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Selected Abstracts

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Azzi et al. 2010. J Mol Genet Med, 4, 252.

Presence of D222E mutation in the haemagglutinin of pandemic (H1N1) 2009 isolates in Tuscany

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Since the first appearance of the pandemic (H1N1) 2009 virus many efforts have been made to monitor the changes of the virus at molecular level with the aim to early detect mutations which could alter its pathogenicity. Some mutations have been observed more frequently in viruses that caused severe or fatal infections than in viruses involved in mild infections. Among these, the most common seems to be the amino acid substitution D222G (or D222E or D222N) in the HA1 sequence. However, to better assess the association of such mutation with pandemic virus pathogenicity a large set of data is required, in addition to other experimental studies.

In our laboratory, as regional reference laboratory in Tuscany, 2350 respiratory specimens have been analysed for pandemic virus detection from May 2009 to May 2010 and 552 out of them showed positive by a real time RT-PCR. Here we report the results of the sequencing of a small region of the HA1 gene, 180 nucleotide long, performed to detect the previously reported mutation in two small groups of our positive samples.

Thirteen out of 18 isolates (72%) from patients with severe disease had the D222E mutation in the HA1 in comparison with 7 out of 26 isolates (27%) from patients with mild disease. In this last group 7 children coming back from a school trip in England were included. Five of them shed a virus with the D222E substitution, suggesting that the mutated virus had been transmitted from a shared source. By cloning the PCR products of some samples from severe case of influenza, the presence of "quasispecies" was observed. Altogether, the 20 isolates with the D222E mutation were obtained from July to November 2009. Our observation confirm that the mutation is more frequently detectable in association with severe forms of influenza and that the mutated virus was easy transmissible.

Keywords: pandemic influenza 2009, pathogenicity marker, haemagglutinin

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Bredholt et al. 2010. J Mol Genet Med, 4, 253.

Re-vaccination with an adjuvanted pandemic influenza H1N1 vaccine provides early protection in healthcare workers

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Healthcare workers were prioritized for pandemic vaccination in the majority of countries to maintain the integrity of the healthcare system. In October 2009, we conducted a clinical trial in 250 frontline healthcare workers vaccinated with a low dose split pandemic H1N1 virus (X179a A/California/7/2009) vaccine adjuvanted with AS03. Vaccination induced a protective level of haemagglutination inhibition (HI) antibodies by 6-7 days post-vaccination, however in 10% of volunteers vaccination failed to induce and maintain a protective HI antibody response at 3 months post-vaccination. These non-responders were offered re-vaccination with the X179a H1N1 vaccine and here we report the kinetics of serum B- and T-cellular response to re-vaccination.

Twelve healthcare workers (8 females and 4 males, average age 40.3 ± 12.7 years) were re-vaccinated with one dose of AS03 adjuvanted pandemic vaccine (3.75 μ g haemagglutinin). The serum antibody response to homologous vaccine strain (X179a) and cross-reactivity to 3 other H1N1 strains (A/Brisbane/59/2007, A/New Caledonia/20/99 and A/Texas/36/91) was evaluated by HI assay. The frequency of antigen-specific antibody secreting cells and memory B-cell responses were detected by ELISPOT assays. The percentage of antigen-specific CD4⁺ T-cells secreting one or more Th1 cytokine (TNF alpha, IFN gamma or IL-2) was analysed by intracellular staining and multiparametric flow cytometry.

There was a significant ($p < 0.001$) increase in X179a-specific HI antibody titre by 7 days post-vaccination (geometric mean titer 284) with 92% of vaccinees having a protective antibody titre of ≥ 40 and the response plateaued up to day 21. Only 1 vaccinee failed to reach a protective antibody level by 21 days after vaccination. The majority of subjects had pre-vaccination HI titres ≥ 40 to A/Brisbane/59/2007, A/New Caledonia/20/99 and A/Texas/36/91 strains and no significant increase in the antibody titre was observed after vaccination. Homologous and cross-reactive IgG-secreting B-cells were detected at 7 days post-vaccination and an increase in the frequency of homologous, IgG-positive memory B cells was detected at day 7 and day 21 post-vaccination (further evaluation of the B-cell response is ongoing). The frequencies of polyfunctional CD4⁺ T-cells simultaneously secreting either one or more Th1 cytokines (TNF alpha, IFN gamma or IL-2) were significantly higher post-vaccination.

Re-vaccination induced a rapid increase in a protective antibody response and a polyfunctional CD4⁺ T cell response in healthcare workers, which would provide early protection against pandemic influenza H1N1 virus.

Keywords: Pandemic H1N1, swine flu, protective antibodies, haemagglutination inhibition, memory B-cells, polyfunctional T-cells

Chaudhry et al. 2010. J Mol Genet Med, 4, 253.

Seroprevalence of avian influenza type A and Newcastle Disease viruses among backyard poultry flocks in Lahore district, Pakistan

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Avian influenza type A and Newcastle Disease viruses cause significant economic losses to commercial poultry in Pakistan and are endemic in this area. Rural poultry contributes 56% of total egg production and 25% of poultry meat in Pakistan. Almost all rural, and 20% of urban, households keep backyard flocks, which are affected by common circulating viruses of poultry. A serological survey was designed to determine the prevalence of these viruses among the backyard flocks in Lahore district during July-August 2009. Two-stage cluster sampling was undertaken. In the first stage, 35 out of

a total of 308 villages in Lahore district were selected on the basis of probability proportional to size (PPS) as clusters. In the second stage, six chickens of >2 months were selected as elementary units. A total of 210 serum samples were collected and examined by the haemagglutination inhibition test for specific antibodies against avian influenza virus (AIV) subtype H9, AIV subtype H5 and ND viruses. The seroprevalence of AIV subtype H9 was 63.8% (95% CI: 55-72%), that of AIV subtype H5 was 18.1% (95% CI: 12-24%) and of Newcastle Disease virus 41.9% (95% CI: 33-50%). The study therefore confirmed the exposure of backyard flocks to these viruses and indicated that AIV subtypes H9 and H5 and Newcastle Disease virus are circulating in backyard poultry. AIV potentially poses a zoonotic risk to human beings, interacting daily with these birds. The spread of these viruses could be due to low biosecurity at farm level or due to mixing of wild and migratory birds with backyard poultry. To control the further spread of AIV and ND, improvement in biosecurity of backyard flocks and ongoing monitoring of their disease status is recommended.

Keywords: Avian influenza type A, seroprevalence, Newcastle disease, haemagglutination Inhibition, backyard flock, AIV H5, AIV H9, cluster sampling

Cheng et al. 2010. J Mol Genet Med, 4, 254.

Knowledge, attitudes, and practices of human H5N1 avian influenza in the Shen Zhen general population

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This study was conducted to understand the knowledge, attitudes and practices of human H5N1 avian influenza (AI) in the Shen Zhen general population. Using a cross-sectional, Proportional Probability Sampling (PPS), face-to-face survey, 2073 Chinese people in Shen Zhen were interviewed anonymously in August of 2007. 90.4% of respondents had heard of avian influenza. The knowledge, attitudes and practices of human H5N1 avian influenza were significantly different among sex, age, education and occupation. 40.5% of respondents who heard of avian influenza had gone to the living chicken market AOR=2.31 (1.61~3.32), and do not touch the cage AOR=0.35 (0.12~0.98). Related AI information primarily came from public newspaper and television. Thus, the AI risk perception is high level, but the level of self-efficacy is low in Shen Zhen. Attention to risk communication and how to increase the self-efficacy should be paid. Timely dissemination of update information is greatly warranted.

Keywords: Human H5N1 avian Influenza, knowledge, attitudes, practices

Chui et al. 2010. J Mol Genet Med, 4, 254.

Memory T cell responses in acute influenza A infection and vaccination in humans

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Influenza virus causes morbidity and mortality worldwide. Neutralizing antibodies are the major correlates of protection against influenza infection and the magnitude of their induction is widely used to evaluate the effectiveness of an influenza vaccine. Despite substantial evidence in animal models which suggest critical roles of T cells in the viral clearance, the role of cellular immunity in humans remained poorly understood. This project aims to determine cellular immune responses in sero-negative human volunteers following nasal challenge with a live virus, and to identify the role of pre-existing T cell responses in the virus shedding and disease severity in the absence of antibody. I have found that pre-existing memory T cells persist in most individuals and predominantly are specific against internal proteins such as nucleoprotein and matrix proteins. The challenge studies identified over 50 peptides and revealed the T cell response to be predominantly CD4-dependent. Seven days after challenge infection, these influenza-specific CD4 cells have greatly expanded (about 10 times) in both breadth and magnitude, and were able to kill antigen-loaded autologous B cell lines *in vitro* by chromium release assay. These acutely expanded T cells were shown to be highly activated (CD38+) and proliferative (Ki-67+). In a separate pandemic H1N1 vaccine trial where 150 healthy volunteers were vaccinated with an inactivated unadjuvanted split virion pandemic H1N1 vaccine, a modest response of influenza-specific T cells could be induced. Responses to both surface (HA and NA) and internal proteins were induced, and specific response measured by HA and NA peptide pools

were also found to be strongly CD4+ dependent. Sixteen peptides were identified in this vaccine cohort. Activated proliferating cells induced during vaccination are of a central memory phenotype. As immune memory forms the basis of protection through natural infection or vaccination, the project will carry on to dissect how these memory CD4+ T cells function and differentiate in an acute infection. Due to the cross-reactive nature of T cell recognition and high degree of homology of different influenza subtypes, it is intriguing to explore the heterotypic immunity of T cell responses in acute influenza infection. This work should provide an insight on how cellular immunity can be targeted in conferring broad protection against different subtypes of influenza A viruses.

Keywords: human seasonal influenza, experimental infection, T cell immunity, influenza inactivated vaccine, pandemic H1N1

Germundsson et al. 2010 . J Mol Genet Med, 4, 255.

Pandemic H1N1 2009 virus in Norwegian pigs naïve to influenza A viruses

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In March-April 2009, a novel pandemic influenza A (H1N1) virus (pH1N1-09v) emerged in the human population. The first case of pH1N1v infection in pigs was reported from Canada in May 2009. In Norway, pH1N1v infection was recorded in a swine herd on the 10th of October of 2009. Here, we report results from the investigation performed during the outbreak and the follow up surveillance performed in the Norwegian pig population.

Nasal swabs were collected from herds i) where pigs had been exposed to persons with verified pH1N1-09v infection or with influenza-like illness (ILI); ii) where pigs showed clinical signs or iii) with a history of close contact with or close proximity to infected herds. In addition, blood samples were collected from nucleus and multiplier breeding herds. Detection of pH1N1-09v was initially performed using a real-time RT-PCR targeted to detect influenza A virus. Positive samples were tested by a pH1N1-09v specific real-time RT-PCR. Blood samples were tested for presence of antibodies against influenza A virus by ELISA (IDVET) and positive samples in the ELISA were tested by haemagglutinin inhibition test using A/California/07/09 as antigen.

From the onset of the outbreak and until 31st of December 2009, the pH1N1-09v was detected in nasal swabs from 54 of 114 herds investigated tested, while 55 of 140 herds tested positive for antibodies against pH1N1-09v. No herd has been tested positive for pH1N1-09v since early January 2010, however, results of the Norwegian surveillance and control programme for specific swine herds for 2010 so far indicates that 40 % of the swine herds (154 herds) are positive for antibodies against pH1N1-09. Serological evaluation of swine herds and detailed back tracking of the outbreak indicated that the virus was introduced in September 2009. The Norwegian swine population has, until the outbreak of pH1N1-09v, been considered free from influenza A virus infection as documented through serological surveillance program running since 1997. Virus isolated from one of the herds positive for pH1N1-09v was fully identical across the full genome to virus isolated from a confirmed human case at the farm. The majority of the positive herds had a history of contact with humans that were diagnosed with pandemic influenza or with ILI. This suggests that infected humans are the most likely source for introduction of pH1N1-09v to the Norwegian pig herds, especially in the early phase of the outbreak.

Keywords: influenza A, pH1N1-09v, pigs, humans

Mbewana et al. 2010. J Mol Genet Med, 4, 255.

Development of an avian influenza H5N1 vaccine in Africa

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Influenza A viruses are responsible for causing annual outbreaks in humans, and the latest pandemic H1N1 virus is an example of the potential for these viruses to recombine. The highly pathogenic avian influenza type H5N1 is the most

virulent influenza virus, reported thus far. Currently Africa does not have the capacity to produce influenza vaccines, thus relies on the goodwill of the developed countries for vaccine stocks. Our aim is to develop a stable and cheap candidate vaccine against H5N1 for Africa by producing it in plants. Recombinant plant expression allows for the production of easy, rapid, inexpensive and infinitely scalable vaccines. We focused on producing two variants of the HA protein derived from the A/Viet/1194/2004 sequence: these were a full-length (H5) and truncated form (H5tr) with the membrane insertion domain removed. These variants were expressed in plants from genes that were optimised for human codon use. We also cloned the HA variant genes into a DNA vaccine vector.

For plant expression, the HA genes (*H5* and *H5tr*) were cloned into four binary plant expression vectors which target recombinant protein into the different subcellular compartments of the host plant cells. These are the cytoplasm, endoplasmic reticulum (ER), the chloroplast and the apoplast. Gene expression was tested by stable transformation of *Nicotiana tabacum* and transient expression in *N. benthamiana* via *Agrobacterium tumefaciens* mediated gene transfer. Western blots of plant extract indicated that both protein variants were successfully expressed in plants. These proteins were purified by diafiltration and His-tag purification via nickel-bound resin. HA protein was produced in plants in high amounts in the ER (H5tr) and apoplast (H5) compared to the other plant compartments. Haemagglutination (HA) and haemagglutination inhibition assays (HI) confirmed that the conformation of both proteins was correct.

For the DNA vaccine, the H5 and H5tr genes were cloned into a mammalian expression vector pTH and both were successfully expressed in HEK293 cells as detected by western blotting. A mouse immunisation trial was conducted followed by immunological analysis. The presence of H5 specific antibodies was detected in the mouse sera by western blotting.

To conclude, the HPAI H5N1 influenza HA variants (H5 and H5tr) were successfully produced in plants. Highest amounts of H5tr were produced in the ER while H5 was best expressed in the apoplast. Mice inoculated with the H5 and H5tr candidate DNA vaccines showed a positive antibody response to both vaccines. These results indicate that vaccines using HA-derived H5 and H5tr are immunologically effective and that production can be made cheaper as they are expressed successfully in plants.

Keywords: Avian influenza H5N1, plant expression, DNA vaccine, Africa

Iorio et al. 2010. J Mol Genet Med, 4, 256.

The new pandemic A/H1N1 influenza virus (pH1N1): cellular and humoral cross-reactive immune responses induced in healthy adults following 2007/2008 influenza vaccination

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The generally mild illness induced by the recent pH1N1 virus and the higher incidence of illness in people younger than 65 years suggested the possible influence of pre-existing cross-reactive immunity against the new virus in the oldest people and probably in people previously vaccinated with seasonal influenza vaccines [1,2]. This study evaluated humoral and ex vivo cellular immune responses in 12 healthy adult subjects vaccinated with the 2007/2008 MF59-adjuvanted trivalent (A/Wisconsin/67/05 (H3N2), A/Solomon Islands/3/06 (H1N1), B/Malaysia/2506/04) subunit vaccine (FLUAD, Novartis). Peripheral blood mononuclear cells (PBMCs) and serum samples, obtained before and respectively 7 and 30 days after vaccination, were frozen and successively used.

Cellular responses were evaluated determining antigen-specific activation of T lymphocytes by FACS enumeration of CD69+ CD3+ or CD69+ CD8+ T lymphocytes and by measuring antigen-induced IFN-gamma release by ELISPOT. PBMCs were stimulated in vitro with the influenza vaccine antigens, the new pH1N1 virus and a haemagglutinin-(HA)-peptide₃₁₇₋₃₄₁, corresponding to a highly conserved sequence of the HA stalk region containing the fusion peptide, a possible candidate for a universal influenza vaccine [3]. After vaccination there was an increase, in most instances significative, in the numbers of activated (CD69+) total T lymphocytes, as well as CD8+ cells and in T lymphocytes IFN-gamma production, following stimulation not only with vaccine antigens, but also with the new pH1N1 virus and with the HA-peptide₃₁₇₋₃₄₁.

Humoral responses were studied determining haemagglutination inhibiting (HI) antibody titres against the vaccine antigens and the new pH1N1 virus in 11 of the 12 volunteers. Nine (82%) volunteers seroconverted at least against one

vaccine antigens, whereas only one against pH1N1. After vaccination the percentages of seroprotected (HI titre ≥ 40) people against the vaccine antigens ranged between 64 and 91%, whereas only one volunteer showed protection against pH1N1.

Overall, these data raised the possibility that, although annual influenza vaccines are primarily aimed to stimulate the generation of anti-HA antibodies, which confer protection against homologous strains, they can induce also some level of cross-reactive immunity, especially cellular immunity. The finding, after 2007/2008 influenza vaccination, of induction of cellular cross-reactivity against pH1N1 virus is consistent with epidemiological studies suggesting effectiveness against pandemic H1N1-associated illness deriving from seasonal vaccination [1,2] and the induction of reactivity against the highly conserved HA- peptide₃₁₇₋₃₄₁ support the possibility of a universal influenza vaccine [3].

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Keywords: Influenza vaccination, cellular and humoral immunity, cross-reactive responses

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Lees et al. 2010. J Mol Genet Med, 4, 257.

The spatial location of mutations in the HA1 domain of Human Influenza A Haemagglutinin and their role in antigenic escape

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Influenza A's escape from human host immunity is generally associated with mutations of the viral surface glycoprotein haemagglutinin, particularly mutations in the membrane-distal HA1 domain. In this study, we examine the spatial location of HA1 mutations between successive epidemic strains and selected intermediates of H1N1 and H3N2 subtypes with reference to X-ray derived crystal structures. We demonstrate, through a computational approach, that the majority of mutations between two such strains can be found to occur in a region whose size approximately matches the typical footprint of a conformational antibody. We suggest the application of this technique as a possible predictive approach for the determination of dominant antibody locations, and apply it to refine our previous work on predictive models of antigenic escape (Lees et al, 2010).

Keywords: Influenza, Virology, Sequence Analysis, Antibody Binding, Haemagglutinin

Lees WD, Moss DS and Shepherd AJ. 2010. A Computational Analysis of the Antigenic Properties of Haemagglutinin in Influenza A H3N2. Bioinformatics, 26, 1403-1408.

Lu et al. 2010. J Mol Genet Med, 4, 257.

Serum antibody response to 2009 H1N1 influenza vaccine among children in Shenzhen

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Since April 2009, the pandemic (H1N1) 2009 caused by a new strain of H1N1 influenza virus has spread all over the world. Massive vaccination is anticipated to have an important role for controlling the transmission. Shenzhen, located in southern China's Guangdong province, is situated immediately north of Hong Kong. Due to its geographical location and massive immigration, Shenzhen became one of the cities dispensing free H1N1 vaccines produced by a domestic company. In this study, we surveyed antibody response in serum samples obtained from children before and after the vaccination.

A total of 286 children without history of a recent respiratory infection were given in a single shot in December 2009. Two serum samples were collected from every child, which were obtained at the time of the vaccination, and two weeks after the vaccination, respectively. Hemagglutination-inhibition (HI) tests were conducted to determine the antibody levels. The seropositive rate (SPR, the percentage with HI titer $\geq 1:40$ post-vaccination) to pandemic (H1N1) 2009 virus and the geometric mean titer (GMT) were calculated. Meanwhile, paired-Samples T test was used to comparatively analyze antibody response before and after the vaccination.

The mean ages of the children were 11.3 ± 2.7 years. Among 286 individuals, 71 were seropositive with a cut-off antibody titer of 1:10, and 20 presented a protecting antibody titer of at least 1:40 before the vaccination. The results indicate that some people might have contracted the H1N1 2009 infection. The SPR of antibodies to pandemic (H1N1) 2009 virus was 62.6%. There was significant difference in GMT antibodies to pandemic (H1N1) 2009 virus between serums collected before and after the vaccination (19.4 vs. 62.4 , $P=0.00$).

In this survey, the antibody level in children to pandemic (H1N1) 2009 virus rapidly rose after a single shot with the vaccine made in China. In addition to vaccination, some people were found to be seropositive after the first wave of the pandemic (H1N1) 2009. Along with the community transmission of H1N1 influenza virus and the campaign of vaccination, more and more people will present with protecting serum antibodies.

Keywords: H1N1 vaccination, Antibodies, Shenzhen

Lycett et al. 2010. J Mol Genet Med, 4, 258.

Adaptive and Compensatory Mutations in Zoonotic Influenza A Subtypes

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The natural reservoir hosts for influenza viruses are thought to be wild waterfowl. Nevertheless transmissions to mammals occur and some lineages have become established in swine and humans. The aim of this work is to investigate mutations in influenza A viruses which are directly associated with adaptation to swine and humans, and distinguish them from mutations that are compensatory.

In this study we focus on zoonotic transmissions in subtypes H1N1 - H3N2 and H5N1, and analyse complete genome sequences from the NCBI database. To gain a better understanding of the diversity and evolution of the pandemic (H1N1) 2009 precursor strains we undertook complete genome sequencing of archived European swine isolates using an Illumina platform. From over 600 archived isolates available, an initial selection of approximately 100 was made to include one isolate per subtype per country per year, prioritising H1N1 sequences from 1990 onwards.

The evolutionary histories of the viral segments in the swine 'flu lineages were investigated using time resolved phylogenetic trees (inferred using BEAST) and rates of evolution were calculated in different lineages. The detailed associations between mutations at amino acid sites and avian, swine and human host changes were inferred using Bayesian Graphical Models (BGMs). A BGM represents the direct conditional dependencies between variables as edges in a network, so distinguishes between direct and indirect interactions. To investigate the effect of host change on the appearance of mutations, and account for founder effects and shared ancestry, we use the mutational histories of the sites as variables. The mutational histories were inferred by using codon models (or host change model) fitted to phylogenetic trees.

Our initial results show that mutations in the receptor binding site in Hemagglutinin are associated with host change (as expected) and also other changes in HA antigenic sites. Several sites in the polymerase complex were found to co-mutate with each other, and there were 7, 4 and 3 sites directly associated with host change in PB2, PB1 and PA respectively (including PB2-627 and PB2-701). For the NS1 protein, we found 4 sites directly related to host change including sites in SH3, RNA and CPSF30 binding domains. The results suggest that some key mutations are needed to adapt avian influenza viruses for mammalian epidemics, and that several compensatory mutations can occur to enable the virus to increase its fitness in its new environment.

Keywords: Adaptation, mutations, modelling, Bayesian, swine influenza

Nazir et al. 2010. J Mol Genet Med, 4, 259.

Use of filter carrier technique to measure tenacity of avian influenza viruses in wet environmental conditions

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Avian influenza viruses (AIVs) have been shown to persist for extended periods of time in water under laboratory conditions. However, estimation of viral persistence in the environment is a difficult task since viruses are mostly associated with particulate matter which has a major effect on their survival. A germ carrier technique was adapted for use with influenza viruses in moist environments. The technique was employed to measure the persistence of 3 low pathogenic AIVs (H4N6, H5N1 and H6N8), one human influenza virus (H1N1) and two model viruses (NDV and ECBO) in lake water at five different temperatures (30, 20, 10, 0 and -10 °C). Persistence of all of the viruses was highest at 0 °C. Lower T-90 values at -10 °C than 0 °C were possibly due to deleterious effect of freeze thawing on infectivity of filter bound viruses leaving the germ carrier technique inappropriate for use at freezing temperatures. Generally, influenza viruses persisted shorter than model viruses while ECBO has the highest survival time in lake water. Individual influenza viruses were inconsistent in their tenacity at all temperatures. A comparison of tenacity of influenza viruses in suspension in lake water and adsorbed to germ carriers showed that the viruses persisted longer adsorbed to germ carriers at all temperatures except -10 °C. This may be important for the actual behavior of the viruses in the environment, as virus shed in fecal and respiratory material may persist longer than free virus. These findings suggest that AIVs can remain infectious in lake water for extended periods of time at low temperatures, allowing persistence of the viruses in the aquatic habitat over winter and possibly over years.

Robertson. 2010. J Mol Genet Med, 4, 259.

The development of vaccine viruses against pandemic A(H1N1) influenza

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Upon the realisation of a pandemic threat in April 2009 from a newly emerged H1N1 influenza virus, a global network of laboratories began the development of candidate vaccine viruses, viruses required by the vaccine industry for efficient vaccine manufacture. By the end of May 2009 several candidates were available, well before the WHO declared a pandemic on June 11. During the ensuing months further improvements were made to these viruses in order to increase the yield of vaccine antigen that could be derived. The above activities were established and practiced well before 2009 as part of pandemic preparedness; further activities in preparedness include the ongoing development of a 'library' of potential pandemic vaccine viruses and research into improving the yield of viral antigen so that the maximum number of doses of vaccine can be produced in the shortest time.

Stech et al. 2010. J Mol Genet Med, 4, 259.

Hemagglutinin cleavage site and beyond: Virulence determinants of HPAIV in HA and other viral genes

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Highly pathogenic avian influenza viruses (HPAIV) differ from all other strains by a polybasic cleavage site in their hemagglutinin (HA) and carry an HA with serotypes H5 or H7 only. In our investigations, we studied the ability of three low-pathogenic avian strains with the subtypes H3N8, H5N1 or H9N2 to transform into HPAIV after introduction of a polybasic HA cleavage site. As HPAIV originate from LPAIV, low-pathogenic H5N1 strains have to be considered potential precursors. Furthermore, the H9N2 strains are also of particular relevance because they became wide-spread across several countries and have been transmitted to humans.

In contrast to their parent viruses, all polybasic cleavage HA site mutants were able to form plaques and replicate in cell-culture in the absence of trypsin. Therefore, in-vitro they resemble an HPAIV. However in chicken, they did not display

high virulence. The H3 cleavage site mutants led only to few temporary clinical symptoms in some chickens accompanied with cloacal shedding whereas the H5 and H9 cleavage site mutants caused temporary non-lethal disease in all animals inoculated. However, a reassortant, derived from LPAIV H5N1 carrying the HA gene of an HPAIV, displayed a lethality of 30% and, furthermore, a reassortant consisting of seven HPAIV genes and the LPAIV HA with engineered cleavage site, exhibited the highest lethality of 80% resembling an authentic HPAIV. Remarkably, a reassortant expressing the H9 HA with engineered polybasic cleavage site and all the other genes from an H5N1 HPAIV is also highly pathogenic in chicken and, with an intravenous pathogenicity index of 1.23, meets the definition of an HPAIV.

Overall, these results demonstrate that acquisition of a polybasic HA cleavage site is only one essential step for evolution of low-pathogenic strains into HPAIV. However, the H5N1 low-pathogenic strains may already have cryptic virulence potential. Beyond the polybasic cleavage site, H5N1 HPAIV carry additional virulence determinants which are located within the HA itself and in the other viral proteins. Furthermore, the finding that an artificial H9 polybasic cleavage site mutant displays the phenotype of an HPAIV, highlights that the H5 and H7 HA have the unique ability to gain a polybasic motif at their cleavage sites.

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The mucosal and systemic immune responses elicited by a chitosan adjuvanted intranasal influenza H5N1 vaccine

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Development of influenza vaccines that induce mucosal immunity has been highlighted by the World Health Organisation as a priority. An influenza vaccine's ability to induce mucosal immunity is important as it is correlated to early protection and protection against drifted influenza strains. The intranasal influenza vaccine mimics the course of a natural influenza infection thus providing the first line of defence. An intranasal vaccine offers a good strategy for efficient mass vaccination as it can be self administered. An efficient mass vaccination regime and dose-sparing strategies will be paramount to reduce morbidity and mortality of a future H5N1 pandemic. This study has investigated the immune response and the dose sparing potential of a chitosan adjuvanted intranasal H5N1 (RG-14) subunit (SU) vaccine. Groups of mice were intranasally vaccinated with one or two doses of a chitosan (5mg/ml) adjuvanted SU vaccine (7.5, 15 or 30µg haemagglutinin (HA)) or with a non-adjuvanted SU vaccine (30µg HA). Another group of mice were intranasally vaccinated with a whole H5N1 (RG-14) virus (WV) vaccine (15µg HA).

We found that chitosan (ChiSys®), which is Archimedes' Proprietary intranasal delivery system, increased the number of double producing CD4⁺ cells and influenza specific antibody secreting cells. Local IgA was boosted by the second vaccine dose and two doses of chitosan adjuvanted vaccine enhanced the serum IgA and IgG response. The production of serum antibodies and the haemagglutination inhibition (HI) response against both the homologous vaccine strain and two heterologous H5N1 strains were also adjuvanted by chitosan. The quality of the B and T cellular response improved with higher doses of adjuvanted vaccine and chitosan showed dose sparing potential down to 7.5µg HA. The WV vaccine elicited the highest frequencies of multifunctional T helper cells (INFγ⁺, IL2⁺, INFα⁺). The cross strain serum reactivity, improved B and T cell responses and dose sparing potential of chitosan shows that a chitosan adjuvanted intranasal influenza vaccine is a strong candidate vaccine to induce a strong and broad protection at lower doses also in humans.

Keywords: Chitosan, dose, H5N1, influenza, intranasal, vaccine

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Internal gene constellation of the viruses possessing H5N1 surface antigens from a highly pathogenic avian influenza virus affects the survival and host gene response of infected chickens

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Pathogenicity of H5 subtype influenza virus in chickens is correlated with the amino acid sequence at the cleavage site of hemagglutinin (HA) protein. It is thought that other factors are involved to viral pathogenicity in chicken as well. Understanding of genetic traits of the pathogenicity would provide a target for prevention and/or control of influenza virus infection in poultries. Aim of our study is to elucidate gene constellations that confer the pathogenicity in chickens and host gene responses against the infection.

Reverse genetic engineered recombinant viruses possessing the HA and NA genes from a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/2004 (Yam; H5N1) were generated. Three of them, designated RGY, YY and YS, had all of internal gene segments derived from Yam, A/chicken/Yokohama/aq55/2001 (Yok; H9N2) or A/whistling swan/Shimane/580/2002 (Shi; H5N3), respectively. Others contained one or two internal gene segments (X) exchanged ones from YY to YS or vice versa (S_X/YY or Y_X/YS). Survival rate and period of the group of chickens infected by reassortants were subjected to the survival analysis. Gene expression in lung collected at 24 hrs pi was investigated by the microarray analysis. Relation between survival period and gene response in lung was analyzed among group of inoculated chickens that were different significantly in the survival analysis.

By the survival analysis among the group of the chickens inoculated with reassorted viruses, they were categorized into three groups with statistical significance. Mean of survival period of YY and YS was 3.87 dpi and 3.33 dpi, and survival rate was 6.67% and 0% respectively. Survival period and rate of chickens inoculated with S_PA/YY and Y_MNS/YS were statistically longer (9.25-10 dpi) and higher (50-100%) than those of YY and YS. All of the chickens inoculated with S_MNS/YY, Y_PB1/YS and RGY died earlier (2-2.25 dpi) than YY and YS. Micro array analysis revealed that expression of 483 genes out of 38681 genes examined was correlated with survival time of the chickens. Gene ontology analysis demonstrated that most of these genes were categorized in either recognition of dsRNA or response to inflammation. These results suggested different constellation of internal gene segments would affect survival period and gene expression of the infected host.

Keywords: Avian influenza, HPAI, pathogenicity, reverse genetics, chicken, gene constellation, microarray analysis