Cross-sectional	Poultry workers and farmers Control: residents from a region without AI	H7N1 (HI≥1:160) 0 (0/1060) H9N2 (HI≥1:160) 1.1 (12/1060)[b] H7N7 (HI≥1:160) 0 (0/407) H9N2 (HI≥1:160) 0 (0/407)	No	4	0	2	0	0	0	0	6

41	M. Wang et al. China	2009	Cross-sectional	Occupationally exposed population Control: general population	H5N2 (HI) 0.2 (4/1890) H9N2 (HI) 5.0 (95/1890)[b*] H7N7 (HI) 0 (0/301) H9N2 (HI) 1.3 (4/301) * significant difference between poultry retailers vs. other occupationally exposed and unexposed control group	H5N2 (VN) 0.2 (4/1890) H9N2 (VN) 5.0 (95/1890)	2	0	5	0	0	0	0	7
42	Y. Yamazaki, et al. Japan	2009	Cross-sectional	Inhabitants region with H5 influenza H5-influenza-free poultry farm workers from region with H5 Control: healthy inhabitants outside of this region	H5N2 (MN≥1:40) 7.0 (8/114) 15.4 (8/52)[b] 4 (4/100)	H5N2 (HI≥1:40) 7.9 (9/114) 3.8 (2/52)	6	0	5	1	0	0	0	12
43	A. Beaudoin et al. USA	2010	Outbreak investigation	Exposed swine workers Control: unexposed swine workers	swH2N3 (MN≥1:40) 6.3 (1/16) 27.3 (3/11)	swH2N3 (HI≥1:40) 6.3 (1/16)	6	1	5	0	1	2	0	15

44	M. Hadipour Iran	2010	Cross-sectional	Occupationally exposed population Hospital population (respiratory symptoms) Control: unexposed population	H9N2 (HI≥1:20) 78.6 (142/180)[b] 35.6 (21/60) 23 (14/60)	No	2	0	2	0	0	0	0	4
45	G. Kayali et al. USA	2010	Cross-sectional	Occupationally turkey exposed population Control: unexposed population	H4N6 (MN≥1:10) 7.5 (7/95)[b] H5N2 (MN≥1:10) 8.6 (8/95) [b] H6N2 (MN≥1:10) 4.3 (6495) [b] H7N2 (MN≥1:10) 0 (0/95) H8N4 (MN≥1:10) 3.2 (3/95) [b*] H9N2 (MN≥1:10) 4.3 (4/95) [b] H10N7 (MN≥1:10) 6.5 (6/95) [b] H11N9 (MN≥1:10) 3.2 (3/95) H4N6, H5N2, H6N2, H8N4, H9N2 (MN≥1:10) 1.3 (1/78) H7N2 (MN≥1:10) 0 (0/78) H10N7 (MN≥1:10) 2.6 (2/78) H11N9 (MN≥1:10) 3.8 (3/78)	No * significant difference small scale growers vs. unexposed control group	4	1	3	0	1	0	0	9

46	A. Krumb- holz et al. Germany	2010	Cross-sectional	Occupationally swine exposed population Control: general population	swH1N1 (HI≥1:80 or 4fold titer increase relative to group mean) 0 (0/50) swH1N2(HI≥1:80 or see swH1N1) 4 (2/50)[b] swH3N2 (HI≥1:80 or see swH1N1) 10 (5/50)[b] 13 out of 18 positive sera were still positive after 6-12 months swH1N1 (see above) 0 (0/118) swH1N2(see above) 0.8 (1/118) swH3N2 (see above) 0 (0/118)	swH1N1(MN≥1:80) 4 (2/50)[b] swH1N2(MN≥1:80) 8 (4/50)[b] swH3N2 (MN≥1:80) 14 (7/50)[b]	4	1	5	0	1	0	2	13
47	P. Z. Qin et al. China	2010	Cross-sectional	Poultry workers Control: unexposed population	H9N2 (HI) 4.6 (116/2772) Unknown (432 control sera)	No	2	0	2	1	0	0	0	5

48	P. Terebuh et al. USA	2010	Cohort study	Swine workers Control: urban blood donors	swH1N1 (HI≥1:40) cH1N1: 12.5 (11/88)[b] trH1N1: 30.7 (27/88)[b] swH1N2 (HI≥1:40) 36.4 (32/88)[b] swH3N2 (HI≥1:40) 3 different types, prevalence ranging from 11.4-28.4 In a 2 year time period subjects showed titer increases for cH1N1 (y1:3%, y2:0%), trH1N1 (y1:7%, y2: 1%) and trH1N2 (y1:7%, y2: 1%)[b] The number of seroconversions for different types of swH3N2 was not significantly higher compared to the control group swH1N1 (HI≥1:40) cH1N1: 1.9 (4/220) trH1N1: 9.5 (20/220) swH1N2 (HI≥1:40) 11.4 (24/220) swH3N2 (HI≥1:40) 3 different types, prevalence ranging from 13.8-32.8	No	4	1	2	0	1	2	2	12

49	F. S. Dawood et al. USA	2011	Cross-sectional and outbreak investigation and case contact study	Veterinary students People exposed to swine at same event as index case Direct contacts Control: unexposed population	swH1N1 (MN≥1:80) 22 (2/9) [b] 40 (17/42) [b] 0 (0/8) 0 (0/10)	swH1N1 (HI≥1:20) 22 (2/9) 40 (17/42) 0 (0/8)	4	0	5	0	1	0	0	10
50	J. G. Donahue et al. USA	2011	Cohort study	Backyard poultry flock handlers	H4N8, H5N2, H6N2, H7N3, H9N2, H10N4, H11N9, H12N5, H13N2 (HI) 0 (0/128) No titer increases in 15 month timeperiod	No	0	0	2	0	1	0	2	5
51	N. A. Gerloff et al. Luxembourg	2011	Cross-sectional	Swine workers Control: unexposed population	swH1N1 (VN≥1:80) 10.0 (21/211)[b] 1.8 (4/224)	No	6	1	3	0	1	0	0	11
52	G. C. Gray et al. USA	2011	Cross-sectional	Bird banders Control: unexposed population	H4N8, H5N2, H6N2, H8N4, H10N4 (MN≥1:10) 0 (0/157) H7N3, H9N2, H11N3 (MN≥1:10) 0.6 (1/157) H4N8, H5N2, H6N2, H7N3 H8N4, H9N2, H10N4 (MN≥1:10) 0 (0/78) H11N3 (MN≥1:10) 1.3 (1/78)	No	4	1	3	0	1	0	0	9

53	M. Hadipour et al. Iran	2011	Cross-sectional	Hospital staff Control: outpatients from same hospital	H9N2 (HI≥1:40) 32.6 (98/300)[b] 2.5 (8/300)	No	2	0	2	0	0	0	0	4
54	M. Hadipour et al. Iran	2011	Cross-sectional	Occupationally poultry-exposed population Hospital population (respiratory symptoms) Control: non- or rarely poultry exposed population	H9N2 (HI>1/8 or at least 3/15 with titer =1/8) 80 (144/180)[b] 46.6 (28/60) 25 (15/60)	No	2	0	2	0	0	0	0	4
55	G. Kayali et al. Lebanon	2011	Cross-sectional	Poultry farmers Control: unexposed population	H4N6(MN≥1:10) 1.5 (3/200) H11N9(MN≥1:10) 1.0 (2/200) H6N1, H7N7, H8N4, H9N2, H10N7, H12N5, H13N6, H14N5, H15N9, H16N3 (MN≥1:10) 0 (0/200) H4N6, H6N1, H7N7, H8N4, H9N2, H10N7, H11N9, H12N5, H13N6, H14N5, H15N9, H16N3 (MN≥1:10) 0 (0/200)	H4N6(MN≥1:10) 1.5 (3/200) H11N9(MN≥1:10) 1.0 (2/200) H6N1, H7N7, H8N4, H9N2, H10N7, H12N5, H13N6, H14N5, H15N9, H16N3 (MN≥1:10) 0 (0/200)	4	1	5	0	1	0	0	11

56	B. P. Khuntirat et al. Thailand	2011	Cross-sectional	Rural villagers	H2N2 (MN≥1:10) 40.2 (322/800) H5N2 (MN≥1:10) 0.1 (1/800) H6N1/ H6N2 (MN≥1:10) 0.2 (2/800) H7N7/ H7N2 (MN≥1:10) 0.1 (1/800) H9N2 (MN≥1:10) 4.7 (38/800) H10N4/ H10N7 (MN≥1:10) 0.1 (1/800) H12N5 (MN≥1:10) 0.1 (1/800) H11N2, H4N6, avH1N1 (MN≥1:10) 0 (0/800)	No	0	1	3	1	1	0	0	6
57	P. Kitikoon et al. Thailand	2011	Cross-sectional	Swine workers Control: unexposed population	swH1N1 (HI≥1:40) 79.5 (62/78)[b] swH1N2 (HI≥1:40) 66.7 (52/78)[b] swH1N1 (HI≥1:40) 5.0 (3/60) swH1N2 (HI≥1:40) 3.3 (2/60)	No	6	1	2	0	1	1	0	11
58	A. Kohls et al. Germany	2011	Cross-sectional	Active falconers	H5, H7 (MN≥1:80) 0 (0/43) H6, H9, H13 (HI) 0 (0/43)	No	0	0	3	0	0	0	0	3

59	J. H. Leibler et al. USA	2011	Cross-sectional	Poultry workers Control: unexposed population	H4N6, H5N2, H6N2, H7N2, H9N2 (MN≥1:10) 0 (0/24) H4N6, H5N2, H6N2, H7N2, H9N2 (MN≥1:10) 0 (0/75)	No	4	0	3	0	1	0	0	8
60	V. Shtjefni et al. Albania	2011	Cross-sectional	Swine farmers	swH1N1 (ELISA 0.4) 0 (0/30) swH3N2 (ELISA 0.4) 13.3 (4/30)	swH3N2 (HI≥1:20) 13.3 (4/30)	0	1	2	0	1	0	0	4
61	G. G. Arzey et al. Australia	2012	Outbreak investigation	Exposed abattoir workers Poultry at the same site	H10N7 (HI) 0 (0/7) Workers reported conjunctivitis (2 also rhinorrhea and 1 a sore throat), including the 2 with confirmed influenza A subtype H10 infection. 45 (27/60)	H10N7 (Neutralization) 0 (0/7)	0	0	5	0	0	3	2	10
62	L. Di Trani et al. Italy	2012	Serial cross-sectional	Poultry workers Control: unexposed population	H5N2/H5N7 (HI≥1:10) 2.1 (4/188) H7N1/H7N3 (HI≥1:10) 5.9 (11/188)[b] H5N2/H5N7 (HI≥1:10) 0.5 (2/379) H7N1/H7N3 (HI≥1:10) 0.8 (3/379)	H5N2/H5N7 (MN≥1:10) 0 (0/188) H7N1/H7N3 (MN≥1:10) 3.2 (6/188)[b]	6	0	5	0	0	2	0	13

63	G. Lopez-Robles et al. Mexico	2012	Cross-sectional	Swine workers Control: unexposed population	swH1N1 (HI≥1:32) 3.2 (2/62) swH3N2 (HI≥1:32) 12.9 (8/62)[b] swH1N1 (HI≥1:32) 0 (0/63) swH3N2 (HI≥1:32) 1.6 (1/63)	No	4	1	2	0	1	0	0	8
64	S. D. Pawar et al. India	2012	Cross-sectional	Poultry workers Control: general population	H9N2 (HI≥1:40) 4.7 (16/338) 0 (0/249)	H9N2 (MN≥1:40) 3.8 (13/338)	2	1	5	0	0	0	0	8
65	S. C. Shafir et al. USA	2012	Serial cross-sectional	Participants of meetings of the American Ornithologist's Union	H7N2, H7N3, H9N2 (MN≥1:40) 0.25 (1/401) H7N2, H7N3, H9N2 (MN≥1:40) 0 (0/401)	No	0	1	3	0	1	0	0	5
66	T. M. Uyeki et al. Vietnam	2012	Cross-sectional	Poultry market workers Control: unexposed population	H5N2, H9N2 (MN≥1:40) 0 (0/200) H9N3 (MN≥1:40) 0.5 (1/200) H9N7(MN≥1:40) 2.5 (5/200) H5N2, H9N2, H9N3 (MN≥1:40) 0 (0/200) H9N7(MN≥1:40) 3.5 (7/200)	H5N2, H9N2 (Westernblot) 0 (0/200) H9N3 (Westernblot) 0.5 (1/200) H9N7 (Westernblot) 2.5 (5/200)	6	0	4	0	1	0	0	11

67	K. K. Wong et al. USA	2012	Case contact study	Suspected cases among attendees of an agricultural fair and a children's agricultural club	swH3N2 (HI ≥1:40 or ≥1:10[a]) 4 seropositive and 16 indeterminate, out of 27 sera	swH3N2 (MN) Unknown	0	1	5	1	0	0	0	7
68	P. Yang et al. China	2012	Cross-sectional	Occupationally duck-exposed population	H7N2 (HI≥1:40) 0 (0/1741) H9N2 (HI≥1:40) 0.7 (12/1741)	No	0	0	2	1	0	0	0	3
69	P. Yang et al. China	2012	Cross-sectional	Participants without poultry-handling practices	H5 (HI) 0.2 (1/605) H7 (HI) 0 (0/605) H9 (HI) 0.8 (5/605)	No	0	0	2	0	0	0	0	2
70	N.A. Luetteke et al. Luxembourg	2012	Cross-sectional	Swine exposed population Control: unexposed population	swH1N1 (NT) Study states that swine workers have more positive antibody titers against pandemic flu and SIV compared to the control population. (no confounding by cross-reactivity) Detailed data not reported.	No	1	0	3	0	1	1	0	6

71	A. Ahad et al. Pakistan	2012	Cross-sectional	Occupationally poultry-exposed population Control: unexposed population	H7N7 (HI≥1:160) 29.0 (135/465)[b] H9N2(HI≥1:160) 44.9 (209/465)[b] H7N7, H9N2 (HI≥1:160) 0 (0/25)	No	2	0	2	0	0	0	0	4
72	T. Bai et al. China	2013	Cross-sectional	Poultry workers	H7N9 (HI≥1:20) 0.5 (17/1544)	H7N9 (MN≥1:20) 0 (0/1544)	0	0	5	0	0	0	0	5
73	P. J. Blair et al. Cambodia	2013	Cross-sectional	Rural villagers (generally exposed to swine and poultry) Control: healthy adults	H1N1, H2N2, H4N6, H5N2, H6N1, H6N2, H7N2, H7N7, H8N4, H10N7, H11N9, H12N5 (MN≥1:10) 0 (0/777) H9N2 (MN≥1:10) 2.7 (21/777) H1N1, H2N2, H4N6, H5N2, H6N1, H6N2, H7N2, H7N7, H8N4, H10N7 (MN≥1:10) 0 (0/4719) H9N2 2.9 (136/4719) H11N9, H12N5 (MN≥1:10) 0 (1/4719)	The two assays showed moderate agreement	2	1	0	1	1	0	0	8

74	A. Coman et al. Rumania	2013	Cross-sectional	Swine workers Control: unexposed population	H4N6, H5N2, H6N1, H7N7, H8N4, H10N4, H11N1 (MN≥1:10) 0 (0/312) H9N2(MN≥1:10) 9.9 (31/312)[b] H4N6, H5N2, H6N1, H7N7, H8N4, H10N4, H11N1 (MN≥1:10) 0 (0/51) H9N2(MN≥1:10) 3.9 (2/51)	No	6	1	3	0	1	0	0	11
75	R. Huang et al. China	2013	Cross-sectional	Poultry workers Control: unexposed population	H9N2 (HI≥1:40) 2.3 (9/382)[b] 0 (0/100)	H9N2 (MN≥1:40) 1.8 (7/382)[b]	2	1	5	0	0	0	0	8
76	N. Khurel-baatar et al. Mongolia	2013	Cross-sectional	Animal exposed population Control: unexposed population	EqH3N8 (MN≥1:10) 1.1 (4/358) H6N1(MN≥1:10) 0.3 (1/358) H9N2(MN≥1:10) 0.8 (3/358) H4N6, H5N2, H7N7, H8N4, H10N4 (MN≥1:10) 0 (0/358) EqH3N8, H4N6, H5N2, H6N1, H7N7, H8N4, H9N2, H10N4 (MN≥1:10) 0 (0/81)	No	4	1	3	0	1	0	0	9

77	W. S. Krueger et al. Thailand	2013	Cohort study	Rural villagers	Follow-up of study no. 56 In a 2 year time period increases in antibody titers were measured for H6N1 (1/800), H7N7 (1/800), H9N2 (y1: 21, y2: 40 /800) and H12N5 (2/800) Seroprevalence of SIV antibodies was high but probably confounded by cross-reactive antibodies against human influenza.	No	0	1	3	1	1	0	2	8
78	J. Okoye et al. Nigeria	2013	Cross-sectional	Poultry exposed participants Control: unexposed population	H5N2(MN≥1:10) 0.3 (1/316) H9N2(MN≥1:10) 1.3 (4/316) H11N1(MN≥1:10) 0.9 (3/316) H4N6, H6N1, H7N7, H8N4, H10N4, H12N5 (MN≥1:10) 0 (0/316) H5N2 (MN≥1:10) 1.8 (1/54) H4N6, H6N1, H7N7, H8N4, H9N2, H10N4, H11N1, H12N5 (MN≥1:10) 0 (0/54)	No	6	1	3	0	1	0	0	11
79	S. Su et al. China	2013	Cross-sectional	Veterinarians Control: unexposed population	H7N3 (HI≥1:80) 1.5 (6/406) H9N2 (HI≥1:80) 3.7 (9/406) H7N3, H9N2 (HI≥1:80) 0 (0/83)	No	2	0	2	0	0	0	0	4

80	Q. Yu et al. China	2013	Cross-sectional	Poultry workers	H9N2 (MN≥1:80) 4.6 (14/305)	No	0	1	3	1	0	0	0	5
81	R. Zu et al. China	2013	Case contact study	Direct contacts Swine in slaughterhouse	swH1N1 (HI≥1:40) 2.7 (3/113) 42.8 (9/21)	No	0	0	2	0	1	2	2	7
82	W. Krueger et al. USA	2013	Cross-sectional	Occupationally dog exposed Control: unexposed population	canineH3N8 (MN≥1:10) 20.7 (63/304) 12.1 (13/101)	canineH3N8 (NI) 19.1 (58/304)	6	1	3	0	1	0	0	11
83	E. Anvar et al. Iran	2013	Cross-sectional	Occupationally poultry-exposed population	H9N2 (HI≥1:160) 1.6 (3/182)	H9N2 (ELISA) 11.5 (21/182)	0	0	4	1	0	0	0	5
84	N. Khurel-baatar et al. Mongolia	2014	Cohort study	Adults, many with occupational exposure to animals Control: unexposed population	Follow-up of study no 77 In a 2-year period titer increases were detected (MN≥1:10) for eqH3N8 (25/351), H6N1 (4/351), H9N2 (11/351) and H10N4 (1/351) Titer rises were not associated with animal exposure	No	2	1	3	0	1	0	2	8

85	A. Krumb-holz et al. Germany	2014	Cross-sectional	Swine exposed population Control: unexposed population	swH1N1 (HI≥1:20) 37.0 (134/362)[b] swH3N2 (HI≥1:20) 59.7 (216/362)[b] swH1N1 (HI≥1:20) 7.7 (20/260) swH3N2 (HI≥1:20) 43.1 (112/260)	No	4	1	2	0	1	0	0	8
86	S. Yang et al. China	2014	Cross-sectional and case studies	Live poultry market workers Confirmed cases Control: Inhabitants of region with previous H7N9 human infections	H7N9 (HI≥1:80) 6.3 (25/396) [b] 60 (27/45) [b] 0 (0/1129)	H7N9 (MN) Unknown, but correlated well.	2	1	5	1	1	0	0	10
87	P. Zhou et al. China	2014	Cross-sectional	Occupationally swine exposed Control: unexposed population	H9N2 (HI≥1:160) 1.8 (37/2006)[b] 0 (0/83)	H9N2 (MN≥1:160) 1.2 (24/2006)	2	0	5	1	0	0	0	8
88	W. Qi et al China	2014	Cross-sectional	Animal workers Control: unexposed population	H10N8 (HI≥1:20) 3.0 (21/710) 0 (0/107)	H10N8 (MN≥1:40) 0.4 (3/710)	4	1	5	0	0	0	0	10
89	H. Zhou et al China	2014	Cross-sectional	Veterinarians	H7N9 (HI≥1:20) 0 (0/400)	No	0	0	2	0	0	0	0	2

90	S. D. Pawar et al India	2014	Cross-sectional	Poultry workers involved in H5N1 outbreaks Control: general population	Data from study no 64 included in H9N2 prevalence H7N9 (HI) 0 (0/466) H9N2 (HI) 8.6 (29/466)[b] H7N9, H9N2 (HI) 0 (0/162)	H9N2 (MN) 4.1 (19/466)	2	0	5	0	1	0	0	8
91	X. Yin et al China	2014	Cross-sectional	Swine workers Control: unvaccinated healthy city residents	swH1N1 (HI≥1:40) 10.7 (124/1162)[b] 0 (0/104)	swH1N1 (NT≥1:40) 7.8 (91/1162)	2	1	5	1	1	0	0	10
92	H. Zhou et al China	2014	Cross-sectional	Swine farm workers Control: healthy city residents	swH1N1 (HI≥1:80) 11.1 (61/546)[b] 4.8 (4/83)	swH1N1 (NT≥1:80) 10.0 (57/546)	2	1	5	1	1	1	0	11

93	A. Coman et al Romania	2014	Cohort study	Swine farm workers Control: unexposed population	Follow-up of study no 74 In a 2-year period titer increases were detected in three individuals (MN≥1:80) for H6N1, H9N2 and H12N5 (3/351). None of them reported poultry exposure. Seroprevalence of SIV antibodies was high but probably confounded by cross-reactive antibodies against human influenza. Only swH3N2 antibodies lacked evidence for cross-reactivity.	No	6	1	3	0	1	0	2	13
94	G. Gray et al Cambodia	2014	Cohort study	Rural villagers (generally exposed to swine and poultry)	Follow-up of study no 73 In a 2-year period titer increases were detected (MN≥1:10) for H4N6 (1/800), H6N1 (3/800), H9N2 (9/800), H11N1 (6/800) and H12N5 (2/800) One 4-fold rise in MN titer was found against H12N5 Seroprevalence of SIV antibodies was high (HI) but probably confounded by cross-reactive antibodies against human influenza.	No	0	1	2	0	1	0	2	6

95	Q. Wang et al China	2014	Cross-sectional	Occupational poultry-exposed population Control: Unexposed population	H9N2 (HI≥1:40) 12.3 (103/840)[b] 2.7 (47/1730)	H9N2 (MN≥1:20) Good correlation with HI assay.	2	0	5	1	1	2	0	11

[a] test results for children <4 years of age were considered seronegative if HI titers to the variant strains were <10, indeterminate if titers were 10 to <40, and seropositive if titers were >40. Test results for children 4–13 years of age were considered seronegative if titers to the variant strains were <10 and indeterminate if titers were >10; [b]Significant difference with the control group

CHAPTER 5

CHANGES IN HETEROSUBTYPIC ANTIBODY RESPONSES DURING THE FIRST YEAR OF THE 2009 A(H1N1) INFLUENZA PANDEMIC

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ABSTRACT

Seropositivity to avian influenza (AI) via low-level antibody titers has been reported in the general population and poultry-exposed individuals, raising the question whether these findings reflect true infection with AI or cross-reactivity. Here we investigated serological profiles against human- and avian influenza viruses in the general population using a protein microarray platform. We hypothesized that higher antibody diversity across recent H1- and H3 influenza viruses would be associated with heterosubtypic reactivity to older pandemic- and avian influenza viruses. We found significant heterogeneity in antibody profiles. Increased antibody diversity to seasonal influenza viruses was associated with low-level heterosubtypic antibodies to H9- and H7-, but not to H5 avian influenza virus. Individuals exposed to the recent 2009 A(H1N1) pandemic showed higher heterosubtypic reactivity. We show that there is a complex interplay between prior exposures to seasonal and recent pandemic influenza viruses and the development of heterosubtypic antibody reactivity to animal influenza viruses.

INTRODUCTION

Influenza virus infection triggers the generation of antibodies as part of the humoral component of the host immune response. These antibodies, produced by specialized B-cells, are predominantly directed against the surface protein hemagglutinin (HA), and to a lesser extent, the neuraminidase (NA) and internal structures, such as the nucleoprotein and the matrix proteins[1]. HA and NA are used to classify influenza viruses into different subtypes. The 16 currently known HA-subtypes, originating from birds, divide into two phylogenetic groups based on their amino-acid composition, and these further segregate into 5 clades. Group 1 consists of three clades spanning ten HA-subtypes (H1, H2, H5, H6; H8, H9, H12; H11, H13, H16), whereas HA-subtypes H3, H4, H14 and H7, H10, H15 form the two clades of group 2[2,3]. The HA consists of three monomers forming the variable globular head (HA1), which contains the receptor-binding site, and the more conserved stem region (HA2). The HA protein plays an important role in infection of host cells through the release of viral RNA into the host cell by means of membrane fusion[4]. Antibodies targeting influenza viruses can have neutralizing- or non-neutralizing ability. Non-neutralizing antibodies play a vital role in the immune response by e.g., inducing phagocytosis, complement-mediated lysis or antibody dependent cellular cytotoxicity (ADCC)[5]. Neutralization of influenza viruses can be achieved in two ways; either by blocking the receptor-binding pocket located in the HA1, or by preventing conformational changes in a region involved in membrane fusion, mainly formed by HA2[6]. The majority of antibodies target the HA1[7]. However, antibodies binding to the HA2 are able to neutralize various subtypes, reduce virus replication and contribute to a faster recovery[8]. Immunoglobulins targeting structures conserved among subtypes are termed as 'cross-reactive'. A number of broadly reactive intra-subtype-, intra-clade-, intra-group- and inter-group specific neutralizing human and mouse monoclonal antibodies targeting the globular head- or the stem region of the HA have been identified (reviewed by Laursen and Wilson[9]). Their possible role in influenza virus infection has become an area of considerable interest since the occurrence of the most recent H1N1 influenza pandemic in 2009 [A(H1N1)pdm09]. Hancock et al.[10] investigated whether seasonal, trivalent influenza vaccines are able to induce cross-reactive antibodies against the A(H1N1)pdm09 virus but did not find such antibodies after vaccination[10]. However, the authors reported on no or little pre-existing antibodies in individuals younger than 30 years of age, whereas in older adults some degree of neutralizing or cross-reactive antibody concentrations was detected in samples collected before the onset of A(H1N1)pdm09 circulation[10]. Wrammert et al.[11] studied the serological response after natural infection with A(H1N1)pdm09 in humans and postulated that broadly cross-reactive antibodies targeting epitopes conserved between different influenza virus strains were induced via the activation memory B-cells. The detected antibodies predominantly targeted the HA2 and to a lesser extent HA1 of pre-pandemic H1 strains. Broadly H1N1-neutralizing antibodies also cross-reacted with avian subtype A(H5N1)[11]. These and subsequent studies showed that cross-reactive antibodies are boosted when infection occurs with a significantly mismatched HA[12].

The effects of broadly-reactive influenza antibodies have not been studied extensively. Specifically, it is unknown if broadly reactive antibodies have any neutralizing effect during an avian influenza (AI) virus infection or if they generate false positive results in seroepidemiological studies on AI viruses. Zoonotic AI viruses pose a threat to public health; for instance, the highly pathogenic (HP) A(H5N1) subtype first crossed the species barrier into humans in 1997[13,14]. Since then, more than 800 human infections of A(H5N1) have been reported to the World Health Organization, of which 53% succumbed to the disease[15]. In recent years, additional HP and low pathogenic (LP) AIs have expanded the list of zoonotic subtypes causing incidental infection, e.g. LP H9N2, H6N2, H10N8, as well as various HP and LP H7 strains. Until recently, H7 strains were associated with mild symptoms in humans[16] but in March 2013 a novel LPAI subtype (H7N9) emerged in China and has caused three waves of human infection associated with severe symptoms and a high case fatality rate[17,18]. Case fatality rates can be inflated if they only capture the most severe cases while mild or subclinical cases are underreported[19,20]. Sero-epidemiological studies are a useful way to shed light on the true extent of a population's exposure to a particular virus. A number of serological studies have put forth evidence of human exposure to AIs in humans that work with animals[21–27] as well as in putatively non-poultry-exposed control groups[28,29]. These findings pose the important question of whether serological reactivity against AI virus antigens reflects true exposure or is caused by cross-reactive antibodies.

In the present study, we investigated serological profiles against different human and AI virus subtypes during the course of the 2009 pandemic in a group of healthy childbearing age women via neonatal heelprick filter cards. Cards were collected continuously over a 100-week period employing a continuous collection study design[30]. Samples were analysed by means of a protein microarray comprising recombinant proteins representing the globular head domain (HA1) of various influenza virus HAs, as described previously[30]. Vaccination history of the mothers was unknown. Understanding serological profiles of healthy humans can help in distinguishing heterosubtypic antibody reactivity from serological response triggered by true infection.

Here, we hypothesized that the profile of antibody reactivities to a range of recent human influenza viruses could be used to explain the presence of cross-reactive antibodies to AI antigens (H5, H7 and H9). We found evidence that supported this hypothesis and showed that cross-reactive antibody levels to AI and ancient influenza virus subtypes significantly increased after the onset of the 2009 H1N1 pandemic.

RESULTS

Exploratory analysis

Characteristics of the study population are shown in Table 1. The majority of samples collected through the heel prick-screening program were submitted from countries located in the northern hemisphere (n=6896), with only 688 samples collected in the southern

hemisphere. Submission periods differed per country and covered the time span from week 40 in 2008 to week 34 in 2010 (Table 1).

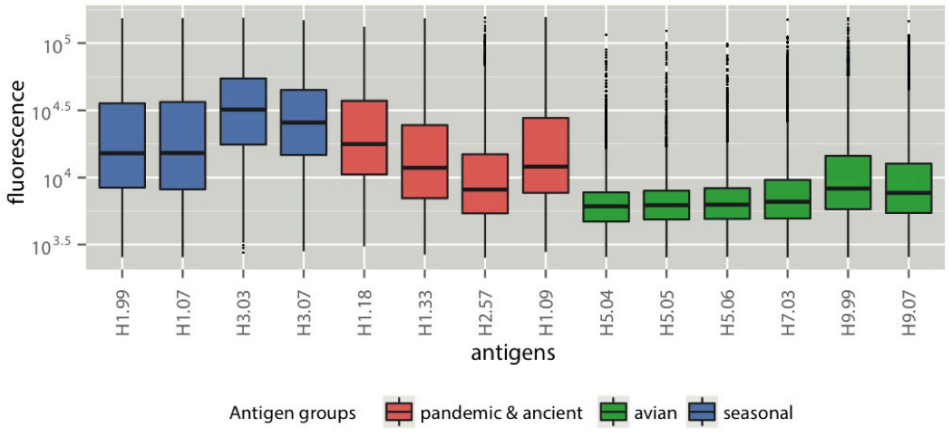
The highest antibody levels were directed to seasonal H1 and H3 antigens, as expected (Figure 1a, blue). Elevated signals against antigen H1.09 were observed (Figure 1b), and their levels were clearly associated with the onset of the pandemic in the second half of the study (Figure 1b). Similarly, raised signals against 1918-lineage influenza strains (H1.18, H1.33) were associated with pandemic onset (see supplementary material S1), as the H1.18 antigen is known to be antigenically similar to A(H1N1)pdm09[31]. In 1957 subtype H2N2 (represented by antigen H2.57) caused the second major human pandemic of the 20th century. In 1968 – before the mothers of our study subjects were born – H2N2 ceased circulating in the human population[32]. Nevertheless, antibody signals to this antigen were raised, albeit at significantly lower levels compared to reactivity against recent H1 and H3 antigens (Figure 1a, red; Wilcoxon signed rank test, p-values<0.001). Antibody signals to H9.99 were similarly raised as those against H2.57 (Wilcoxon signed rank test: p-value=0.15). Fluorescence levels against H9.07 and H7.03, although also elevated, were significantly lower compared to H2.57 (Wilcoxon signed rank test, p-values<0.001). Reactivity against these AI antigens was most likely caused by cross-reactive antibodies[33,34]. No noteworthy reactivity against H5-antigens was found (Figure 1a, green).

Antibody diversity across human influenza A antigens and its relation with heterosubtypic reactivity

To examine antibody diversity in our study subjects, we introduced the adapted Shannon diversity index (ASDI; see Methods and supplementary material), which aims to represent the number of antigens to which an individual has a high titer response. The influenza antigens included in the ASDI measure represented recent seasonal influenza viruses H1.99, H1.07, H3.03 and H3.07. ASDI values were calculated per individual and ranged from 0.64 to 4.0, with a value of 4.0 meaning that the individual had high titer responses to all four antigens in the ASDI measurement. We arbitrarily divided the ASDI range into four categories to assess corresponding serological profiles and investigate the association between heterosubtypic responses and increasing ASDI. The majority (~77%) of individuals had antibody signals to 1.5 to 3.5 antigens (ASDI categories 2&3; Table 1). The category with lowest diversity (0-1.5; n=416) was characterized by the lowest level of H3 responses when compared to other ASDI categories and comprised slightly raised signals to H1.18 and H1.09 antigens (Figure 2). A similar pattern was observed for category two (1.5-2.5; n=2548) with predominant seasonal H3 signals, together with somewhat elevated seasonal and pandemic H1 responses. We also observed high H3 responses in the third category (2.5-3.5; n=3272), albeit in combination with markedly increased H1 signals compared to lower categories. The fourth category (3.5-4; n=1348) comprised individuals with the highest antibody diversity and reactivity against H1.09. Pandemic, seasonal H1, and seasonal H3 antigens were approximately equally strong in this group, partly reflecting saturated luminescence signals in the assay. A total of 161 individuals (2.1% of the total population; 0.8% and 9.9% of diversity category 3 and 4,

respectively) had saturated fluorescence values for H1.09 and all four seasonal antigens; all but one of these individuals were sampled after pandemic onset.

Figure 1. a) Overall antibody reactivity against different antigens for the entire study period (week 40, 2008 to week 34, 2010) including all countries. **b)** Development of A(H1N1)pdm09 over time for all countries combined. Pandemic onset and -course per country were previously described in de Bruin et al [30]. Both y-axes represent fluorescence values on a log10-scale.



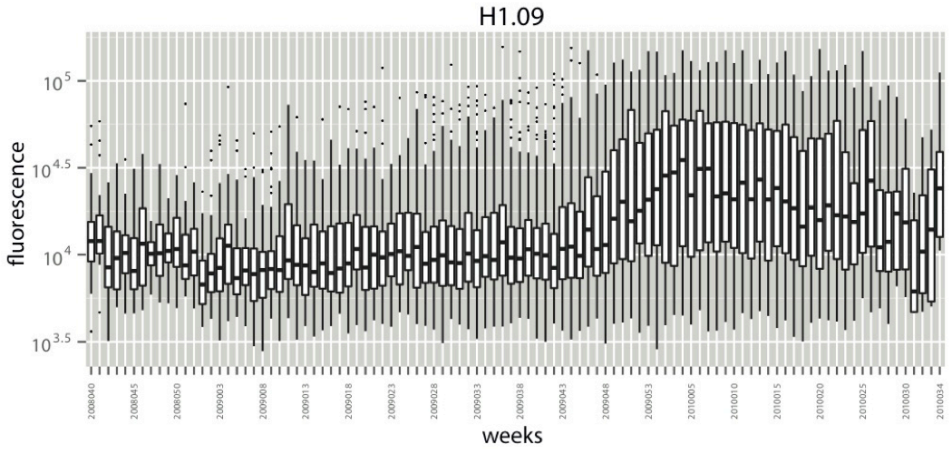


Table 1. Characteristics of study population and number of samples submitted per country and time period. Diversity index categories reflect percentage of individuals within the respective category.

						Diversity index categories			
						1	2	3	4
Continent	**Country**	**Country totals**	**Row totals**	**Pandemic onset[1,2]**	**Sampling period (year/week)**	**0-1.5**	**1.5-2.5**	**2.5-3.5**	**3.5-4**
North America	**Canada**	913	444	Pre	08/40-09/32	6.8	34.7	45	13.5
			469	Post	09/33-10/26	1.1	17.1	48	33.9
	Mexico (central)	579	272	Pre	09/26-09/45	21	40.4	32.7	6.2
			307	Post	09/46-10/24	8.1	41.7	36.2	14
	Mexico (northern)	432	432	Pre	09/01-09/044	18.8	50.9	26.6	3.7
			0	Post	NA				
	USA	520	130	Pre	09/28-09/40	0.8	24.6	47.7	26.9
			390	Post	09/41-10/26	0.3	8.7	38.7	52.3
Europe	**The Netherlands**	559	188	Pre	09/27-09/45	1.6	46.8	40.4	11.2
			371	Post	09/46-10/30	0.5	32.1	50.1	17.3
	Portugal	479	130	Pre	09/28-09/40	13.8	54.6	28.5	3.1
			349	Post	09/41-10/23	5.2	33.8	44.4	16.6
	Sweden	868	526	Pre	08/40-09/40	2.5	46.4	44.3	6.8
			342	Post	09/41-10/22	0.3	20.2	49.1	30.4
	Switzerland	637	180	Pre	09/23-09/40	3.3	31.1	52.2	13.3
			457	Post	09/41-10/34	3.5	33.5	50.3	12.7
	UK	568	190	Pre	09/27-09/45	7.9	39.5	40	12.6
			378	Post	09/46-10/30	2.6	27.5	47.4	22.5
Asia	**India**	474	120	Pre	09/28-09/40	4.2	31.7	51.7	12.5
			354	Post	09/41-10/25	3.4	34.5	48.3	13.8
	Japan	530	140	Pre	09/27-09/40	1.4	21.4	51.4	25.7
			390	Post	09/41-10/26	0.3	13.6	47.7	38.5
	Lebanon	337	337	Pre	09/02-09/44	11.3	49.3	34.4	5
				Post	NA				
Africa	**South Africa**	276	248	Pre	09/29-10/15	12.1	48	35.1	4.8
			28	Post	10/16-10/21		42.9	42.9	14.3
South America	**Argentina**	412		Pre	NA				
			412	Post	09/27-10/16	6.6	37.1	43.4	12.9
Total range of study weeks:					08/40-10/34				
Totals per pandemic period	pre: 3337	post: 4247							
Totals per diversity category						416	2548	3272	1384
Total									7584

[1]Pandemic onset of A(H1N1)pdm09; [2]pre- and post pandemic onset of circulation of A(H1N1)pdm09 per respective country, percentages are represented within pandemic period; NA: not available

Analysis of Broad responders

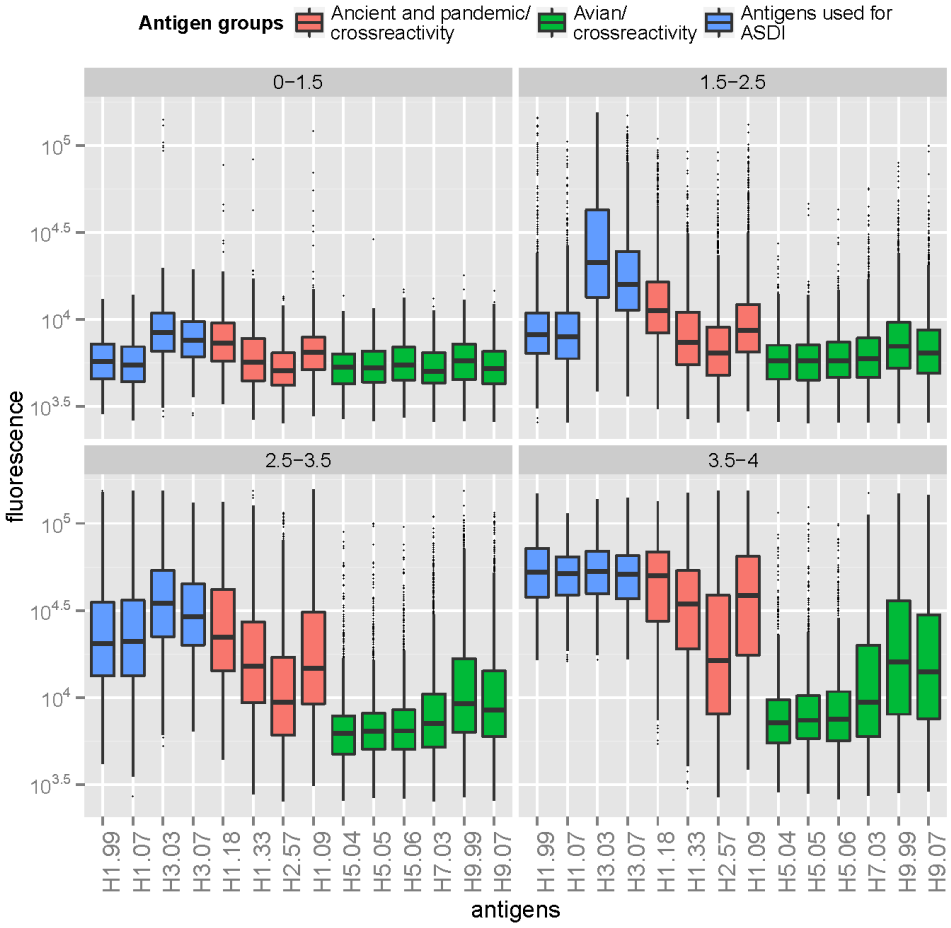
Based on the high and broad signals in the fourth category (3.5 < ASDI ≤ 4.0), we designated subjects in this category as 'broad responders', i.e. individuals showing serological responses to between 3.5 and 4 antigens. Approximately half of the individuals in the fourth category (52%, n=702) were from the northern hemisphere (Canada, USA, Sweden, United Kingdom and Japan) and had been sampled after pandemic onset (Table 1).

Consistent with our hypothesis, we observed a gradual elevation in heterosubtypic reactivity against avian influenza antigens with increasing diversity categories (Figure 2, green). Broad responders (3.5 < ASDI ≤ 4.0) showed the highest reactivity against avian antigens, which was most pronounced for H9, followed by H7; reactivity to H5 antigens remained low (Figure 2, green). However, some cross reactivity was found in each diversity category, and we found statistically significant Spearman's rank correlation coefficients between diversity indices and AI HA1 reactivities (0.25-0.29 for H5, 0.35 for H7 and 0.42-0.43 for H9; all $p < 10^{\wedge -15}$). Similarly, some heterosubtypic reactivity against ancient and older pandemic human strains (H1.33 and H2.57) was detected with increasing diversity categories (Figure 2, red).

Explaining heterosubtypic reactivity by serological responses to recent seasonal and pandemic strains

We performed a multivariable linear regression analysis using reactivity to human seasonal and recent pandemic influenza HA1 antigens as explanatory variables to explore the relationship between serological responses to AI antigens and infection with recent human influenza virus strains (Table 2). Antibody reactivities to most included antigens (i.e. explanatory variables) were able to explain the variation observed in H7- and H9 signals to some extent (Table 2), as these reactivities predominantly tended to be positively correlated (Table 2). However, antibody reactivities to the most recent human influenza strains H1.09 and H3.07 had a larger relative effect on the variation of H7 and H9 signals compared to signals against other antigens, indicating that recent infection with these viruses can explain part of the low-level heterosubtypic antibody reactivity to AI virus antigens. Nevertheless, the models could only explain between 28% and 38% of observed variation in H7 and H9 avian responses, suggesting a more complex relationship (Table 2). Multicollinearity between explanatory variables was not an issue as variance inflation factors for all explanatory variables in all models remained below 10 (range: 1.41-3.59). Likewise, testing model assumptions revealed no overt violations of homoscedasticity and deviation from normality of residuals after log-transformation. Regression analysis was not attempted for H5, given the low antibody signals.

Figure 2. Serological profiles based on adapted Shannon diversity index (ASDI) categories. Recent seasonal influenza virus antigens were used to calculate ASDI per individual to summarize individual antibody profiles in one measure (blue). Assumed cross-reactive antibody responses are depicted in red (ancient- and older pandemic influenza virus strains) and green (avian influenza virus strains). Fluorescence values representing serological reactivity per antigen (x-axis) are shown on a log10-transformed y-axis.



The influence of A(H1N1)pdm09 on the level of cross-reactive antibodies

To further examine whether exposure to the novel pandemic influenza strain A(H1N1)pdm09 was associated with increased cross-reactivity, we divided the data set into two periods of before (n=3337) and after pandemic onset (n=4247) (Table 1). We observed a clear shift in proportion of broad responders (category 4) towards higher diversity categories after pandemic onset (Chi-squared test, p-value<0.001) (Table 3a). Proportions of H1.09-seropositive individuals gradually increased with increasing diversity index categories for

pre- and post-pandemic periods. Within each category, changes in proportions between pandemic periods according to seropositivity status were significant for all but the lowest category, which could not be tested due to too few observations (Chi-squared test, p-values< 0.001, Table 3b). The vast majority of H1.09-seropositive individuals were sampled in the post-pandemic period (3.3% pre-, versus 29% post pandemic, Table 3b).

Table 2. Regression coefficients calculated on log2-transformed data spanning pre- and post-pandemic periods. Outcome refers to reactivity against heterologous antigens on which serological responses against recent human influenza virus antigens were regressed (explanatory variables). The number of asterisks indicates level of significance.

	Estimates with standard errors (SE)						
Outcome	**Intercept**	**H1.99**	**H3.03**	**H1.07**	**H3.07**	**H1.09**	**Adjusted R2**
H7.03	5.99	-0.03	0.05	0.05	**0.16**	**0.25**	0.28
SE	0.14	0.013	0.011	0.013	0.012	0.009	
	***	*	***	***	***	***	
H9.99	3.90	-0.12		0.14	**0.29**	**0.35**	0.38
SE	0.15	0.014		0.014	0.011	0.010	
	***	***		***	***	***	
H9.07	4.22	-0.05	0.08	0.11	**0.16**	**0.32**	0.37
SE	0.15	0.013	0.011	0.014	0.013	0.011	
	***	***	***	***	***	***	

Significance codes: 0 '***'; 0.001 '**', 0.01 '*', 0.05 '.', 1' '

Next, we only included data from persons sampled after the pandemic onset in the analysis, to ensure that serological responses were truly triggered by H1.09 infection or vaccination (Table 3b). With exception of the lowest ASDI category that only comprised four individuals in the H1.09 positive category (Table 3b), within higher ASDI categories, we generally observed higher heterosubtypic antibody responses among H1.09-positive individuals compared to negative ones (Figure 3). Post-pandemic onset, 57% of broad responders (highest diversity category 4) were seropositive for H1.09 (Table 3b). In this category, we also observed significantly higher levels of H7 and H9 antibodies compared to H1.09-negative persons (Figure 3; Wilcoxon rank sum test, p-value<0.001).

Heterosubtypic reactivity and its consequences of for seroprevalence studies of avian influenza in humans

When estimating the proportion of individuals with titers of approximately higher than 80 against AI antigens (based on an arbitrary cut-off applied to fluorescence values as described in the Methods section), we found that overall, about 1% (n=53-80) of individuals had antibody titers >80 for H5 antigens, and 4.3% (n=329), 9.5% (n=720) and 7.5% (n=571) of individuals showed titers >80 to H7.03, H9.99 and H9.07, respectively. The majority thereof were sampled in the post-pandemic period [1% (n=49-76), 6.5% (n=276), 13.7% (n=580) and 10.8% (n=460), respectively, Figure 4].

Figure 3. Antibody profiles of samples collected after pandemic onset, stratified according to adapted Shannon diversity index (ASDI) and seropositivity status to A(H1N1)pdm09.

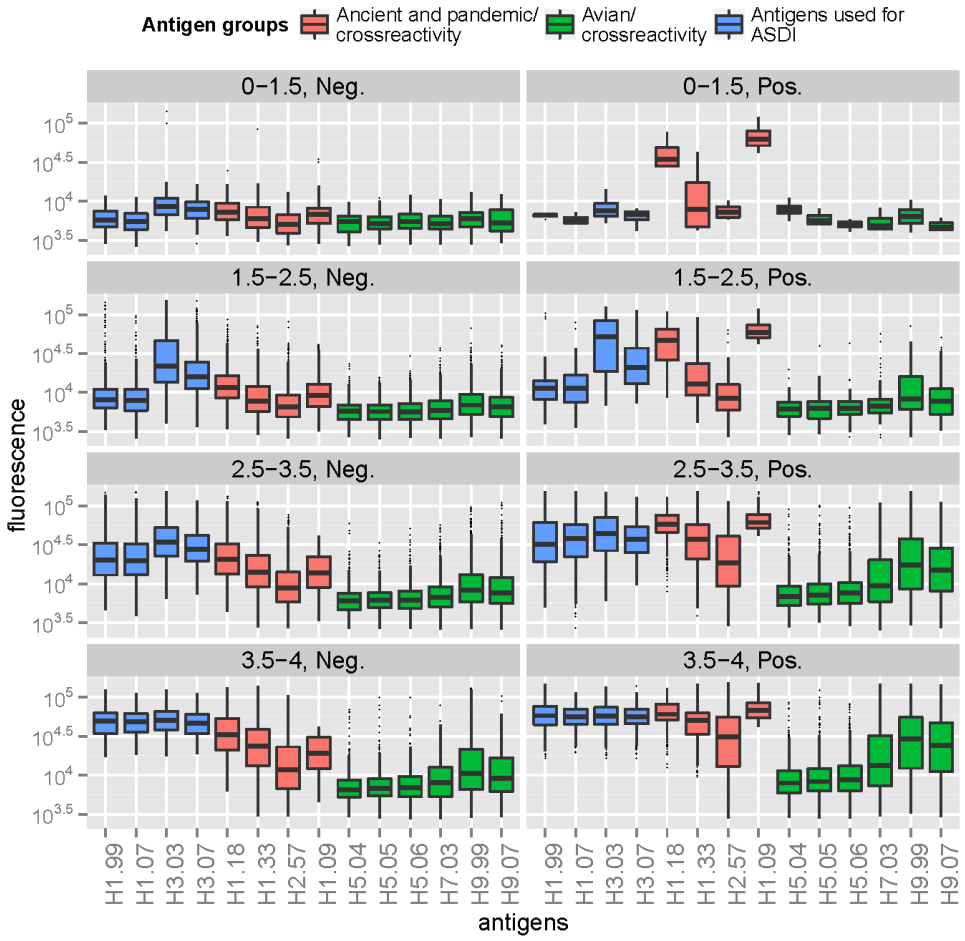


Table 3. A) Number and proportions of individuals per category based on adapted Shannon diversity index (ASDI) versus pre- and post-pandemic periods. B) Number and proportion of H1.09-positive and -negative individuals per diversity category before (n=3337) and after (n=4247) pandemic onset, respectively.

			Diversity index category								Total	
			1		2		3		4			
		ASDI range:	0-1.5		1.5-2.5		2.5-3.5		3.5-4			
		Pandemic onset:	pre	post	pre	post	Pre	post	pre	post	pre	post
A)	Per diversity category & pandemic period	N	298	118	1403	1145	1319	1953	317	1031	3337	4247
		% pre/post, by diversity category	71.6	28.4	55.1	44.9	40.3	59.7	23.5	76.5		
		% in diversity category, by pre/post	8.9	2.8	42	27	39.5	46	9.5	24.3	44 (100%)	56 (100%)
B)	H1.09 antibody reactivity	N negative	298	114	1395	1067	1265	1391	270	443	3228	3015
		%	100	96.6	99.4	93.2	95.9	71.2	85.2	43.0	96.7	71
		N positive	0	4	8	78	54	562	47	588	109	1232
		%	0	3.4	0.6	6.8	4.1	28.8	14.8	57	3.3	29
		N within diversity category	416		2548		3272		1348		7584	
		Total %	5.5		33.6		43.1		17.8		100	
		Total N H1.09-negative/ positive (%)									6243 (82.3)	1341 (17.7)

*Category of 'Broad responders', defined as showing the highest antibody diversity across recent seasonal human influenza viruses (H1.99, H1.07, H3.03, H3.07), expressed by the Adapted Shannon diversity indices (ASDI).

DISCUSSION

In this study, we investigated serological profiles against human influenza virus subtypes in healthy humans from around the world and studied associations between serological responses to seasonal influenza viruses and heterosubtypic reactivity, defined as presence of antibodies to influenza viruses that have not circulated among humans. A validated microarray platform[35] comprising recombinant HA1 proteins, i.e. the globular head of the HA, was used to provide standardized serological profiles to a range of influenza A virus antigens in this population-based study.

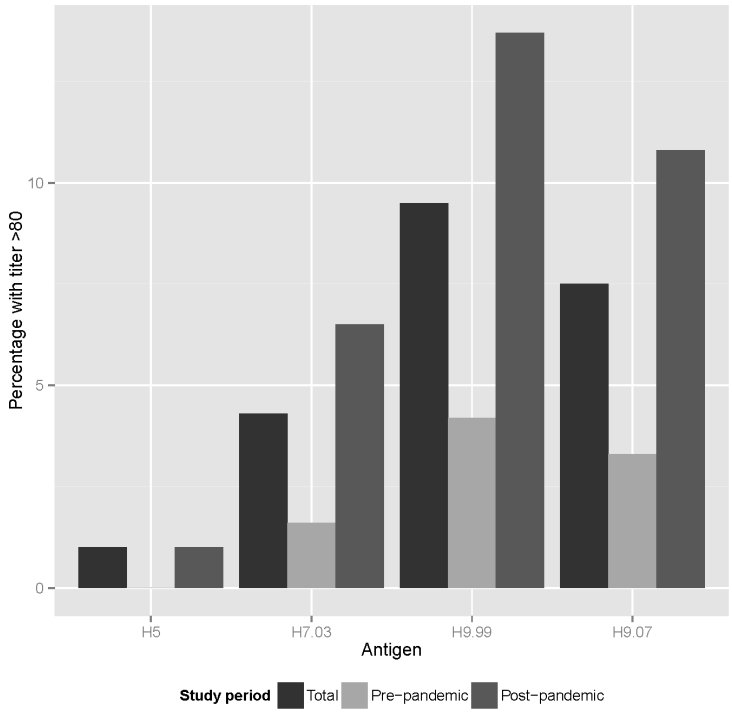
The highest antibody titers were observed against H3-virus antigens [A(H3.03), A(H3.07)]. This subtype emerged in the human population in 1968, and in 1977, subtype A(H1N1) re-emerged and co-circulated with A(H3N2) until 2009[32]. Given the assumed range of birth years of our study population (between 1968 and 1990), a significant proportion of individuals was probably primed (i.e. experienced their first influenza infection) by subtype A(H3N2) influenza viruses. These findings are consistent with the observation of original antigenic sin[36,37], a phenomenon by which an individual's first influenza infection imprints a high life-long specific antibody titer in that individual. Under this hypothesis, infections with recent strains thereby serve as a booster for antibodies to the `original' strain, whereas specific antibodies to the recent strain itself may be detected at low or moderate levels.

A number of serosurveillance studies conducted on high risk groups revealed serological evidence for avian viruses[23–25,38–40], although the possibility of cross-reactive antibodies for most studies was raised in a commentary article[41]. The level of seropositivity may also be influenced by the type of assay and respective cut off levels used (e.g. hemagglutination inhibition- (HI), microneutralization (MN)- or pseudotype-based assay)[42]. In this study, we investigated cross-reactive antibodies to AI virus antigens in the general population, and its relation to antibody diversity against recent human influenza virus strains by developing an adapted Shannon diversity index (ASDI) as a summary measure describing both antibody diversity and total antibody concentration. Consistent with our hypothesis, we demonstrated a positive association between increasing antibody diversity and heterosubtypic reactivity against AIV antigens. These observations are consistent with previous publications that have found raised antibody titers to AI antigens in the general population[28,33,39,43,44]. A population-based studies on heterosubtypic immunity of intravenous immunoglobulins (IVIG) from blood donors from Australia, Malaysia and Europe clearly showed binding of heterosubtypic antibodies against H9 and H5, but negligible binding against H7 subtypes by immunoblotting. In IVIG formulations from all regions neutralizing ability could be confirmed for H5 subtypes using cell culture[45]. Consistent with our findings, a study conducted in the general population from rural and urban locations in Vietnam, using the same protein microarray, reported similarly elevated antibody titers to H9 and to a lower extent H7 and H5 antigens[33]. Similarly, depending on the cut-off used (ranging from 80 to 20), 0.25% to 9.4% and 1.8% (cut-off of 20) of the general population in Wuhan, China, tested seropositive for H9 and H7 by HI-assay, respectively[43]. Serological evidence for antibodies to influenza A(H5N1)

was also detected in the Italian general population by single radial hemolysis, but could not be confirmed by HI- or MN-assay[44].

With the onset of the A(H1N1) pandemic, pre-existing immunity and heterosubtypic antibody responses to AI viruses in the general population became of significant interest[10–12]. For this reason, we focussed our analysis of cross-reactive antibody patterns on this cohort, to perform inference on AI antibody concentrations only, and to describe associations with other covariates. During the unfolding of the pandemic, overall cross-reactive responses to AI viruses increased. Multiple linear regression analysis suggested that H1.09 responses could explain part of this heterosubtypic reactivity but there was considerable heterogeneity in antibody profiles, with persons responding differently to similar challenges, most likely due to differences in exposure history. Therefore, the history of exposures to human seasonal and pandemic influenza exposures, natural or vaccine-induced, can influence levels of antibodies that bind to animal viruses [46].

Figure 4. Estimated proportions of individuals with titers to avian influenza virus antigens of higher than approximately 80. Proportions are presented for the total study period and split according to pre- and post pandemic periods. Reactivity to the three H5 antigens is combined.



The present study design had some limitations that need to be weighed when looking at the effects of human influenza infections on heterosubtypic antibody response. First, we were unable to discern whether serological reactivity against H1.09 was triggered by natural infection or by vaccination with the novel pandemic strain, as the onset of circulation of A(H1N1) pdm09 coincided with the beginning of vaccination campaigns in the majority of participating countries[30]. A recent study in pregnant women demonstrated that immune response elicited by vaccination to A(H1N1)pdm09 was significantly higher than after natural infection. This observation was also reflected in newborns of vaccinated mothers, with 89.5% showing antibodies to the pandemic strain, compared to 15.8% of infants born to naturally infected mothers[47]. Second, a limitation is that all study participants were of childbearing age. Although this subset of the general population represents an unbiased sample, extrapolation of these conclusions to the general population, including young infants and older age groups, should be made with caution. The assumption that our study participants were between 20 and 40 years of age, seemed appropriate. Estimated mean ages of women at childbearing ranged from 26.3 to 30.9 between 2005 and 2010 for the participating countries[48]. However, we cannot fully exclude that a small proportion of individuals was older and experienced natural infection with H2N2, a subtype that may generate additional cross-reaction to AI. Furthermore, no information on poultry exposure was available. Given the near global distribution of AI, we cannot exclude that part of the study population might have been exposed to and possibly infected with AI viruses. However, only five participating countries (the Netherlands, USA, Canada, India, Japan) reported AI outbreaks (H5, H7) in birds during the period of our study[49].

Finally, whereas the microarray platform serves as an excellent screening tool to investigate population exposure to various influenza virus HA1s in a standardized fashion, it cannot provide information on functionality of heterosubtypic antibodies against avian antigens detected in our study (i.e. neutralizing ability), neither can it measure cross-reactive antibodies against the HA2 stem region of the HA. Previous experiments using the entire recombinant HAs of different subtypes in the microarray platform had low discriminatory ability, as the HA2 is more conserved between different influenza virus subtypes. Using HA1s, therefore, provides a better resolution of antibodies targeting the more variable globular head of the HA, thereby allowing subtype discrimination. Although we cannot directly generalize antibody reactivities against the HA1 to the entire HA, we previously showed good correlation between antibody titers measured by HI and by microarray HA1 proteins[33,35]. Longitudinal studies examining heterosubtypic responses using functional assays would shed light on this issue. This is in fact the major limitation in all human serological studies of AI - it is unknown whether assay results correlate to any level of severity or protection from infection. Given the pandemic potential of AI virus subtypes, investigating the protective effect of cross-reactive responses to AI viruses in the general population would aid pandemic preparedness by providing information on herd immunity[50]. Ascertaining, whether antibody responses against avian influenza viruses reflect true exposure or cross-reactivity, remains a challenge. To address this issue further, establishing

antibody profiles from humans exposed to avian influenza viruses during AI outbreaks could be considered, while also systematically expanding population-level serological studies by including exposure and vaccination history. Such an approach would allow studying kinetics of low-level heterosubtypic antibody responses and comparison of serological profiles in high- versus low risk populations, thereby potentially aiding unbiased interpretation of such findings. For these purposes, the microarray platform could serve as a broad first screening assay, which could be followed by additional serological tests, such as the HI- or MN-assay to ascertain functionality of the detected antibodies.

METHODS

Study population

In a study conducted to monitor the progression of the A(H1N1)pdm09 in different parts of the world, 13 countries from five continents contributed more than 7000 anonymized, filter cards containing dried blot spots from heel prick sampling[30] (Table 1) through neonatal screening programs. This collection method, originally implemented to test for hereditary diseases in new-borns, can also be used to measure maternal antibodies conferred via the placenta[51]. As samples could only be collected when anonymized, we assumed the age of the mothers to be between 20 to 40 years, translating to birth years ranging from 1968 to 1990. We furthermore hypothesized that study subjects in our data set reflect a segment of the general population with unknown prior poultry exposure, thus providing an unbiased systematic population sample.

Ethical approval

As previously described in de Bruin et al.[30], participants were included in the study in accordance with local medical ethical rules. Samples were collected within neonatal screening programs and parents provided informed consent for using residual samples (anonymized) for research purposes. The study was approved by the Japanese Institutional Review Board of the Sapporo City Institute of Public Health (reference number 09-010) and the American NYS DOH Institutional Review board (protocol number #09-045). Participating laboratories collected 10 randomly selected anonymized filter paper cards per week, concordant with regulations of local ethical committees.

Protein microarray technique

IgG levels against different human and avian influenza HA types were measured using a protein microarray platform as described previously[30,35]. Briefly, recombinant proteins of the HA1 part of HA of different influenza virus subtypes (see supplementary material S2) were printed onto nitrocellulose-coated glass slides (64pad, Oncyte Avid, Grace Biolabs, Bend, USA) using a non-contact Piezorray spotter (Perkin Elmer, Waltham, USA). Subsequently, dried blood spots were eluted as described previously and samples were tested at a 1:80 dilution[30]. A Dylight649-labelled goat-anti-human IgG (Fc-fragment specific, Jackson

ImmunoResearch) was used to bind to serum antibodies and fluorescence was quantified by means of a microarray scanner (ScanArray, Perkin Elmer). The protein microarray technique allows simultaneous and standardized detection of antibodies against different influenza subtypes in a minute serum quantity. It has also been used to measure influenza IgG titers in humans[35].

Data analysis

Data analysis was performed in R (version 3.1.0, R Statistical Computing, Vienna, Austria). For all statistical analyses, a p-value of less than 0.05 was considered statistically significant. All samples were normalized to a mean background fluorescence of 5000. Correction for day-to-day variation between microarray slides was achieved based on H1.09 signals against an international standard positive control as described before[30].

For exploratory data analysis, overall fluorescence values between different antigens were compared using the Wilcoxon signed rank test. To characterize antibody profiles and study possible profile-specific heterosubtypic reactivities against AI antigens, we summarized individual serological responses against multiple antigens using the Shannon diversity index, which is a measure frequently applied in ecological studies to quantify biodiversity of species within habitats (see supplementary material S3). For our purpose, we adapted the Shannon diversity index (ASDI) so that we could detect both diversity and magnitude of an antibody response (a traditional Shannon diversity index only describes magnitude). To achieve this effect, we included a dummy serological response with a fluorescence value of 50.000 to ensure that low-and-broad antibody profiles receive a low ASDI score (see supplementary material S3). Only seasonal human influenza antigens that circulated in the 10 years prior to the sample collection were included in the ASDI calculation (H1.99, H1.07, H3.03, H3.07; Table S1). The most recent pandemic strain H1.09 was not included in the ASDI calculation, so that we could investigate its effect on antibody profiles within ASDI categories. The dummy strain's contribution was subtracted off, and the ASDI can thus be thought of as the number of strains to which an individual has a high antibody response; the maximum diversity being 4.0. For the purposed of presentation, we divided the ASDI range into four arbitrary categories ranging from '0-1.5', '1.5-2.5', '2.5-3.5', and '3.5-4'. Associations between ASDI and avian fluorescence signals were evaluated using a Spearman correlation coefficient.

We used R packages 'psych'[52] and 'ggplot2'[53] for exploratory analysis and to create figures, respectively. Package 'MASS'[54] was used for a multivariable log-log linear regression model (with backward elimination using function 'stepAIC') to investigate whether antibody reactivity against recent human antigens (H1.99, H1.07, H3.03, H3.07, H1.09) could explain serological reactivity against avian antigens. Repeating the analysis using a 'forward selection' algorithm yielded the same results. The full model included all recent human H1 and H3 antigens as explanatory variables. Final models included only significant explanatory variables presented in this table. R package 'car'[55] was used to calculate the variance inflation factor to check for multicollinearity between explanatory variables.

For comparison of mean ranks of fluorescence levels per antigen during the A(H1N1) pandemic of 2009/2010, we used the non-parametric Wilcoxon Rank-Sum test with continuity correction on data stratified by pre- and post-pandemic sampling periods. For this, "time of pandemic onset" was selected based on country-specific pandemic curves as shown in de Bruin et al. [30] (Table 1). To determine a cut-off for the pandemic H1.09 HA1 antigen signals, we used H1.09 data of samples collected before the official onset of the pandemic in April 2009. The cut-off was established using mean fluorescence levels plus three standard deviations. Using this cut-off, we reported proportions of H1.09-seropositive individuals within ASDI categories.

To investigate the effect of the A(H1N1)pdm09 on the proportions of broad responders, a Pearson's chi-squared test was used to test for changes in proportions before and after pandemic onset.

As samples of the study population were tested in a one-point dilution (1:80), we used this arbitrary cut off to approximate proportions of individuals with an estimated antibody titer of higher than 80 to avian antigens. Based on prior studies[35], a fluorescence cut-off point of ~30.000 corresponds to an antibody titer of higher than approximately 80.

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COMPETING FINANCIAL INTEREST

MFB has been a paid consultant to Visterra Inc in Cambridge, MA. MFB is an Academic Editor at Nature Scientific Reports. The authors declare no other competing financial interests.

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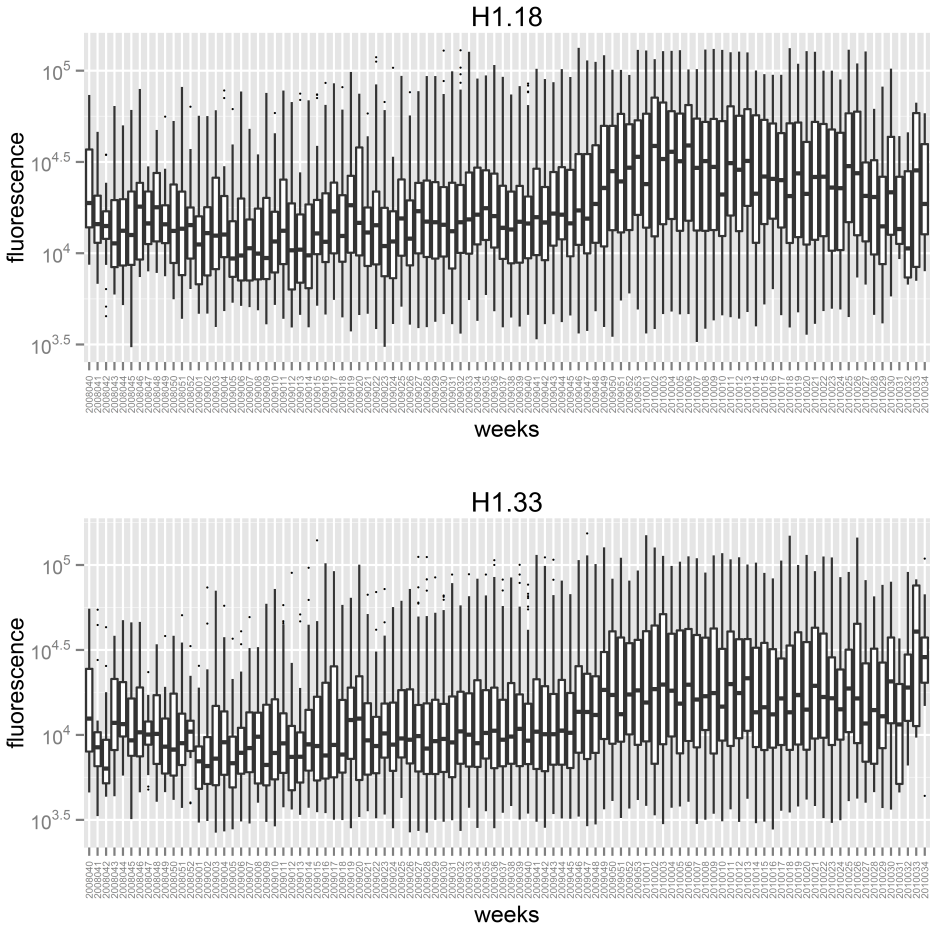
SUPPLEMENTARY MATERIAL

S1. Serological responses to H1.18 and H1.33 were associated with the development of the H1N1 2009 pandemic in the second half of the study. Fluorescence values are depicted over the entire study period (expressed in weeks on the x-axis) for all countries combined. Y-axes represent fluorescence values on a log10-scale.



S2. Table depicting recombinant proteins used for the production of protein microarray slides used in this study [1].

Antigen	Influenza virus strain	Manufacturer
H1.18	A/South Carolina/1/18	Immune Technology Corp
H1.33	A/WS/33	Immune Technology Corp
H1.99	A/New Caledonia/20/99	Immune Technology Corp
H1.07	A/Brisbane/59/2007	Immune Technology Corp
H1.09	A/California/6/2009	Immune Technology Corp
H2.57	A/Canada/720/05*	Immune Technology Corp
H3.03	A/Wyoming/3/03	Immune Technology Corp
H3.07	A/Brisbane/10/2007	Immune Technology Corp
H5.04	A/Vietnam/1194/2004	Immune Technology Corp
H5.06	A/Turkey/15/2006	Genscript
H5.05	A/Indonesia/5/2005(H5N1)	Genscript
H7.03	A/Chicken/Netherlands/1/03	Immune Technology Corp
H9.99	A/Guinea fowl/Hong Kong/WF10/99	Immune Technology Corp
H9.07	A/Chicken/Yunnan/YA114/2007	Genscript

*Influenza virus strain of subtype H2 was isolated in 2005 in course of a laboratory accident

S3. Adapted Shannon Diversity index (ASDI)

There are several different diversity measures. Each of them has a different range and all their values have different interpretations. In general, these values can be represented as the number of responses of equal magnitude so as to provide an intuitive measure that everyone may understand. Furthermore, this approach collapses many diversity measures onto each other, since they lead to the same species diverity when mapped back to the number of equally-abundant species (for details, see [2]).

The key to this approach is the transformation of the diversity measures to species:

$$ {}^{q}D = \left(\sum_{i=1}^{S} (p_i)^q \right)^{\frac{1}{1-q}} $$

where *D* is the diversity measure of order *q*, *S* is the number of species, and

$$ p_i = \frac{\{s_i\}}{\sum_i^S s_i} $$

i.e., the fraction of each species in the data, where s_i is the set of s_1, s_2, ...

The interpretation of this index is diversity equivalent to that number of equally abundant species. For instance, a diversity of 3 means: 3 equally abundant species.

Adaptation to antibody measurements:

The index is adapted to be used for evaluating antibody measurements, i.e., fluorescence. To quantify diversity of serological responses, this index can be used. However, to down-weigh the influence of low fluorescence values, we included a parameter that sets the minimal level of what is considered an effective response. By adding an element to *si*, the index has an offset or *anchor* to distinguish between background and real responses:

$$ {}^{q}D' = \left(\sum_{i=1}^{S} (p'_i)^q \right)^{\frac{1}{1-q}} - 1 $$

where

$$ p'_i = \frac{\{k, s_i\}}{k + \sum_i^S s_i} $$

with

$$ k = \max(k_0, s_i) $$

Properties: For $\max(s_i) \sim k_0$, $qD' \to qD$.

For $\max(s_i) \ll k_0$, $qD' \to 0$.

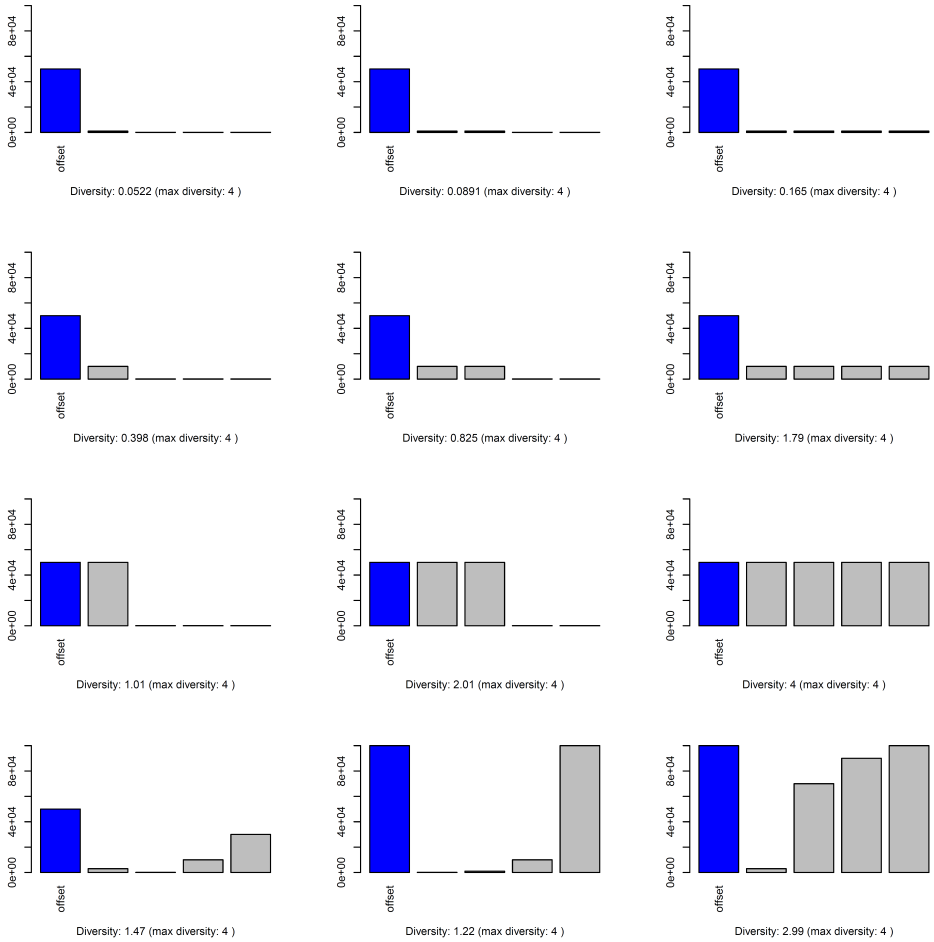
NB: Please note that the anchor is not a cut-off, as the values below it are given less weight, but are still taken into account.

Implementation in R

```
qDp = function(values, q=2, cutoff = 0, offset=50000){

 ## the offset prevents patients with all-round low values
 ## from having high diversity scores

 ## prevent the offset from
 ## pulling down the diversity score when high
 if(max(values)>offset) offset = max(values)

 values = c(values, offset)
 pp = values/sum(values)
 Div = (sum(pp^q))^(1/(1-q))

 return(Div-1) ## subtract 1 to remove the offset again.
```

Examples of diversity scores

Some examples of diversity scores are included below. The grey bars indicate antibody fluorescence values to different antigens, the blue bar indicates the anchor. The maximum diversity is the number of grey bars (n=4).



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CHAPTER 6

SEROLOGICAL EVIDENCE OF INFLUENZA A VIRUSES IN FRUGIVOROUS BATS FROM AFRICA

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ABSTRACT

Bats are likely natural hosts for a range of zoonotic viruses such as Marburg, Ebola, Rabies, as well as for various Corona- and Paramyxoviruses. In 2009/10, researchers discovered RNA of two novel influenza virus subtypes – H17N10 and H18N11 – in Central and South American fruit bats. The identification of bats as possible additional reservoir for influenza A viruses raises questions about the role of this mammalian taxon in influenza A virus ecology and possible public health relevance. As molecular testing can be limited by a short time window in which the virus is present, serological testing provides information about past infections and virus spread in populations after the virus has been cleared. This study aimed at screening available sera from 100 free-ranging, frugivorous bats (*Eidolon helvum*) sampled in 2009/10 in Ghana, for the presence of antibodies against the complete panel of influenza A haemagglutinin (HA) types ranging from H1 to H18 by means of a protein microarray platform. This technique enables simultaneous serological testing against multiple recombinant HA-types in 5µl of serum. Preliminary results indicate serological evidence against avian influenza subtype H9 in about 30% of the animals screened, with low-level cross-reactivity to phylogenetically closely related subtypes H8 and H12. To our knowledge, this is the first report of serological evidence of influenza A viruses other than H17 and H18 in bats. As avian influenza subtype H9 is associated with human infections, the implications of our findings from a public health context remain to be investigated.

INTRODUCTION

Bats are likely reservoirs for a range of zoonotic viruses, such as rabies and other lyssaviruses (family *Rhabdoviridae*), Ebola- and Marburg viruses (*Filoviridae*), Hendra- and Nipah viruses (*Paramyxoviridae*), as well as severe acute respiratory syndrome (SARS) virus (*Coronaviridae*) [1]. In 2009/10, influenza A expanded the list of viral pathogens found in bats, when RNA of two novel influenza A virus (IAV) subtypes (*Orthomyxoviridae*), H17N10 and H18N11, was discovered in frugivorous bats from Guatemala and Peru, respectively [2,3]. Until then, sixteen hemagglutinin (HA)- and nine neuraminidase (NA) types, two surface proteins utilized to classify IAV into subtypes, had been previously described. Water- and shore birds have been known to be the only relevant reservoir hosts for IAV [4]. Several IAV subtypes originating from birds have established stable lineages in birds, pigs and humans. Other avian (e.g., H5N1, H6N2, H7N9, H10N8) and swine influenza virus subtypes (e.g., most recently H3N2v) occasionally cause human infection, resulting in mild- to severe disease and occasional death [5].

Although the two newly discovered subtypes were recently found to have no zoonotic potential [6], the aim of this study was to investigate the role of bats as potential mammalian reservoirs for possibly zoonotic influenza A viruses, by screening for serological evidence against all currently known influenza virus HA-types in frugivorous bats from Ghana.

METHODS

Ethics statement

As described previously [7], all animals used in this study were captured and sampled with permission from the Wildlife Division, Forestry Commission, Accra, Ghana. Geographic coordinates of the sampling site in Kumasi/Ghana were N06°42´02.0´´ W001°37´29.9´´. Capturing was conducted under the auspices of Ghana authorities. Following anesthesia using a Ketamine/Xylazine mixture, skilled staff exsanguinated all bats (permit no. CHRPE49/09; A04957). Samples were exported under a state contract between the Republic of Ghana and the Federal Republic of Germany. An additional export permission was obtained from the Veterinary Services of the Ghana Ministry of Food and Agriculture (permit no. CHRPE49/09; A04957). Materials of all sacrificed animals were used for various studies [8–11].

Sample Analysis

Serum samples (n=100) from straw-colored fruit bats (*Eidolon helvum,* Pteropodidae*)* were collected in 2009 (n=81) and 2010 (n=19) in Kumasi Zoo in Ghana. Although sampling was performed at Kumasi Zoo, all bats included in this study belonged to a wild, migratory colony roosting in trees on site at the time of sample collection.

For serological testing, we used a modification of the protein microarray (PA) technique as previously described by Koopmans et al. [12] and Freidl et al. [13]. 31 recombinant proteins of influenza A viruses [globular head domains (HA1)] were printed in duplicates onto

nitrocellulose Film-slides (16 pad, ONCYTE AVID, Grace Bio-Labs, Bend, Oregon, USA). Selected proteins comprised various strains of all presently known influenza A virus HA-types. Reactivity and optimal working concentration of the proteins were determined by means of checkerboard titrations using specific rabbit antisera homologous to the antigens used (Table 1). Printed slides were stored in a desiccation chamber until further use.
Prior to analysis, we inactivated all bat sera in a water bath at 56°C for one hour. Four-fold sample dilutions were prepared in Blotto Blocking Buffer (Thermo Fisher Scientific Inc., Rockford, MA, USA) containing 0.1% Surfactant-Amps (Thermo Fisher Scientific Inc.). We used five microliters of serum for a starting concentration of 1:40.

Serum dilutions were incubated for one hour in a moist, dark box at 37°C. Antibody binding was detected using an unconjugated goat-anti-bat whole immunoglobulin G (IgG) (Bethyl Laboratories Inc., Montgomery, TX, USA) at a dilution of 1:800, in combination with a Fc-fragment specific AlexaFluor647-labelled rabbit-anti-goat IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) at 1:1100. Both conjugates were titrated to determine the optimal working concentration prior to sample analysis. Each incubation period followed three washing steps using wash buffer (Maine Manufacturing, Maine, USA).
Fluorescent signals were measured with a Powerscanner (Tecan Group Ltd, Männedorf, Switzerland) and converted into titers as described before [12,13]. The detection capacity of the PA spanned a titer range from 40 to 2560. Samples showing no antibody reactivity were regarded as negative and were assigned a titer of 20 (half of the starting dilution). As we were unable to formally calculate a cut-off based on confirmed influenza A-positive and negative bat sera due to unavailability of such materials, an arbitrary cut-off of 40 was chosen, similarly to previous work [12]. Hence, samples displaying a dilution curve resulting in a titer of ≥40 were interpreted as positive. Comparisons of seropositivity between sexes, age groups, sampling seasons and -years, respectively, were performed using Chi^2- or Fisher's exact test in RStudio (Version 0.98.507, Boston, MA, USA) with a significance level of 0.05.

RESULTS

Of the 100 bats tested, 67 (67%) were negative against all antigens (Fig. 1), whereas 33 bats showed titers against one or more antigens [2009: 26 (32%); 2010: 7 (37%)]. Four antigens showed slightly elevated geometric mean titers (GMT): H8.79: 21, H9.97: 29, H9.09: 29, H12.91: 23, whereas the GMT against the remaining antigens was 20 (Fig. 1). Thirty bats (30%) showed antibody titers higher than 40 against at least one H9-antigen included on the PA. Thereof, 21 (21%) bats were positive for H9.97 (range: 43-1388) and 20 (20%) for H9.09 (range: 41-1048), respectively. Eleven animals showed antibody reactivity against both H9-antigens (11%), whereas ten individuals (10%) selectively reacted with H9.97, and nine (9%) solely with H9.09. Of the H9-positive bats, 24 were sampled in 2009 (29.6%) and 6 in 2010 (31.6%), respectively.
Ten of the samples from H9 positive bats also bound to the H12.91 protein (range: 53- 509), and four in addition bound to H8.79 protein (range: 42-129). Serum from one of these four

bats had additional reactivity to H11.02 antigen. Similarly, two other H9.97-positive individuals, reacted against H7.12 (titer: 46) and H15.83 (51), or with H6.07 (43). Unique reactivity to single proteins was observed for H3 (n=1), H5 (n=1) and H8 (n=1, titer: 42). We found no significant differences in seropositivity between sexes, age groups, sampling season and –year, respectively (Table 2).

Table 1. Recombinant HA1-proteins included in the protein microarray.

#	Code	Subtype	Strain
1	H1.18	H1N1	A/South Carolina/1/18
2	H1.77	H1N1	A/USSR/92/1977
3	H1.07	H1N1	A/Brisbane/59/2007
4	H1.09	H1N1	A/California/6/2009
5	H2.05	H2N2	A/Canada/720/05
6	H3.68	H3N2	A/Aichi/2/1968(H3N2)
7	H3.10	H3N2v	A/Minnesota/09/2010
8	H3.07	H3N2	A/Brisbane/10/2007
9	H4.02	H4N6	A/mallard/Ohio/657/2002
10	H5.97	H5N1	A/Hong Kong/156/97 (HP, clade 0) [a]
11	H5.02	H5N8	A/duck/NY/191255-59/2002, LP [b]
12	H5.10	H5N1	A/Hubei/1/2010 (HP, clade 2.3.2.1) [a]
13	H5.06	H5N1	A/Turkey/15/2006 (HP, clade 2.2) [a]
14	H5.07	H5N1	A/Cambodia/R0405050/2007 (HP, clade 1) [a]
15	H5.05	H5N1	A/Anhui/1/2005 (HP, clade 2.3.4) [a]
16	H6.07	H6N1	A/northern shoveler/California/HKWF115/2007
17	H7.03	H7N7	A/Chicken/Netherlands/1/03 (HP)[a]
18	H7.13	H7N9	A/chicken/Anhui/1/2013 (LP)[b]
19	H7.12	H7N3	A/chicken/Jalisco/CPA1/2012 (HP)
20	H8.79	H8N4	A/pintail duck/Alberta/114/1979
21	H9.97	H9N2	A/chicken/Hong Kong/G9/97 (G9 lineage)
22	H9.09	H9N2	A/Hong-Kong/33982/2009 (G1 lineage)
23	H10.07	H10N7	A/blue-winged teal/Louisiana/Sg00073/07
24	H11.02	H11N2	A/duck/Yangzhou/906/2002
25	H12.91	H12N5	A/green-winged teal/ALB/199/1991
26	H13.00	H13N8	A/black-headed gull/Netherlands/1/00
27	H14.82	H14N5	A/mallard/Astrakhan/263/1982new
28	H15.83	H15N8	A/duck/AUS/341/1983
29	H16.99	H16N3	A/black-headed gull/Sweden/5/99
30	H17.09	H17N10	A/little yellow shouldered bat/ Guatemala/153/2009
31	H18.14	H18N11	A/flat-faced bat/Peru/033/2010

Proteins were obtained from Immune Technology Corp. (NY, USA) or Sino Biological Inc. (Beijing, China); [a]highly pathogenic; [b]low pathogenic

Figure 1. Titers of individual Ghanaian bats plotted against all recombinant proteins included on the microarray. Horizontal bars represent geometric mean titers per antigen including a 95% confidence interval. Sera below fluorescence values of 31.268 (half of the fluorescence spectrum) were regarded as negative and were assigned a titer of 20 (half of the starting dilution of 1:40). Sera above 40 (dashed line) were regarded as positive. Arrows indicate antigens grouping within the same phylogenetic cluster.

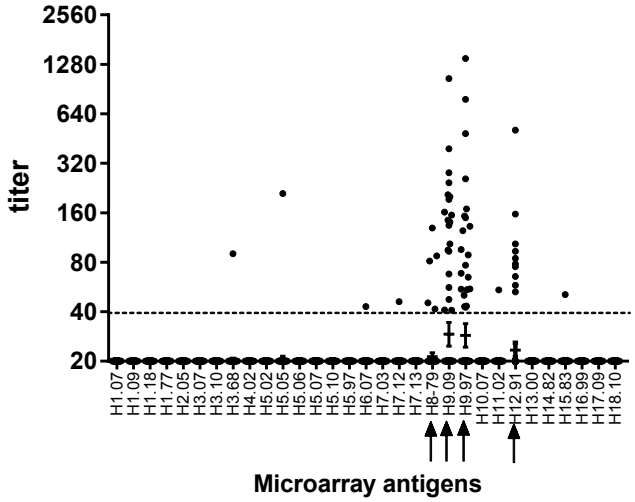

Table 2. Serological findings versus sex, age group, sampling year and –season.

		serology			
		negative	**positive**	Row total	p-value
sex[a]	male	51	22	73	0.4463
	female	16	11	27	
age group[b]	adult	54	31	85	0.134
	juvenile	13	2	15	
year[a]	2009	55	26	81	0.9008
	2010	12	7	19	
season[b,c]	dry	6	4	10	0.7259
	rainy	61	29	90	
	Column total	67	33	**100**	

Comparisons showed no significant differences. Counts also reflect percentages (n=100). [a]Pearson's Chi^2-test with continuity correction. [b]Fisher's exact test. [c]dry: December to February, rainy: March to July and September to November.

DISCUSSION AND CONCLUSIONS

In this study, we report on serological evidence of influenza A viruses in straw-colored fruit bats from Ghana. We found no reactivity against H17 antigens such as recorded in bats from Guatemala (38% [2] or H18 antigens like in bats from Peru (27% [3]. However, we studied a not closely related bat species from a different continent, and found about 30% antibody detection rate against HA-type H9. Sonntag et al. [14] and Fereidouni et al. [15] screened Central European bats for genomic traces of influenza virus using generic RT-PCR assays but found no such evidence. For the Central and South American bats influenza virus RNA was detected in a low percentage (0.9%) of the Guatemalan (3/316) and Peruvian (1/110) bats by pan-influenza conventional RT-PCR [2,3]. However, molecular detection is limited by a short duration of virus excretion making it impossible to exclude virus presence on population level. Serological techniques can shed light on the infection history even after the virus has been cleared by the immune system. Still, serological tests are limited in their specificity due to the existence of cross reactive antibodies [16]. As with every attempt to study infection in novel host systems, our techniques are not finally optimized for use with bats due to limited availability of reagents (including confirmed influenza A positive- and negative bat sera) [17,18]. Moreover, there was insufficient sample material for additional analyses such as microneutralization- or hemagglutination inhibition assays due to utilization of materials in prior studies [8–11]. Our results are preliminary in this regard, leaving the possibility that antibodies in bats may not be directed against typical avian-origin H9 HA lineages, but outlier viruses yet to be discovered.

Nevertheless, several of the bat sera showing H9-reactivity also reacted with antigens H8 and H12. This supports the credibility of our findings as there is clear phylogenetic relatedness of these particular subtypes[19]. Similar intra-clade reactivity was previously observed in chickens naturally infected with subtype H9N2 [13]. No significant association in seropositivity between sex, age groups, sampling year and season, respectively, could be found. However, as sample size was small we cannot rule out that potentially significant associations might have been missed.

Influenza A viruses of HA-type H9 have a wide geographical distribution in birds and are recognized as possible candidates to cause a future pandemic [20]. In addition, this subtype is associated with human infection causing mostly mild symptoms, which likely leads to an underestimation of cases [5]. Limited surveillance in birds allow unnoticed reassortment events between circulating avian or potentially human influenza virus strains, resulting in variants with yet unknown zoonotic potential [21]. Given the relatively high seroprevalence found in bats in both sampling years and the clinically healthy status at the time of sample collection, we cautiously suggest that bats – as for other emerging viruses [22] – might constitute asymptomatic mammalian carriers of influenza A viruses. In summary, we present serological evidence of influenza A viruses in Old World fruit bats that have been shown to be biologically relevant reservoirs of pathogenic viruses such as Henipaviruses, Coronaviruses, Lyssaviruses and Filoviruses. It is conceivable that there might be a link between serological evidence of influenza A virus in bats and migratory birds, as their flyways overlap with the

geographic distribution of *E. helvum* [23,24]. However, in the absence of molecular data this hypothesis remains speculative. As *E. helvum* are widely consumed as bush meat in West Africa [25], the implications of the findings from a public health perspective remain to be investigated. Serological studies in humans consuming bats (including suitable control groups) would be useful to shed light on possible spillover events.

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Conflict of interest

The authors have declared that no competing interests exist.

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CHAPTER 7

GENERAL DISCUSSION

The aim of this thesis was to contribute to emerging disease preparedness by developing novel serological screening tools for the use in a range of hosts, and to address current challenges with respect to surveillance at the human-animal interface. Suggestions as to how to potentially tackle part of the challenges are outlined in this discussion.

TOWARDS AN INTEGRATED 'ONE HEALTH' SURVEILLANCE APPROACH AT THE HUMAN-ANIMAL INTERFACE

Our first literature review on human cases with reported virological evidence of avian influenza (AI) virus infection provided a comprehensive overview of avian- and swine influenza subtypes reported to have crossed the species barrier to humans (chapter 2). As outlined in the introduction of this thesis, the current influenza sentinel surveillance system for human influenza viruses has limited capability to detect human cases infected with animal influenza viruses, and therefore a more integrated surveillance approach from both, the veterinary and human health side, under the umbrella of 'One Health' could be considered to target populations at risk of AI virus infection. Suggestions as to how to possibly expand the current surveillance system to capture exposures at the human-animal interface in the light of emerging disease preparedness are discussed in the following sections.

Surveillance for avian influenza viruses on the veterinary side – current limitations and suggestions for improvement

Current surveillance programs for AI on the veterinary side in the European Union (EU) and beyond consist of active and passive components [1,2]. Active-, or serological surveillance is periodically executed and uncovers subclinical circulation of AI viruses in wild birds and domestic poultry and -waterfowl by using antibody detection methods, thereby complementing existing early warning systems. Active surveillance can be carried out either via risk-based surveillance or surveillance based on stratified sampling. Passive surveillance, on the other hand, is syndrome-based and conducted when increased morbidity or mortality is observed in domestic and wild birds, which consequently are tested for AI virus presence. Both surveillance components are focused on AI types H5 and H7 [2]. If samples are positive for influenza viruses with H5 or H7 HA types [irrespective of low- (LP) or highly pathogenic (HP) phenotype], control measures are initiated, i.e. culling of infected animals. In contrast, if samples test positive for influenza A virus but H5/H7 AI types can be excluded, it is currently not obligatory under EU regulations to determine which other AI subtype caused the infection [2].

Although from an economic perspective the focus of the veterinary surveillance system on H5 and H7 AI type viruses is justified – as these subtypes are associated with high mortality in poultry and are therefore notifiable to the World Organization for Animal Health (OIE) –, from a public health/ emerging disease preparedness point of view, such narrow monitoring precludes establishing a comprehensive picture of AI epidemiology and identification of AI

subtypes with (yet unknown) zoonotic potential directly at the source. As evolution of influenza A viruses is highly unpredictable [3], early warning through timely identification of potentially zoonotic viruses could ultimately reduce the impact of a new pandemic or novel emerging variant, which should be monitored through systematic surveillance [4].

Low pathogenic (LP) AI subtype A(H7N9) constitutes a recent example of a novel AI virus, which emerged in 2013 in China [5] following prior reassortment events in wild and domestic birds [6]. Whereas A(H7N9) viruses spread silently in the poultry population, this subtype has caused severe symptoms and death in humans [7], which challenged the notion that LPAI viruses pose a negligible human health risk. Although LPAI A(H7N9) would have been picked up through current H5- and H7-focused veterinary surveillance regimes, other zoonotic LPAI viruses, e.g. recent A(H6N1) and A(H10N8), are currently missed and highlight that broader surveillance for LPAI viruses at the animal source is indicated from a public health perspective [8].

To achieve the goal of such a 'One Health'-based surveillance, novel technologies could be integrated in the current veterinary-based surveillance system. Multiplex PCRs have already been developed for the simultaneous detection of H5 and H7 [9,10], and also including H9 [11], hence future work could address the expansion of existing PCR protocols to capture additional AI subtypes potentially relevant for public health. Another detection method called PanHA RT-PCR uses five degenerate primers targeting the cleavage site of all 16 hemagglutinin types which allows simultaneous pathotyping of H5 and H7- (determination of HP or LP) as well as subtyping of non-H5/H7 viruses through sequencing [12]. Importantly, co-infection/ circulation of various subtypes in a commercial poultry flock or in wild birds could be identified, which might be overlooked by the current surveillance system. Considering that non-H5/H7 LPAI viruses served as gene donors for zoonotic viruses, findings of co-circulating subtypes could trigger additional molecular investigations, e.g. sequencing, to study the zoonotic potential of novel reassortants. For instance, LPAI A(H9N2) – itself a zoonotic subtype – served as gene segment donor for subtypes HPAI A(H5N1) [13], LPAI A(H7N9) [6] and LPAI A(H10N8) [14], whereas an LPAI A(H6N6) and HPAI A(H5N1) exchanged genetic material to form the recently discovered HPAI zoonotic subtype A(H5N6) [15]. The importance of identification of co-circulating AI viruses to assess the human health risk was also emphasized by a recent study that found co-circulation of subtypes A(H7N9) and A(H9N2) on a poultry farm in China. Five reassortant genotypes were identified of which one caused human infection [16].

Molecular surveillance, aimed at the detection and identification of influenza virus subtypes and characterization of virological traits based on sequence information, has played a vital role in human and animal surveillance systems during the past 25 years [17]. Learning from the complication of absent prior surveillance data during the emergence of A(H7N9) and the pandemic swine influenza strain A(H1N1) in 2009 [A(H1N1)pdm09], an expanded approach to molecular surveillance is indicated which would facilitate pinpointing the location, exact time and host species of newly emerging, zoonotic AI variants [8,18]. Globally, sequence-

based surveillance data for AI- and, particularly, for swine influenza viruses is lacking, with only a few countries actively contributing to molecular surveillance by sharing sequences, as was shown by a study conducted in 2012. The majority of the shared sequences is limited to subtype A(H5N1) [19], whereas information on other subtypes is scarce. The study furthermore revealed that countries with large poultry populations only contribute a relatively small amount of sequences, leaving many 'black boxes' with weak veterinary systems which could potentially constitute cradles for new, silently emerging viruses [19]. Another issue hampering 'real-time surveillance' is delayed sharing of sequences. The construction of a global surveillance network, consisting of key sentinel sites in regions and countries most at risk (e.g. countries with high poultry density or areas with high density of migratory wild birds), which could systematically provide real-time data, as well as the implementation of an overall global agency responsible for flu surveillance which oversees efforts by the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) are possible cost-effective solutions to the current patchy and event-driven surveillance approach [19,20]. Similarly, predictive modelling could help identify regions likely constituting a cradle for the next emerging zoonosis on which surveillance efforts could subsequently be focused on [21]. A benefit sharing system as suggested in the Pandemic Influenza Preparedness Framework by WHO [22] – partly leaning on capacity building and providing access to antivirals and vaccines for A(H5N1) – could encourage countries and farmers, which might not yet conduct surveillance or do not report AI surveillance results due to trade reasons or lacking financial compensation, to participate [23].

On the other hand, as financial resources can be limiting and likely preclude broad molecular surveillance as standard method in the veterinary sector, a more upstream implementation of targeted, risk-based molecular surveillance could be considered by placing a focus on wild birds in hotspots with high viral diversity, such as stopover points along flyways, as was described before [24,25]. Cui et al. [26] similarly described a risk-based surveillance approach in wild birds based on presence and abundance of 'high-risk' bird species. In 2005, highly pathogenic subtype A(H5N1) spread globally affecting large numbers of domestic poultry following an outbreak in migratory waterfowl at Qinghai lake, a migration hotspot in China [27,28]. In addition, the emergence of HPAI subtype A(H5N8) in 2014 and its introduction into domestic poultry farms in South Korea [29,30], China [31], Japan, Germany, the Netherlands, the United Kingdom, Canada and the United States [32] provides a more recent example highlighting the importance of monitoring wildlife in order to timely detect and effectively prevent/ contain outbreaks in poultry. Verhaegen et al. [33] investigated the epidemiology of HPAI A(H5N8) subtype and highlighted that serological methods are valuable for surveillance, as AI virus excretion is short and would necessitate screening of high amounts of birds [34]. As reservoirs for influenza A viruses, wild birds are vital for maintenance, spread and introduction of flu viruses into the commercial poultry sector, hence investing in sustained, cost-effective surveillance in key locations (e.g. migration stopover sites and hotspots for reassortment [35] would constitute a more effective investment rather than

sporadically mobilizing large amounts of financial resources in response to outbreaks [25]. Targeted monitoring of diversity and early detection of changing dynamics of AI viruses in reservoir populations would ultimately serve public health and pandemic preparedness through, e.g. providing input for potential vaccine development, determine virus origins and assess potential for virus dispersal through trade and migration [25].

As outlined earlier, the active component of AI surveillance picks up ongoing and prior circulation of AI viruses that are not detected through syndrome-based events as described above. If serological evidence of influenza A viruses is found through general ELISA screening, the current screening regime could similarly be expanded by employing novel serological multiplex techniques to capture subtypes other than H5 and H7, which are already developed. For instance, Bucokovski et al. [36] reported on a multiplex approach for AI surveillance in mallards and the protein microarray technique for serological AI screening in chickens is described in chapter 3 of this thesis. To our knowledge, a modification of the latter multiplex serologicical screening technique has already been implemented at the Central Veterinary Institute (CVI) in the Netherlands which is currently used for surveillance [37]. Additional serological screening for other AI subtypes can often be limited by serum constraints when conventional serological testing methods, such as the hemagglutination inhibition (HI)- and microneutralization (MN) assay, are used. Multiplex methods can overcome this limitation by necessitating only a small quantity of serum from which information on multiple subtypes can be deduced and therefore warrant further evaluation and optimization. Combining sero-surveillance with targeted, periodic risk-based molecular surveillance within wild birds would elucidate circulation of subtypes of public health relevance, which would in turn guide public health decision making and could provide input as to when to update or include additional antigens in such multiplex screening platforms, to monitor spill over events into poultry and associated risks for human health.

Avian influenza virus outbreaks and the human health side

In 2007, the WHO published guidelines for the investigation of human cases infected with HPAI A(H5N1). Such investigations are usually launched following confirmation of HPAI A(H5N1) in domestic or wild bird populations, if (i) two or more persons present with fever and acute lower respiratory symptoms (ALRS) within two-weeks and in the same geographical area of the outbreak, (ii) if healthcare workers develop abovementioned symptoms after caring for an A(H5N1) confirmed patient/ patient with unexplained ALRS and fever, (iii) if people occupationally exposed to birds/animals fall ill, or (iv) if abnormalities in seasonal influenza surveillance are observed [38]. In the Netherlands, in case of an AI suspicion or confirmed AI outbreak, the Community Health Services (GGD), which are informed either via the Netherlands Food and Consumer Product Safety Authority (NVWA) or the National Coordination of Infectious Disease Control (LCI), are encouraged to cover the public health aspects by attending to people involved in the outbreak response [39].

The response on the human health side differs based on the subtype and pathogenicity phenotype identified in poultry. When HPAI virus subtypes (H5, H7) are diagnosed, the current regimen includes that humans with prolonged or intense exposure to AI-infected poultry (e.g. poultry farmers and their families, farm workers, cullers, veterinarians, etc.) receive personal protective equipment, advice on hygiene, and are offered the antiviral drug oseltamivir as prophylaxis (neuraminidase inhibitor), as well as seasonal influenza vaccination, depending on the season in which an outbreak occurs. Furthermore, individuals exposed to HPAI infected poultry are requested to monitor potential development of disease symptoms [39]. This guideline was applied during a major outbreak with HPAI A(H7N7) in commercial poultry in 2003 in the Netherlands, which caused one human fatality [40,41]. Active case finding based on case definitions comprising conjunctivitis and/ or ILI identified additional 89 human cases with molecular evidence of infection [40]. Subsequent studies on the extent of subclinical infection by an adapted HI-assay using horse red blood cells (RBC) revealed a staggering 49% of 508 individuals exposed to AI-infected poultry as seropositive, but no neutralizing activity could be detected [42]. Although the findings generated by the modified HI were initially questioned by others [43], the credibility of the findings was recently confirmed on a subset of samples by an alternative assay (in press).

For LPAI outbreaks irrespective of subtype, poultry-exposed humans similarly receive advice on hygienic measures and monitor development of symptoms, whereas wearing personal protective equipment is not mandatory and seasonal influenza vaccination is also advised based on the season [39]. The rationale for this recommendation/ advice is based on the severity of symptoms caused in humans, and the relatively low amount of human cases infected with LPAI viruses previously reported in the published literature, for which, among others, our literature review was consulted [39]. However, the emergence of the novel, zoonotic LPAI A(H7N9) subtype which has caused severe disease in humans calls these recommendations into question. Similarly, targeted serosurveillance studies in AI-exposed populations reported on *serological* evidence of human infection to a range of avian influenza viruses (reviewed in chapter 4 of this thesis), which shows that the number of human LPAI cases with *virological* evidence described in the scientific literature likely only reflects the tip of the iceberg. These arguments raise the questions on whether the current guidelines need to be revised and whether the response regarding the human health side during AI outbreaks needs to be re-assessed.

Unveiling subclinical AI spillover events to humans – Widen testing focus to AI viruses other than H5 and H7

From an emerging disease preparedness perspective – and supported by an increasing body of scientific studies on the zoonotic potential of other AI subtypes [44–47]–, addressing unapparent spill-over events of AI viruses other than H5 and H7 to humans remains an interesting field for research that deserves further attention. On the other hand, with respect to identification of human infection with subtype A(H5N1) based on serological methods, there are also open questions still that could in turn also be translated to other AI subtypes.

For instance, recent reviews on serological evidence of human infection with A(H5N1) sparked discussions [48,49] on the credibility of low-level titers which lead to debates on whether the case-fatality rate for A(H5N1) reported by the WHO – currently amounting to about 60% – is truly that high or constitutes a gross overestimation [50–52]. Other points that were raised in course of this discussion touched on observed heterogeneity in laboratory methods between studies and lacking uniform guidelines for detection of antibodies to A(H5N1) in sero-surveillance settings, unknown antibody kinetics (increase, drop, persistence) following infection with A(H5N1) and to what extent influenza viruses other than A(H5N1) are able to trigger cross-reactive antibodies [53].

These questions could be addressed by complementing the current surveillance approach with systematically including humans with substantial exposure to AI-infected poultry, irrespective of subtype, in active testing which would shed light on subclinical spill-over events. Such new findings could put current public health actions, which differentiate based on AI subtype and pathogenicity type, into perspective. An evident approach to generate first pilot data could be to actively collect paired serum samples of humans exposed to AI-infected poultry – i.e. directly at the source of infection – rather than relying on self-monitoring and -reporting on the development of symptoms of disease. That monitoring of human infections based on self-reporting has its limitations was demonstrated during two outbreaks with LPAI A(H7N3) viruses in Italy [54]. In this study, researchers found clear evidence of infection in 7 out of 185 (3.8%) individuals by HI- and/or MN assay, as well as Western Blot. However, only one of the seropositive individuals reported symptoms of conjunctivitis, whereas the remaining six cases reported no symptoms. In contrast, six- and 14 individuals reported conjunctivitis and ILI, respectively, but had no serological evidence of infection [54], which points out that relying on a symptom-based approach for the identification of cases has its limitations.

Real-time information sharing between veterinary (NVWA) and human health side (GGD) would facilitate a comprehensive and swift joint investigation at the human-animal interface, thereby actively applying the 'One Health' concept. For instance, upon confirmation of an outbreak by the veterinary side, the GGD could initiate immediate collection of acute serum samples of poultry exposed humans (and preferably their household contacts), followed by a second serum sample ~ three weeks later to investigate potential seroconversion, defined as a 4-fold antibody titer rise [55]. However, a limitation of this approach might be that the time of human exposure is difficult to pinpoint and that paired serum samples might not be informative. The incubation period for AI, irrespective of subtype is broadly estimated to range from a few hours in individual birds to 3 to 5 days [56], with a maximum incubation period of 21 days [57]. For HPAI A(H5N1) experimental infection of chickens resulted in a mean incubation period of 2.1 days before symptoms develop [58]. Modelling based on experimental infection with HPAI A(H7N7) showed that approximately two weeks can pass until elevated mortality is observed in a flock [59]. A similar length of the infectious period was found for natural infection occurring during the large-scale outbreak with HPAI A(H7N7)

in the Netherlands, which decreased to about seven days after implementation of control measures. The model furthermore showed that a flock can be infectious for up to 26 days [60]. During this period, people directly involved in animal care and handling are potentially at risk of infection, as animals are already infectious before elevated mortality is noticed [59,61] later showed that persons actively involved in control activities during the HPAI (H7N7) outbreak have a high probability of infection, particularly when handling poultry for clinical inspection in the protection- and surveillance zone and during culling activities. To our knowledge, for LPAI viruses such data is lacking. Given that LPAI viruses do not cause apparent symptoms in poultry, humans can possibly be exposed for prolonged periods of time which warrants further investigation.

As an alternative to collecting paired serum samples during an ongoing outbreak or when a flock tests seropositive for influenza virus during active surveillance, one serum sample could be taken from exposed humans and compared to region- and age matched controls. Upon positive serological findings in poultry-exposed individuals, sampling could be expanded to also investigate potential human-to-human transmission by including epidemiologically linked contacts, as was previously conducted in studies primarily focusing on H5 and H7 subtypes [62–64]. Through such a standardized approach, unbiased estimates of human infection with AI viruses could be acquired – rather than relying on self-monitoring and reporting of symptoms – which would allow calculation of unbiased case-fatality rates and would also shed light on the understudied area of subclinical and mild cases caused by animal influenza viruses. Another advantage of systematic sampling would be that varying perception of symptoms of disease could be circumvented [65]. Furthermore, studies on 'antibody landscapes' and so-called 'antigenic seniority' have provided valuable insights into longitudinal dynamics of human serological responses within one influenza virus subtype, seasonal A(H3N2) [66–68]. However, comparable data on human antibody titers to avian influenza viruses is scarce. The proposed approach would allow to study antibody kinetics (e.g. persistence, waning) in subclinically infected persons, thereby complementing existing knowledge of antibody titer dynamics in symptomatic patients infected with AI types H5 and H7 [69–71]. Such information would also be useful to guide interpretation of low-level serological findings in sero-surveillance studies at the human-animal interface.

In conclusion, these arguments show that making public health actions dependant on symptom development in AI-exposed risk groups is flawed in the light of emerging disease preparedness. The generation of unbiased, standardized data on the frequency of zoonotic infections in AI hotspots could be implemented by setting up dedicated networks and would provide comprehensive, evidence- and risk-based guidance for public health decision making, by serving as baselines for risk-assessments [19]. Given their role as mixing vessel for influenza viruses [72], a similar monitoring approach could be considered for swine flu viruses and associated human exposure. However, as cross-reactivity between animal- and human-origin influenza viruses can be problematic for interpretation of antibody findings,

research efforts into development of serological assays able to disentangle viruses of human and animal origin would be needed.

SEROLOGY – CURRENT CHALLENGES

Subclinical and mild human AI-infections - Need for investigating alternative definitions for seropositivity?

Whereas human infection detected through virological testing is considered thorough evidence of infection (reviewed in chapter 2), serological findings of animal influenza virus infection can be more challenging to interpret. A number of sero-surveillance studies conducted in high risk populations reported low-level antibody titers to various animal influenza virus subtypes (chapter 4). Thereof, a subset applied serological methods and criteria for seropositivity against AI viruses in humans as described in the WHO guidelines, whereas others defined their own criteria for seropositivity [38,73]. The currently available WHO guidelines were established to specify laboratory protocols for the diagnosis of human infection with the particular AI subtypes A(H5N1) and A(H7N9). The MN assay is considered the method of choice to detect antibodies induced by AI viruses due to improved sensitivity and a ≥4-fold antibody titer rise between acute and convalescent serum samples is considered proof of infection. Alternatively, when only a single serum sample is available – preferably collected 14 days after symptom onset or later – a MN-antibody titer result of ≥80 indicates infection, if confirmed by an additional serological method, e.g. Western Blot specific for the respective H5 or H7 subtype, or by a HI assay resulting in an antibody titer of ≥160 using horse RBC [38,73]. However, as case definitions for A(H5N1)- and A(H7N9) encompass a certain exposure history and clinical presentation, e.g. fever in combination with other respiratory symptoms – i.e. require a patient to be clinically ill – it remains an area for future research as to whether the diagnostic criteria for seropositivity defined in the current guidelines, established for acutely infected, clinically ill patients, can directly be translated to mild or asymptomatic human AI cases, i.e. the use of serology to study exposure. Hence, the question whether adapted criteria for seropositivity after mild or subclinical human infection are necessary could be investigated and developed through pilot data generated during outbreak settings. In this respect, further research into immunogenicity of different AI viruses is needed. As was shown for virologically confirmed human patients, infection with H7 AI viruses might only elicit a weak or undetectable neutralizing antibody response in human patients [42,74,75]. Children have been described to mount weak antibody responses to A(H5N1) [76]. Also, recent research on combining serological assays for the detection of human A(H7N9) infections also showed that antibody responses in children are weaker than in the elderly and in adults [71]. In this study, the authors similarly suggest adjustments of serological criteria for diagnosis of A(H7N9) infection. Multiplex serological testing can potentially help to unravel to what extent pre-existing immunity from infection or vaccination with human influenza viruses might play a role in mounting a robust or weak antibody response to AI viruses.

In chapter 4 we found high heterogeneity between studies reporting on serological findings to animal influenza viruses, which limited comparability of results. Therefore, future sero-surveillance studies investigating exposure to animal influenza viruses at the human-animal interface for subtypes other than H5 and H7 would benefit from the development and implementation of uniform guidelines tailored to study subclinical and mild human infections with animal viruses in a standardized fashion.

Heterosubtypic immunity – up and downsides

In chapter 4 we reviewed publications reporting serological evidence of human infection with avian and swine influenza viruses. A number of these studies acknowledged the issue of cross-reactive antibodies, raising the question whether low-level antibody titers to avian influenza viruses are truly evidence of infection or are resulting from cross-reactive antibodies. In recent years, a number of cross-reactive, neutralizing monoclonal antibodies were detected in humans and mice [77–89] and protective effects of broadly neutralizing antibodies have become of interest for universal vaccine development to prevent infection with influenza A viruses, e.g. in risk populations exposed to AI viruses or prevent the occurrence of the next pandemic [90]. Heterosubtypic neutralizing antibodies were similarly detected in the general population [91,92] and individuals recently infected or vaccinated with seasonal, pandemic or avian influenza A viruses [87,88,93–96]. In chapter 5, we similarly found serological responses to AI viruses in the general population, particularly H9 and H7, and showed that heterosubtypic reactivity increased after the onset of the A(H1N1)pdm09. These findings are important to take into account when studying exposure at the human-animal interface. Comparing heterosubtypic reactivity in animal exposed and -unexposed populations and addressing the question whether it is possible to distinguish antibody titers triggered by true exposure versus such elicited by cross-reactivity constitutes an interesting area for further research.

To begin with, recording a study subject's history, including detailed information on the nature of exposure to animal influenza viruses, age and vaccination history would provide important background information which would aid correct interpretation of serological results. Age is a particularly important factor (more refs). For instance, Todd et al. [97], showed that in a human cohort from Vietnam seropositivity to A(H9N2) rapidly increased with age, faster than titers to H7 and H5. As the authors and others [98–100] suggested that human antibody titers to A(H9N2) are likely triggered by cross-reactions and should be interpreted with caution, more systematic research into this phenomenon would contribute to an improved understanding of cross-reactivity. To generate reliable and comparable results in future human-animal interface studies, the proposed guidelines, as outlined in the earlier section, should include advice on how to address the issue of confounding of antibody titers to animal viruses by crossreactivity triggered by exposure to human influenza viruses or influenza virus vaccination. For instance, cross-reactivity could be addressed in course of the sample analysis, e.g. via pre-adsorption of sera with human influenza viruses

[38,101,102] or in course of the data analysis stage, e.g. by including serological data on human influenza viruses as covariate [103,104].

The knowledge that recent vaccination with seasonal influenza A viruses can influence the generation of broadly cross-reactive antibodies is not only relevant when studying exposure to zoonotic influenza viruses to avoid false classification of results, but could also influence policy making. For instance, cross-reactive antibodies to avian HA types were elicited in healthy volunteers and in migratory bird handlers upon seasonal influenza vaccination [87,105,106]. Such findings could result in a general advice to administer seasonal influenza virus vaccination to high risk groups; a point that was previously raised and advocated for by others [107–109]. However, there is also contrary evidence from a small pilot study showing that seasonal influenza vaccination did not elicit heterosubypic antibodies [110], so systematic investigation of the benefit of current seasonal influenza vaccines with respect to heterosubtypic, humoral immunity is indicated.

INFLUENZA VIRUSES AND THEIR EXPANDING HOST RANGE: BATS AND THE HUMAN ANIMAL INTERFACE

Avian influenza viruses crossed the species barrier multiple times in the past, with infections reported in aquatic- (e.g. cetaceans and pinnipeds) and terrestrial mammals (e.g. pigs, horses, mink, civets, tigers, leopards, dogs and cats) [111]. Recently, bats expanded the list of potential hosts for influenza viruses when Tong et al. [112,113] discovered RNA of two novel influenza virus subtypes, A(H17N10) and A(H18N11). Bats form the second largest order of mammals [114] and play an important role as reservoirs for a number of emerging and zoonotic viruses, e.g. rabies virus, Ebola virus, Marburg virus, SARS- and MERS Corona virus, as well as Nipah- and Hendra virus [115]. The versatility of bats to live in structures created by humans (e.g. tunnels, houses) as well as human encroachment of natural bat habitats for plantations or animal husbandry purposes create direct interfaces for viral spill-over opportunities to humans [115,116]. Alternatively, bat-borne pathogens can come in close proximity to humans via intermediate hosts serving as bridging links, such as, e.g. civet cats for SARS-Corona virus, horses for Hendra virus, pigs for Nipah virus and most recently, dromedary camels for MERS Corona virus [115]. The discovery of RNA of the two novel influenza virus subtypes in Middle- and South American fruit bats [112,113] raised questions about the role of bats as mammalian reservoir for potentially zoonotic influenza viruses. Although later investigations showed that the novel subtypes were considered to be of little threat for human health [117], the question still remained whether there is evidence for other potentially zoonotic influenza A viruses in this taxon. To explore this question further, we investigated antibody responses to a comprehensive antigen panel of previously known influenza A viruses (H1-H16) and bat-derived H17 and H18 in straw-coloured fruit bats (*Eidolon helvum*) from Ghana (chapter 6). Our predominant finding of H9 antibodies, an avian-originating subtype known to be zoonotic, open new research avenues into the ecology of influenza viruses in bats. For that multiplex serological screening can be

advantageous as sample quantities are limited. Similarly, recent advances in molecular biotechnology, such as high throughput sequencing and metagenomics [118,119] have facilitated discovery of hundreds of novel bat-borne viruses, currently spanning 28 virus families [120] largely with yet unknown zoonotic potential [121].

A study on commodity chains of bat bush meat in southern Ghana showed that an estimated figure of at least 128.000 bats of the species *Eidolon helvum* – the same species we tested for the presence of influenza antibodies in chapter 6 – are sold every year for consumption in this region alone [122]. Bats were found to be primarily sold in marketplaces but hunters also reported keeping a large number of bats for personal consumption. With hunters, vendors and consumers being part of the commodity chain – comparable to avian influenza virus transmission dynamics – such marketplaces would constitute interesting sites to study zoonotic spill-over events. A recent investigation on risk perceptions with respect to contracting disease through bush meat activities in Ghana showed that 23% of the participants, consisting of hunters, vendors and consumers, were aware of the potential disease risks. Bat consumption was considered significantly more risky to contract infectious disease, than preparing or hunting, and awareness was significantly lower in rural- compared to urban areas [123].

To date, there is a comprehensive body of literature reporting on serological evidence of zoonotic pathogens in various bat species [124–129]. However, targeted studies at the human-animal interface, i.e. also including testing of humans exposed to bats, are scarce [130–132] and would therefore offer an interesting future research field. Multiplex serological methods such as the protein microarray, or more advanced and novel serological techniques using synthetic viromes, termed VirScan, would be advantageous for such purposes. VirScan, developed for the use in humans, enables unravelling of exposure histories against an array of viral pathogens, currently comprising 206 human virus species and more than 1000 strains, in less than 1µl of blood [133]. Adapting such novel screening methods to study exposure to zoonotic diseases in bat-exposed humans and potential animal hosts could be promising for future research. Collaborations with bush meat hunters could facilitate easy accessible blood sample collection by using filter paper cards for surveillance of zoonotic pathogen circulation as well as for serological and virological screening, as has successfully been carried out by Global Viral [134], formerly known as the Global Viral Forecasting Initiative for screening of bush meat and hunters in Central Africa. Whereas unfocused virus discovery can quickly exhaust available resources, surveillance efforts should be concentrated to reservoirs and vectors with increased risk of interspecies transmission to humans, as informed by hotspot modelling [135] and human animal interfaces [136]. Data from such a surveillance approach can aid quick response in the early phase of an emerging pathogen, with respect to identification of the source and swift development of diagnostics [21]. In combination with multiplex molecular and serological techniques such a sampling strategy would not only widen our understanding of infectious disease dynamics and ecology at the human-animal

interface, but would also allow efficient spending of funding, rather than focusing on one specific disease [21].

CONCLUDING REMARKS

In conclusion, expanding the current surveillance system for AI and swine influenza towards a concerted 'One Health' approach by involving AI-exposed humans in active sampling in targeted locations would be needed to provide reliable data on the true frequency of zoonotic spill-over events. Such complete scientific evidence can in turn be used to guide public health policy making. The use and further development of multiplex molecular and serological laboratory techniques would enable the generation of serological profiles and addressing heterosubtypic humoral responses, thereby steering away from focusing on select pathogens/ subtypes. Such advances open research opportunities with respect to advancing our understanding of serological responses and complex infectious disease dynamics. As public health interventions have to be weighed in light of cost versus benefit, the proposed actions could first be explored during a pilot/ trial period utilizing funding raised through existing international networks.

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Summary

Zoonoses, i.e. diseases that are transmissible from animals to humans, have received increasing scientific and public attention in recent years. A comprehensive literature review identified 60% of human pathogens as zoonotic, of which 12% constitute so-called 'emerging infectious diseases'. This term denotes previously unknown diseases that cause outbreaks or known diseases that cause significantly more infections in humans today than in the past two decades. This thesis investigates animal influenza viruses as a topical example of zoonotic, emerging infectious diseases. I argue that surveillance at the human-animal interface can be improved by implementing novel serological screening tools. Furthermore, I will recommend that a One Health-based surveillance system, spanning both human and animal health, needs to be put in place.

Water and shore birds are known to act as asymptomatic reservoirs for influenza A viruses. These viruses are classified into different subtypes based on their surface proteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA types were identified in birds, whereas genetic material of two novel subtypes – A(H18N11) and A(H17N10) – was recently discovered in bats from Central and South America. In the past, avian and swine influenza A viruses repeatedly crossed the inter-species barrier with humans. Reassortants thereof – i.e. influenza viruses whose genetic makeup consists of gene segments of more than one influenza virus – have even successfully established themselves in the human population, causing seasonal epidemics and occasional pandemics.

A number of factors are known to promote the occurrence of emerging infectious diseases: increasing contact between humans and animals, animal trade, intensified food production, and enhanced connectivity between humans through international travel. For instance, movement of poultry played an important role in the emergence of the low pathogenic avian influenza (LPAI) virus subtype A(H7N9) in China in 2013. Likewise, air travel contributed significantly to the spread of the swine flu [subtype A(H1N1)pdm09] in the early stages of the pandemic in 2009.

The emergence of the swine-originating A(H1N1)pdm09 was nevertheless rather unexpected; until 2009, avian influenza (AI) viruses – in particular highly pathogenic (HPAI) A(H5N1) and LPAI A(H9N2) – were considered likely candidates for the next pandemic. At the time, these subtypes had already repeatedly crossed the species barrier to humans. To systematically assess the pandemic potential of different animal influenza virus subtypes, the European Food Safety Authority (EFSA) commissioned the 'FLURISK' project, dedicated to the development of a methodological risk assessment framework for potentially pandemic influenza virus strains. As part of the FLURISK project we conducted a comprehensive literature review of zoonotic influenza virus infections in humans. We divided cases based on the type of evidence of infection: direct, virological evidence (when the virus itself could be isolated or viral RNA was detected, **Chapter 2**) versus indirect, serological evidence (when antibodies against animal influenza viruses were found, **Chapter 4**). Of all human cases for which we had direct, virological evidence, 45.7% were caused by HPAI virus subtype A(H5N1)

– a subtype associated with mortality rates of up to 100% in poultry and approximately 60% in humans based on criteria established by the World Health Organization (WHO). Another quarter (26.4%) of human cases were infected with other animal influenza viruses carrying H7 (HPAI and LPAI) or the LPAI HA types H6, H9, and H10. The remaining 27.9% were identified to have been infected with different swine influenza virus subtypes A(H1N1), A(H1N2) or A(H3N2) (**Chapter 2**).

Summarizing results from cases with serological evidence was more challenging, as substantial heterogeneity between studies impeded direct comparability (**Chapter 4**). We therefore developed a scoring system to grade reliability of the reported serological evidence based on a number of factors, such as study design and laboratory methods used. Using this scoring system, we identified studies reporting reliable evidence of past infection with swine influenza virus subtypes A(H1N1), A(H1N2) and A(H3N2) in swine-exposed humans. A number of studies reporting evidence of human infection with avian influenza virus subtypes H5, H7 and H9 also received high scores. Interestingly, we found that studies with lower scores generally reported a higher seroprevalence of antibodies against avian influenza viruses. Our review also revealed that screening efforts to detect antibodies against avian influenza viruses in humans largely targeted HA-types H5, H7 and H9. However, since it is known that other influenza virus subtypes also have zoonotic potential (**Chapter 2**), it is important to expand future seroprevalence studies at the human-animal interface to these understudied subtypes.

On the veterinary side, avian influenza surveillance is primarily focused on subtypes with H5 and H7 HAs. This focus is motivated by the large economic impact associated with these subtypes: these are the subtypes that can mutate from low to highly pathogenic forms, causing high mortality in poultry. However, as our literature review revealed, monitoring circulation of other (potentially) zoonotic subtypes is also important from a public health perspective. We therefore developed a screening tool – protein microarray – that allows simultaneous detection of antibodies for multiple influenza virus subtypes in chickens (**Chapter 3.1**). We included thirteen of the sixteen known HA-types (H1-H9, H11-H13, H16) in the array setup. Validation of the protein microarray using sera from experimentally and naturally infected chickens demonstrated a high sensitivity and specificity, which makes it an attractive screening tool for surveillance at the human-animal interface.

For large-scale sero-surveillance studies or outbreak situations a high number of screening assays is required. To determine the shelf life of spotted microarray slides, we monitored the stability of several antigen classes representing influenza A- and measles viruses and of bacterial *Streptococcus pneumoniae* polysaccharides, over a period of 22 months under four different storage conditions (temperature-controlled, desiccated; room-temperature, non-desiccated; frozen, desiccated; 37°C, desiccated; **Chapter 3.2**). We showed that the currently used standard storage regimen [temperature controlled (21°C) and desiccated (0% relative humidity)] provided the best storage condition for all antigen classes, with spotted slides

remaining stable for the duration of the experiment. The other three conditions produced acceptable results for influenza A and *S. pneumoniae* antigens, but proved less suitable for the measles antigen formulation.

When we reviewed the literature for serological evidence of human infection with animal influenza viruses, we found publications reporting on antibody titers to HA types H1-H13 (**Chapter 4**). However, low-level serological findings against animal influenza viruses need to be interpreted with caution, given the possibility of cross-reactive antibodies triggered by prior infection or vaccination with human influenza viruses. To study the occurrence of cross-reactivity more in depth we analysed maternal antibodies in blood samples from new-borns collected via filter cards through the neo-natal heel prick-screening program (**Chapter 5**). Thirteen countries from five continents provided approximately 7000 samples. All samples were serologically screened using the protein microarray platform comprising a selection of avian influenza virus subtypes as well as recent seasonal and older pandemic human subtypes. A previous study used this dataset to chart the worldwide unfolding of the swine flu pandemic in 2009. In our study, we hypothesised that higher antibody diversity across recent human influenza virus HA types H1 and H3 would result in increased occurrence of cross reactivity. In other words, higher exposure to recent human influenza viruses through infection or vaccination leads to higher heterosubtypic reactivity to avian (H5, H7, H9) and older human pandemic influenza virus subtypes (H1, H2). Overall, we found significant heterogeneity in antibody profiles, but showed that higher antibody diversity to recent human influenza viruses was indeed associated with low-level reactivity to H7 and H9, but not to H5 antigens. Individuals exposed to A(H1N1)pdm09 through natural infection or vaccination showed higher heterosubtypic reactivity. Hence, our study demonstrates that there is a complex interplay between prior exposures to recent seasonal and pandemic influenza viruses and the development of heterosubtypic reactivity against animal influenza viruses.

Finally, in **Chapter 6**, we investigated the role of bats as potential mammalian reservoirs for influenza A viruses. This study was triggered by the discovery of RNA-traces of two novel influenza virus subtypes A(H17N10) and A(H18N11) in bats in 2012/13. As this was the first time that influenza A viruses were found in this taxon, questions were raised about their zoonotic potential. Until then, bats had been already known hosts for a number of zoonotic viruses, such as Rabies, Ebola, Marburg, Hendra and Nipah viruses. Although later studies found no evidence of zoonotic potential of the novel influenza subtypes, these findings prompted us to further investigate whether bats constitute possible carriers for other – potentially zoonotic – influenza A viruses. For this we adapted the protein microarray platform and screened 100 straw-coloured fruit bats (*Eidolon helvum*) from Ghana for the presence of antibodies against known hemagglutinin types H1-H18. Overall, we found a seroprevalence of about 30% against subtype H9, with low-level cross-reactivity to the phylogenetically closely related subtypes H8 and H12. This study was the first to report serological evidence of influenza A viruses other than H17 and H18 in bats. Since avian

influenza virus subtype H9 is associated with human infections, these findings could have potential public health implications that should be the topic of future study.

In conclusion, this thesis contributed to preparedness and surveillance for emerging infectious diseases by developing novel serological screening tools and by addressing benefits and challenges of serology at the human-animal interface. I suggest that integrating screening platforms such as the protein microarray into routine avian influenza surveillance would be a useful complement to the current veterinary-based screening focus on subtypes H5 and H7. This combined approach would constitute a 'One Health'-based surveillance system. Since the benefits of public health actions have to be weighed against their costs, the proposed expansion of the current surveillance system should at first be explored during a pilot/trial period using funding raised through existing international networks.

NEDERLANDSE SAMENVATTING

Zoönoses, oftewel ziektes die van dieren op mensen overgaan, staan sinds enkele jaren in het middelpunt van wetenschappelijke en publieke belangstelling. Recentelijk identificeerde een literatuuroverzicht 60% van alle menselijke pathogenen als zoönoses, en 12% daarvan als zogenoemde 'emerging infectious diseases'. Dit zijn onbekende ziektes die uitbraken veroorzaken, of bekende ziektes die nu aanzienlijk meer infecties in mensen veroorzaken dan in de afgelopen twee decennia. Dit proefschrift is een studie van zoönotische, emerging infectious diseases met dierlijke influenza A (griepvirus) als voorbeeld. Ik stel dat surveillance op het raakvlak van mens en dier verbeterd kan worden door nieuwe serologische screening methodes. Daarnaast beveel ik aan dat er een op 'One Health'-gebaseerd surveillance systeem wordt geïmplementeerd, dat zowel dierlijke als menselijke gezondheid omvat.

Water- en waadvogels staan bekend als asymptomatische reservoirs voor griepvirussen van type A. Deze virussen kunnen verder worden onderverdeeld in subtypes op basis van de oppervlakte eiwitten hemagglutinine (HA) en neuraminidase (NA). Tot op heden zijn 16 HA en 9 NA types geïdentificeerd in vogels. Ook is er onlangs genetisch materiaal van twee nieuwe subtypes – A(H18N11) en A(H17N10) – ontdekt in vleermuizen uit Centraal- en Zuid-Amerika. In het verleden zijn vogel- en varkensgriepvirussen van het A-type meermaals de soortengrens met de mens overgestoken. Reassortanten daarvan, oftewel griepvirussen met genetische materiaal van meer dan één griepvirus, is het zelfs gelukt om permanent te circuleren in menselijke populaties met tot gevolg, seizoensgebonden epidemieën en enkele pandemieën.

Er zijn een aantal factoren bekend die bijdragen aan het ontstaan van opkomende infectie ziekten; (i) toenemend contact tussen mens en dier, (ii) handel van dieren, (iii) geïntensiveerde voedselproductie, en (vi) verhoogde connectiviteit tussen mensen door internationale reizen. Zo speelde pluimveevervoer een belangrijke rol in de verspreiding van de laag pathogene aviaire influenza subtype A(H7N9) in China in 2013. Een ander voorbeeld is de bijdrage door de luchtvaart aan de verspreiding van varkensgriep [subtype A(H1N1)pdm09] in het beginstadium van de pandemie.

De opkomst van A(H1N1)pdm09 uit varkens kwam onverwacht: tot 2009 ging men ervan uit dat de volgende pandemie veroorzaakt zou worden door vogelgriepvirussen, in het bijzonder door de hoog pathogene subtypes (HPAI) A(H5N1) en LPAI A(H9N2). Deze subtypes waren toentertijd al enkele malen de soortengrens met de mens overgestoken.
Om het pandemisch potentieel van verschillende dierlijke virustypes in kaart te brengen, heeft de Europese Autoriteit voor Voedselveiligheid (EFSA) opdracht gegeven voor het 'FLURISK' project. Deze is belast met de ontwikkeling van een raamwerk voor risicoanalyse van potentieel pandemische virustypes. Als onderdeel van het FLURISK project hebben wij een uitgebreide literatuurstudie verricht naar infecties bij mensen door verschillende dierlijke griepvirus subtypes. We hebben de onderzochte infecties onderverdeeld op basis van bewijssoort: direct en indirect. Voor direct virologisch bewijs is er gekeken naar het virus zelf en/of viraal RNA kon worden gedetecteerd (**Hoofdstuk 2**). En serologische bewijs als

indirect, waarbij antilichamen aangetoond worden als bewijs voor blootstelling (**Hoofdstuk 4**). Van alle gevallen van infectie bij mensen waarvoor we direct, virologisch bewijs hadden, was 45,7% veroorzaakt door het HPAI virus subtype A(H5N1) – een subtype dat geassocieerd wordt met een sterftepercentage van 100% in pluimvee en 60% in mensen. Nog een kwart (26,4%) van alle gevallen van infectie bij mensen was door virussen van het type H7 (HPAI en LPAI) of de LPAI HA types H6, H9, en H10. Van de overige 27,9% kon worden vastgesteld dat ze geïnfecteerd waren met de varkensgriep subtypes A(H1N1), A(H1N2), of A(H3N2) (**Hoofdstuk 2**).

Van de gevallen waarvoor serologisch bewijs beschikbaar was, bleek het moeilijker om de resultaten onderling te vergelijken. De aanzienlijke heterogeniteit tussen de studies belemmerde een directe vergelijking van resultaten (**Hoofdstuk 4**). Om deze reden hebben wij een puntensysteem opgezet waarmee de betrouwbaarheid van serologisch bewijs kan worden bepaald aan de hand van diverse factoren, zoals het onderzoeksopzet en de gebruikte laboratorium methodieken. Met behulp van dit puntensysteem hebben we studies geïdentificeerd die betrouwbaar bewijs leveren van infecties met varkensgriep-subtypes A(H1N1), A(H1N2) en A(H3N2) in mensen die blootgesteld zijn geweest aan varkens. We hebben ook hoge aantal punten kunnen toekennen aan een aantal studies die bewijs leverden van menselijke infecties met vogelgriepvirus-subtypes H5, H7, en H9. Verrassend genoeg bleek dat studies met lagere scores over het algemeen melding maakten van een hogere seroprevalentie van antilichamen tegen vogelgriepvirussen. Onze literatuurstudie wees ook uit dat inspanningen om mensen te screenen op antilichamen tegen vogelgriepvirussen voornamelijk gericht was op de HA-types H5, H7, en H9. Aangezien het bekend is dat andere virus-subtypes ook zoönotisch potentieel hebben (**Hoofdstuk 2**), is het van belang om toekomstige seroprevalentie-studies op het grensvlak van mens en dier uit te breiden naar deze onderbelichte subtypes.

In de veterinaire context is de surveillance van vogelgriep vooral gericht op de subtypes met H5 en H7 HA's. Deze focus is ingegeven door commerciële belangen: dit zijn de subtypes die hoge sterftepercentages kunnen veroorzaken onder pluimvee, door te muteren van laag-pathogene naar hoog-pathogene varianten. Voor de volksgezondheid is het echter ook van belang om de verspreiding van andere (potentiële) zoönotische subtypes in kaart te brengen. Wij hebben daarom een serologische test ontwikkeld om antilichamen tegen meerdere griepvirus subtypes tegelijkertijd te kunnen detecteren in kippen (**Hoofdstuk 3.1**). Deze test is een aanpassing van de proteïne microarray, waarmee menselijk serum op antilichamen tegen griepvirussen getest kan worden. Onze array-opstelling maakt het mogelijk om simultaan te testen op dertien van de zestien bekende HA types (H1-H9, H11, H16). Validatie van de proteïne microarray met behulp van sera van geïnfecteerde kippen toonde een hoge sensitiviteit en specificiteit aan. Dit maakt de proteïne microarray tot een aantrekkelijke test voor surveillance op het grensvlak van mens en dier.

Een hoog aantal screening analyses is vereist in het geval van uitbraken of voor sero-surveillance studies op grote schaal. Om de houdbaarheid van gespotte microarray slides te bepalen, hebben we de stabiliteit onderzocht van een aantal groepen antigenen die griep A-virussen, mazelen, en bacteriële *Streptococcus pneumoniae* representeren. Daarvoor hebben we de gespotte microarray slides gedurende 22 maanden bewaard onder vier verschillende condities, namelijk: gedroogd en op gecontroleerde temperatuur; niet-gedroogd en op kamertemperatuur; gedroogd en bevroren; gedroogd en op 37°C (**Hoofdstuk 3.2**). We hebben aangetoond dat het gebruikelijke opslagregime – temperatuur-gecontroleerd (21°C) en gedroogd (0% relatieve vochtigheid) – de beste stabiliteit bood voor alle groepen antigenen; gespotte slides bleven stabiel gedurende het verloop van het experiment. De drie andere condities leverden acceptabele resultaten voor influenza A en *S. pneumoniae*, maar bleken minder geschikt voor mazelen antigenen.

In onze literatuurstudie naar serologisch bewijs van menselijke infectie door dierlijke griepvirussen vonden we publicaties die verslag maakten van antilichaam titers tegen HA types H1-H13 (**Hoofdstuk 4**). Lage serologische titers moeten echter behoedzaam worden geïnterpreteerd, omdat ze mogelijkerwijs berusten op kruisreagerende antilichamen veroorzaakt door eerdere infectie of door vaccinatie met menselijke griepvirussen. Om kruisreactiviteit beter te kunnen bestuderen hebben we bloedmonsters van pasgeborenen getest. Deze bloedmonsters werden verkregen met behulp van 'filter cards' uit nationale hielprik-programma's van dertien landen op vijf continenten (**Hoofdstuk 5**). In totaal hebben we 7000 bloedmonsters gescreend op maternale antilichamen tegen griepvirussen met behulp van het proteïne microarray platform. De microarray omvatte en een selectie van vogelgriepvirus subtypes en recente seizoensgebonden en pandemische subtypes die onder mensen circuleerden. Dezelfde dataset is in een eerdere studie gebruikt om het ontvouwen van de in varkensgriep pandemie van 2009 in verschillende landen in kaart te brengen. De hypothese van onze studie was dat een hogere diversiteit van antilichamen tegen het recente menselijke griepvirus HA-types H1 en H3 zou resulteren in een toename van kruisreagerende antilichamen. Met andere woorden, we verwachtten dat een hogere blootstelling aan recente menselijke griepvirussen door infectie of vaccinatie zou leiden tot een hogere heterosubtypische reactiviteit tegen vogelgriep (H5, H7, H9) en oudere menselijke pandemische griepvirus subtypes (H1, H2). Onze resultaten lieten een significante heterogeniteit onder antilichaamprofielen zien. We hebben aangetoond dat een hogere diversiteit van antilichamen tegen recente menselijke griepvirussen gepaard gaat met een lichte verhoging in reactiviteit tegen vogelgriep antigenen H7 en H9, maar niet tegen H5 antigenen. Daarnaast hebben een hogere heterosubtypische reactiviteit geconstateerd bij individuen die blootgesteld waren aan A(H1N1)pdm09 door natuurlijke infectie of vaccinatie. Kortom, onze studie toont aan dat er een complexe wisselwerking bestaat tussen voorgaande blootstelling aan seizoensgebonden en pandemische griepvirussen en de ontwikkeling van heterosubtypische reactiviteit tegen dierlijke griepvirussen.

Tot slot bespreken we in **Hoofdstuk 6** de resultaten van ons onderzoek naar de mogelijke rol van vleermuizen als zoogdierreservoirs voor griepvirussen van het type A. Deze studie werd ingegeven door de ontdekking, in 2012/13, van RNA-sporen van twee nieuwe griepvirus subtypes A(H17N10) en A(H18N11) in vleermuizen. Dit was de eerste keer dat griep A virussen in dit taxon werden aangetroffen, wat vragen opriep over hun zoönotische potentieel. Eerder was al wel bekend dat vleermuizen gastheren zijn voor een aantal andere zoönotische virussen, zoals rabiës, ebola, Marburg, Hendra en Nipah virussen. Hoewel latere studies geen bewijs vonden voor zoönotisch potentieel van de nieuwe influenza subtypes, gaven deze bevindingen ons aanleiding om te onderzoeken of vleermuizen mogelijk dragers zijn van andere, potentieel zoönotische griepvirussen van het type A. We hebben hiertoe de proteïne microarray gebruikt, waarmee we 100 palmvleerhonden (*Eidolon helvum*) uit Ghana gescreend hebben op antilichamen tegen hemagglutinine types H1-H18. Onze resultaten lieten een seroprevalentie van ongeveer 30% tegen subtype H9 zien, en een lage kruisreactiviteit met de fylogenetisch nauw verwante subtypes H8 and H12. Dit was de eerste studie die serologisch bewijs leverde van de aanwezigheid van andere griep A virussen in vleermuizen dan H17 en H18. Omdat vogelgriepvirus subtype H9 geassocieerd wordt met menselijke infecties hebben deze resultaten mogelijk implicaties voor de volksgezondheid. Dit vergt nadere studie.

Tot besluit concludeer ik dat dit proefschrift heeft bijgedragen aan de surveillance van – en waakzaamheid voor – opkomende infectieuze ziektes, zowel door ontwikkeling van nieuwe serologische screening tools als door het in kaart brengen van de bruikbaarheid van serologie op het grensvlak van mens en dier. Op basis hiervan beveel ik aan om screening platforms zoals de proteïne microarray te integreren in routine surveillance van vogelgriep. Dit zou een belangrijke aanvulling zijn op het screenen op H5 en H7, waar vandaag de dag de nadruk op ligt in de veterinaire sector. Een dergelijke combinatie zou leiden tot een 'One Health' surveillance systeem. Omdat de voordelen van volksgezondheidsmaatregelen gewogen moeten worden met hun kosten, moet de voorgestelde uitbreiding van het huidige surveillance systeem eerst middels een pilot worden verkend. Om de financiële middelen hiervoor te verkrijgen moeten bestaande internationale samenwerkingsverbanden worden ingeschakeld.

PhD PORTFOLIO

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Research School: Molecular Medicine

PhD period: 03/2011-10/2015

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Education

2011-2015	PhD training at the National Institute for Public Health and the Environment (RIVM), Bilthoven/ Erasmus Medical Center, Rotterdam, the Netherlands (NL)
2012-2015	Postgraduate Epidemiology Master with specialization in 'Epidemiology of Infectious Diseases', Utrecht University, Utrecht, NL

Courses

2011 Summer school: Principles of Research in Medicine, ErasmusMC, Rotterdam, NL
2011 Summer school: Introduction to Global Public Health, ErasmusMC, Rotterdam, NL
2011 Beginner's R course, RIVM, Bilthoven, NL
2011 Use R Course, RIVM, Bilthoven, NL
2011 Meta-analysis course, RIVM Bilthoven, NL
2011 Research Management, ErasmusMC, Rotterdam, NL
2011 Basic Course on SPSS, ErasmusMC, Rotterdam, NL
2011 Course on Molecular Diagnostics, ErasmusMC, Rotterdam, NL
2012 Course in Virology, ErasmusMC, Rotterdam, NL
2012 HKU-Pasteur Virology Course – Viral Zoonoses (*invited*), Hong Kong S.A.R., China
2012-2014 Courses followed for postgraduate Epidemiology Master's program:

- Introduction to Epidemiology
- Introduction to Statistics
- Study Design in Etiologic Research
- Classical Methods in Data Analysis
- Presentation and Writing of Research Proposal
- Modern Methods in Data Analysis
- Research Ethics and Society
- Clinical Epidemiology
- Epidemiology of Infectious Diseases
- Public Health Epidemiology
- Molecular Epidemiology of Infectious Diseases- the pathogen side
- Multivariate Analysis
- Mixed Models

- Generalized Linear Models
- Basics of Mathematical Modelling of Infectious Diseases
- Advanced Modelling of Infectious Diseases

2013 Workshop: *'Loskomen van de plakkende vloer – dat doe je zelf'*, ErasmusMC, Rotterdam, NL

2013 Workshop: Debating, RIVM, Bilthoven, NL

2014 Biomedical English Writing Course, ErasmusMC, Rotterdam, NL

2014 Masterclass: Scientific Integrity, Utrecht University, Utrecht, NL

2014 Workshop Storytelling, VENA, Erasmus University, Rotterdam, NL

2014 Zoobiquity Conference, Amersfoort, NL

2015 Masterclass: 'Women in Medicine – how can you take a leadership role?', Erasmus Centre for Women and Organisations, Rotterdam, NL

Scientific Conferences and Meetings

2011 The 4th ESWI Influenza Conference, St. Julian, Malta (poster presentation)

2013 2nd ISIRV International Symposium on Neglected Influenza Viruses (poster presentation), Dublin, Ireland

2014 7th International Conference on Emerging Zoonoses, Berlin, Germany (oral presentation)

2014 International Meeting on Emerging Diseases and Surveillance (IMED), Vienna, Austria (poster presentation)

2014 Young Antigone Meeting, Scheveningen, the Netherlands (poster presentation)

2014 Antigone meeting, Scheveningen, the Netherlands (oral presentation)

I gave additional oral presentations in the context of national and international collaborations throughout the PhD training period.

Teaching

2012 Workshop: 'Indonesian-Dutch cooperation project – Control of Avian Influenza H5N1', Jakarta Indonesia

2013 Supervision of MLO student

Other activities

2014-2015 Reviewer for 'Journal of Clinical Virology'

2015 Volunteered to support diagnostic activities during the Ebola Virus Outbreak, Sinje, Liberia

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CURRICULUM VITAE

Gudrun Stephanie Freidl was born on 28 December 1984 in Wolfsberg, Austria. She trained as a veterinarian at the University of Veterinary Medicine Vienna, Austria. She pursued part of her clinical training abroad, at the Faculty for Veterinary Medicine of the University of Pretoria, South Africa, at the University of Cambridge, United Kingdom and at the Budapest Zoo and Botanical Gardens in Budapest, Hungary. In course of her studies she specialized in 'Small Animal Medicine' and 'Conservation Medicine' during which she developed an interest in zoonotic diseases and population health. After graduating in 2010, she worked for the University of Veterinary Medicine Vienna at the Institute for Wildlife Ecology (FIWI). In 2011, she moved to the Netherlands to pursue a PhD in Public Health Virology at the National Institute for Public Health and the Environment (RIVM), and the Erasmus Medical Center in Rotterdam. She was involved in two international, EU-funded projects: 'Antigone' and 'FluRisk'. In parallel to her doctoral research she earned a Master's degree in Epidemiology with a specialization in Infectious Disease Epidemiology at Utrecht University. In early 2015, she volunteered to work in a mobile laboratory in Liberia to provide diagnostic support to the Ebola outbreak. In June 2015, the European Centre for Disease Prevention and Control (ECDC) awarded Gudrun with a fellowship in the European Programme for Intervention Epidemiology (EPIET, EU-track). She pursues this fellowship at the department for Epidemiology and Surveillance at the RIVM.

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'It is good to have an end to journey toward, but it is the journey that matters most in the end'
~ Ernest Hemingway

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