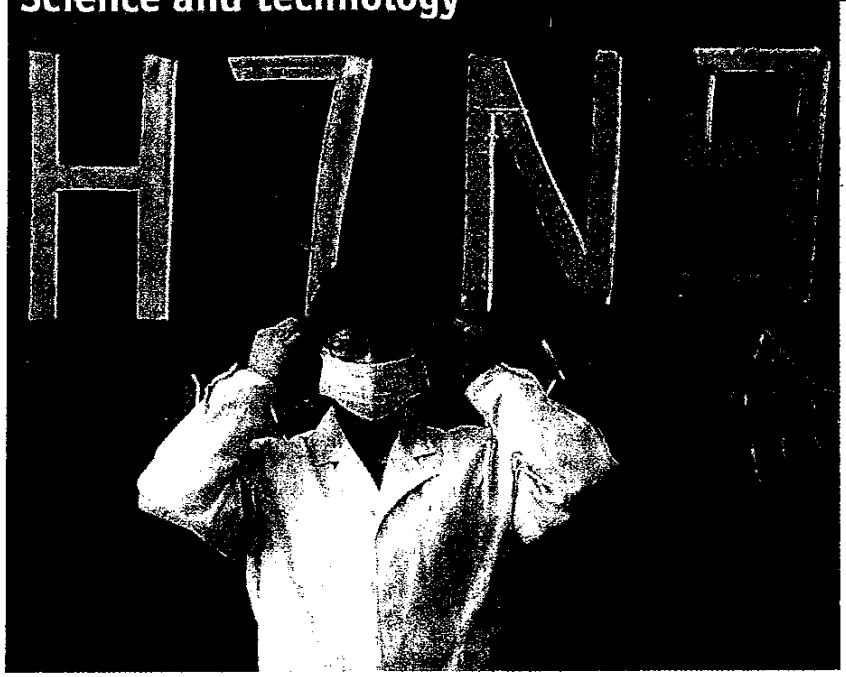

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Pre-empting pandemics

An ounce of prevention

BANGKOK AND NEW YORK

As new viruses emerge in China and the Middle East, the world is poorly prepared for a global pandemic

IN FEBRUARY an 87-year-old man was admitted to hospital in Shanghai. What started as a cough progressed to a fever. One week later, unable to breathe and with his brain inflamed, he died. Shortly afterwards, a 27-year-old pork butcher was admitted to the same hospital with similar symptoms. He died too, within a week. A 35-year-old housewife who went to hospital in Anhui on March 19th lasted only slightly longer. On March 31st officials confirmed these were the first three cases of a strain of influenza, H7N9, that had never before been seen in humans.

The government responded quickly—a far cry from its reaction, ten years ago, to a similar cluster of cases in Guangdong. That infection turned out to be SARS (severe acute respiratory syndrome). At first, officials tried to hide that disease. The deceit served to ensure its spread and it went on to kill nearly 800 people. Much has changed in the past decade. This time officials quickly posted H7N9's genetic sequence, then published a detailed report in the *New England Journal of Medicine*.

Even so, H7N9 has infected at least 82 people and killed 17 of them. The virus's path of transmission is not well understood. The recent detection of H7N9 in a boy with no apparent symptoms suggests people can carry the virus unwittingly. Meanwhile a new coronavirus (the family of viruses that SARS belongs to) is circulating in the Middle East. It has killed 11 people since it was noticed in September. Though Saudi Arabia has welcomed some foreign investigators, other scientists claim the country should be more transparent.

Be prepared

These cases illustrate both how far the world has come, and how far it still has to travel, on the journey towards building a system that can identify new infectious diseases and snuff them out before they become threatening. As the case of AIDS shows, a novel pathogen that spreads around the world unnoticed by the medical authorities can wreak havoc. More recently, cheap air travel has proved a boon to pathogens keen for a global tour. Fortunately the world has learned from the cases of SARS, H5N1 bird flu (in 2005) and H1N1 swine flu (in 2009). Systems are being put in place to spot potentially pandemic diseases and stop them quickly. These systems, though, are still piecemeal. At present it looks unlikely that either H7N9 or the new coronavirus will become pandemic. But if they do—or if some other powerful new virus or bacterium emerges—it is unclear whether the world will be ready.

SARS and H5N1 gave people a shock, and in their wake a lot of progress has been made—and not only in China. In 2005 the members of the World Health Organisation (WHO) agreed on a new set of International Health Regulations, with rules for responding to outbreaks that are of global concern. For example, all members must alert the WHO to any risky-looking pathogen that might move beyond their borders. The regulations also include measures to dissuade people from imposing unnecessary restrictions on travel and trade. In the past, fear of such bans discouraged governments from reporting outbreaks. Meanwhile individual countries have started making their own plans for dealing with a pandemic. As of 2011, 158 had official provisions in place.

Surveillance has moved on by leaps and bounds, too. PROMED and HealthMap, two online reporting programmes at the International Society for Infectious Diseases and Boston Children's Hospital respectively, use a range of sources to provide quick information on emerging threats. Google Flu Trends, run by the eponymous internet firm's charity, monitors flu-related searches to estimate the disease's prevalence. Such electronic systems complement conventional epidemiology, rather than replacing it (and are not always reliable; in America's most recent flu season Google Flu Trends overestimated the number of those sickened). But traditional surveillance methods have improved, too.

Improved techniques, for instance, allow segments of DNA to be amplified rapidly, so viruses can be identified quickly. The cost of full genetic sequencing continues to fall. And countries' surveillance efforts are now better co-ordinated. A model ▸▸

of co-operation can be seen north of Bangkok, where Thailand's health ministry includes a National Influenza Centre. As one of the WHO's designated regional laboratories, it tests samples from the whole of South-East Asia. This laboratory has also been supported for the past decade by America's Centres for Disease Control and Prevention (CDC), an organisation well equipped to identify new medical threats. (It was the CDC that spotted AIDS.)

Many countries are better equipped than before to respond to an outbreak. America leads the way. The authorities have stockpiled 68m courses of antiviral drugs, 18m respirators and 31m face masks, and are investing in research to create better ones. The Biomedical Advanced Research and Development Authority (BARDA), an American government agency, contracts with companies to develop new ways to counteract biological threats. It has 130 products in development, including 45 for influenza.

The world's biggest cities, often with aid from national governments, have honed their strategies, too. Shanghai watches for 15 categories of infectious disease at more than 5,700 sentinel sites. It has several emergency plans, tailored for outbreaks of different intensities. New York collects data from hospitals, laboratories and even pharmacies, to look for signs of new infections. The city cannot forcibly vaccinate its citizens, but it can order the unvaccinated to stay at home.

Even such extraordinary measures, though, may be ill-matched for a virus. H1N1 proved how much can go wrong. It was contagious but not particularly deadly, so officials were confused about how to convey its risks. Research published in the *Public Library of Science* on April 15th estimates that half the Tamiflu (an antiviral drug made by Roche) prescribed in England at the time of the H1N1 outbreak went unused, based on an analysis of traces of the drug in sewage. Vaccines took months to deploy, delayed by fundamental problems (the time needed to develop them) and trivial ones (American shipments had to wait for the pallets carrying vaccines to receive a fumigation certificate). An independent committee issued a discouraging review of the WHO's response. "They made it very clear", says Keiji Fukuda, the WHO's top influenza official, "we are not ready for anything big."

Since then the WHO and others have tried to improve things. In 2011 the WHO's members created a new framework for sharing flu viruses—in 2006 Indonesia refused to share samples of H5N1 with the WHO out of concern that companies would use an Indonesian virus to develop treatment unaffordable to Indonesians. GlaxoSmithKline is the first company to sign a deal with the WHO under the new framework. The pharmaceutical giant will donate 7.5% of its vaccine production in the event of a pandemic. A further 2.5% will be sold at tiered prices, depending on a country's income.

There is also new capacity to make the vaccines themselves. Last year BARDA awarded contracts for three new centres, to be led by Novartis, Emergent BioSciences (a firm in Maryland) and Texas A&M University, in collaboration with GlaxoSmithKline. These will develop and manufacture medical countermeasures, including vaccines. In November Novartis won approval for the first flu vaccine made from cultured cells rather than eggs—a technology that will help produce vaccines more quickly. There has also been progress in poor countries. The WHO has given grants to flu-vaccine manufacturers in 14 countries. Four of these are ready to go.

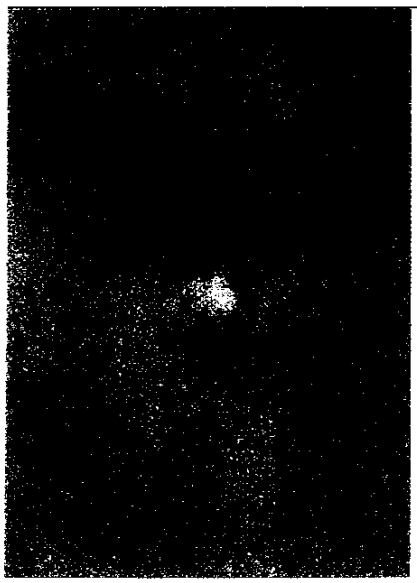
And researchers continue to test new tools that may help. For example, Marta González of the Massachusetts Institute of Technology has modelled how diseases spread by plane. In the early days of an outbreak, such models may help officials decide which routes to cancel to contain a virus. America is paying scientists to patrol rapidly changing environments in Africa, Asia and Latin America, where viruses are prone to hopping from beast to man. The hope is that the scientists will find dangerous viruses early, before they spread.

Next candidate...

There remains, nevertheless, much to be done. Many countries now have plans to prepare for a pandemic, but it is unclear which are operational. Dr Fukuda estimates that the world has the capacity to make enough vaccine for about 2 billion people—an improvement, but still short of what might be needed. Adding to the problem, governments are hardly flush with cash. The WHO has an influenza budget of $7.7m, less than a third of what the city of New York devotes to public-health emergencies. The main question is whether progress will continue, and whether it will pre-empt a more serious threat.

It is hard to say if either H7N9 or the new coronavirus will be pathogens that put the world to the test. The coronavirus in particular is still poorly understood. Ron Fouchier of the Erasmus Medical Centre in the Netherlands was the first to sequence its genome. He annoyed Saudi Arabia by patenting the result (though gene patents are a controversial area anyway—see "Natural justice" at the end of this section). He argues that the Saudi government should be more forthcoming with information. Saudi officials say they are working as quickly as possible and are collaborating with foreign epidemiologists.

The work around H7N9 has been relatively transparent. China has already shipped samples of the virus to laboratories all over the world. But the virus itself is still spreading in China, and people continue to die. Vaccines, once developed, may be ineffective. "H7" and "N9" refer to particular types of two proteins, haemagglutinin and neuraminidase, that help influenza viruses invade host cells. Other H7 vaccines have not created a strong immune response, according to Michael Osterholm of the University of Minnesota. It is also unclear if the virus may become more contagious—at the moment it does not seem to jump from person to person. Last year two groups of scientists, one led by Dr Fouchier, described specific mutations that might make H5N1 transmissible from human to human. H7N9 contains mutations implicated in this switch. If the virus mutates further, it might become airborne. And if that did happen, the world's pandemic-protection system might be put to the test quite suddenly. ■



The first spacecraft to land on Mars

At the centre of this picture is a blob thought to be the parachute from the first mission to survive a trip to Mars's surface—the Soviet Union's *Mars 3*, which arrived in 1971. The picture is part of a larger image taken by *Mars Reconnaissance Orbiter*, an American craft that has been circling the planet since 2006. The work of hunting through the 1.8 billion pixels in the original image was done by Russian space enthusiasts. Besides the parachute, they reckon they have found *Mars 3*'s heat shield and the probe itself. It is the latest development in the nascent field of space archaeology, which studies the history of space flight. In March a team led by Jeff Bezos, the boss of Amazon, made the biggest discovery yet, when it raised engine parts from one of America's Saturn V moon rockets from the floor of the Atlantic Ocean.

Replikins Ltd.

Executive Summary - April 22, 2013

Pioneering synthetic vaccines, and helping predict infectious disease outbreaks

In the last decade, outbreaks of SARS, H5N1 (bird flu), H1N1 (swine flu), and now H7N9 bird flu (see attached) have increased awareness of the need for rapid vaccine development in response to emerging viral threats. Replikins, Ltd has made substantial breakthroughs in the surveillance of global disease threats and synthetic vaccine development.

The Challenge

Lethal outbreaks occur without advance warning. In the past, there has been insufficient time to prepare counter-measures and specific response before a virus has struck or escalated into a pandemic. The inability to predict outbreaks or their spread is faced repeatedly annually when there are, for example, international gatherings like the Hajj and the Olympics, involving up to one to two million people bringing a variety of infectious diseases and returning to their respective countries with new infectious diseases. The country, and the institution, which becomes the centre for surveillance and response to this problem will make a large contribution to world health and successfully address a major business segment.

The Solution: Prediction and Surveillance

Prediction → Outbreak → Real-Time Tracking

Replikins' software-driven technology is the first ever to predict oncoming outbreaks one to two years in advance, permitting earlier development of vaccine candidates as well as rapid (7 days rather than 8 months) production of vaccines for immediate clinical trials. Correct predictions of specific outbreaks and their geographic location have been made to date in 12 of 12 tests, with no false positives or false negatives. Replikins disease surveillance software measures the infectivity and lethality of a strain, and tracks and predicts the geographic location of outbreaks as well as which species the disease may affect.

Replikins believes that prediction alone provides a clear competitive advantage to companies and governments who possess this information. This advantage has in fact already been demonstrated. Leveraging our 2008 prediction of the 2009 H1N1 pandemic, a major international pharmaceutical company was able to produce and successfully sell H1N1 vaccine in advance of both its competitors and the outbreak. In addition, in 2002, we were able to predict the SARS outbreak and its rapid decline in 2003.

Replikins are a class of closely related peptide sequences, with compositions described by our patents covering both Replikins themselves and a range of applications. The United Kingdom Trade and Investment Department has 'green-lighted' Replikins Ltd's subsidiary Bioradar UK, Ltd and UK embassies worldwide have begun to help to disseminate information on Replikins technology. The United Nations Food and Agriculture Organization (FAO), in reviewing the risk of an H5N1 pandemic, has noted the accuracy of Replikins predictions, and the promise of Replikins technology in driving appropriate public health pandemic prevention measures.

Figure 1: Prediction and First Real-Time Tracking of a Pandemic:
Replikin Software: Report of Replikin Counts in the H1N1 Virus in Humans Before and During the 2009 Pandemic. **Infectivity Gene (red), Lethality Gene (black)**

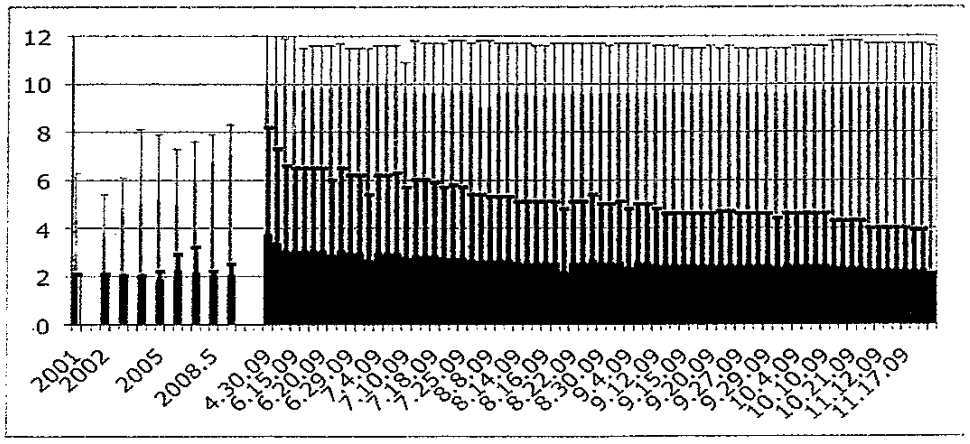
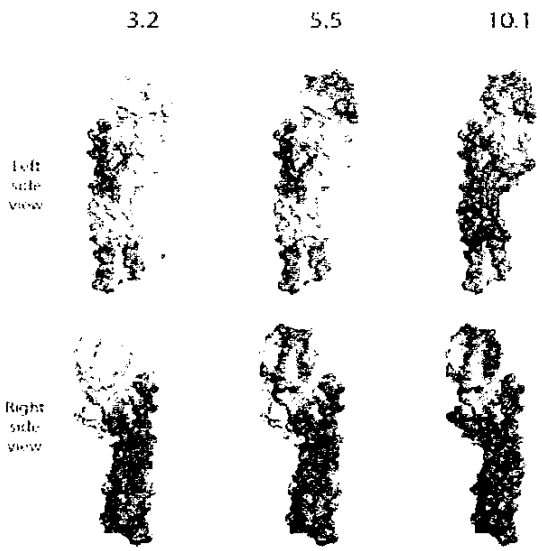


Figure 2: Replikins can be seen and counted in the Genome

H1N1 Virus HA Gene Replikin Counts (3.2, 5.5, and 10.1) and corresponding 3D gene structure showing the observed spread of Replikins sequences (blue) on the surface of the HA Gene of the H1N1 virus before and during the H1N1 pandemic of 2009.



REPLIKINS VACCINES

Replikins synthetic vaccines

Replikins vaccines are synthetic, do not require refrigeration and have been manufactured in as little as 7 days instead of 8 months. Production of our vaccines is straightforward to ramp up, since it involves standardized peptide manufacturing techniques already in use across multiple industries, with accompanying economies of scale. By contrast, nearly all other vaccines sold today are manufactured using early-20th century technologies, such as using live chicken eggs as incubators for tiny batches of highly impure doses. Previous vaccine manufacturing techniques are beset by limitations such as difficulties ramping up production volume, contamination of vaccines by a range of antigenic impurities, and even difficulties accurately targeting disease strains, as in influenza, whose composition changes from year to year. Replikins synthetic vaccines in influenza and other infectious are new, highly targeted vaccines from crafted peptide sequences, which are much more effective than classical vaccines, without the side effects to date. Initial trials against H5N1 in chickens and Taura Virus in shrimp have been highly successful. A new Replikins vaccine has just been offered against H7N9 influenza, now on course to becoming a pandemic in China.

Increases in concentration of Replikin sequences in virus genomes have been found to be associated with rapidly replicating pathogens. These corresponding sequences are consistently present in a wide range of rapidly replicating viruses, providing many promising candidates for synthetic vaccines for animal as well as human health. We believe there may be direct applicability to vaccine markets in as many as 80 diseases.

The market opportunity

In the last flu pandemic of 2009, no more than 1.25 billion doses of vaccine were delivered for the world's 7 billion inhabitants, with timing 6+ months later than optimal. Replikins, Ltd. believes that addressing this gap represents a massive and under-served business opportunity, one of the largest in the modern biotech/pharmaceutical space.

According to the US GAO, the price to manufacture one dose in the 2009 pandemic was about $11; our estimate for the cost of goods for a synthetic Replikin-based vaccine dose would be less than 1% of this at scale. Adjacent opportunities also exist in animal health, where farming on a global scale has led to growing markets for technologies that can maintain the health of fowl, livestock and marine stock.

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APPENDIX B: REPLIKINS H5N1 VACCINE TRIAL

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***Avian Diseases:* Article In Press**

Efficacy of a Replikin Peptide Vaccine Against Low Pathogenicity Avian Influenza H5 Virus

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SUMMARY. In this study, the sequence of the H5 and PB1 genes of the low pathogenic avian influenza virus (LPAI) A/Black Duck/NC/674-964/06 isolate were determined for replikin peptides and used to design and chemically synthesize a vaccine. The vaccine was used to immunize specific pathogen free leghorn chickens held in Horsfal isolation units by the upper-respiratory route, at 1, 7, and 14 days of age. The birds were challenged at 28 days of age with 1 x 106 EID50 of the LPAI Black Duck/NC/674-964/06 H5N1 virus per bird. Oropharyngeal and cloacal swabs were collected at 2, 4 and 7 days post-inoculation (PI) for virus detection by real time RT-PCR. Serum was collected at 7, 14 and 21 days PI and examined for antibodies against avian influenza virus (AIV) by the enzyme linked immunosorbant assay (ELISA) and hemagglutination inhibition (HI) tests. Tissue samples for histopathology were collected from 3 birds per group at 3 days PI. The experimental design consisted of a negative control group (not vaccinated and not challenged) and vaccinated group, a vaccinated and challenged group and a positive control group (challenged only). None of the non-challenged birds, the vaccinated birds, and the vaccinated and challenged birds showed overt clinical signs of disease during the study. A slight depression was observed in the non-vaccinated challenged birds on day 2 post-challenge. Although the numbers of birds per group are small, no shedding of the challenge virus was detected in the vaccinated and challenged birds, whereas oropharyngeal and cloacal shedding was detected in the non-vaccinated and challenged birds. Hemagglutination inhibition (HI) antibodies were detected in the vaccinated and non-challenged group as well as in the vaccinated and challenged group, but rising antibody titers indicating infection with the LPAI challenge virus were not detected. Rising HI titers were observed in the non-vaccinated and challenged group. In addition, no antibodies were detected in the non-challenged birds. Noteworthy microscopic lesions were not observed in the vaccinated and challenged birds whereas non-vaccinated challenged birds had microscopic lesions consistent with infection with LPAI viruses. **Taken together, these data indicate that a replikin peptide vaccine specifically made against the H5N1 Black Duck/NC/674-964/06 and administered 3 times to the upper-respiratory tract, was capable of protecting chickens from infection and shedding of the homologous virus, which is extremely important because reduced virus shedding and transmission decreases the potential for H5 LPAI viruses to become HPAI viruses. The study is also important because it shows that the vaccine can be effectively mass delivered to the upper-respiratory tract.**

Figure 14

Efficacy of a Replikin Peptide Vaccine against Low Pathogenicity Avian Influenza H5 Virus

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Noteworthy microscopic lesions were not observed in the vaccinated and challenged birds whereas non-vaccinated challenged birds had microscopic lesions consistent with infection with LPAI viruses. Taken together, these data indicate that a replikin peptide vaccine specifically made against the H5N1 Black Duck/NC/674-964/06 and administered 3 times to the upper-respiratory tract, was capable of protecting chickens from infection and shedding of the homologous virus, which is extremely important because reduced virus shedding and transmission decreases the potential for H5 LPAI viruses to become HPAI viruses. The study is also important because it shows that the vaccine can be effectively mass delivered to the upper-respiratory tract.

Key words: Avian influenza virus, H5N1, vaccination, replkin peptide vaccine, low pathogenicity.

Abbreviations: AIV= avian influenza virus, LPAI= low pathogenicity avian influenza, HPAI= high pathogenicity avian influenza, PBS= phosphate buffered saline, RT-PCR= reverse transcriptase-polymerase chain reaction.

Avian influenza viruses (AIV) continue to be an enormous economic burden on the commercial poultry industry worldwide because they are highly infectious, have extensive genetic diversity, a short generation time, and a high mutation rate (3, 18). There are two main pathotypes of AIV, low pathogenic avian influenza (LPAI) and high pathogenic avian influenza (HPAI), which were originally characterized by their ability to kill experimentally infected chickens (18). The HPAI viruses are further defined by genetic and biological characteristics as presented in the World Organization for Animal Health (OIE) Terrestrial Animal Health Code (http://www.oie.int/eng/info_ev/en_ai_notification.htm). Currently there are 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes of influenza A virus recognized, and all are known to infect birds. In addition, some H subtypes of the virus can infect other animal hosts as well as humans (2, 6). Wild anseriform (ducks, geese and swans) and charadriiform (gulls and shorebirds) birds are known to be the natural host reservoir of AIV, and those birds play a key role in viral evolution, pathogenesis, and transmission both among wild birds and to domestic poultry (9, 18). In addition, the LPAI H5 and H7 subtypes have the potential to become HPAI viruses in chickens (18). And for this reason, in addition to HPAI viruses, the OIE Terrestrial Animal Health Code also lists all low pathogenic influenza A viruses of H5 and H7 subtype as "notifiable AI" (http://www.oie.int/eng/info_ev/en_ai_notification.htm). Control of AIV in commercial chickens in some countries, including the USA, is through monitoring and eradication of infected flocks. This involves a stamping out strategy where infected flocks are killed and surrounding flocks are vaccinated and monitored to differentiate infected from vaccinated animals (DIVA) (7). In other countries where AIV is endemic, poultry are routinely vaccinated for AIV (17). Killed vaccines are typically used, but two recombinant vaccines, a fowl poxvirus vector and a Newcastle disease virus vector both containing an

AIV H5 gene insert have been licensed and are also available for use in poultry (8, 14, 20). It is extremely important to control LPAI H5 and H7 viruses in commercial poultry because they have the potential to become HPAI viruses (15). Although current vaccines against AIV can protect against mortality, clinical signs, and can reduce shedding and transmission of the virus in poultry, none of the currently licensed vaccines can prevent infection or completely stop virus replication and inhibit shedding (15, 16, 20). In addition, there is evidence that many of the recombinant vaccines reported in the literature also do not protect against virus replication and shedding in chickens (5, 8, 11, 22). Subunit vaccines consisting of all or portions of the HA, NA, and M1 proteins have been reported to induce partial protection against challenge (8, 10, 11). Virus shedding and transmission to other susceptible birds or neighboring flocks increases the potential for the H5 and H7 LPAI viruses to become HPAI viruses. An optimal vaccine against AIV would not only protect against disease and mortality, it would also prevent virus shedding and spread. Peptides are short sections of a protein that can be used as a vaccine when they contain epitopes capable of inducing an immune response. For a review, see Bae *et al.* (4). Since they are rarely immunogenic by themselves, due to their small size, they are usually attached to a large carrier protein such as bovine serum albumen or keyhole limpet hemocyanin. Multiple antigenic peptides have also been produced where the peptides are linked to a core lysine molecule. Peptide vaccines contain only a portion of the disease agent, and are therefore not capable of causing the disease. An epitope based vaccine against four-conserved virus epitopes was reported to induce protection against lethal challenge of HPAI H5N1 virus in mice (1). In another study, researchers vaccinated mice with a peptide vaccine prepared against the M2 protein and showed that they were protected from lethal challenge (21). In this study, we examined the efficacy of a synthetic peptide vaccine against the LPAI A/Black Duck/NC/674-964/06 H5N1 virus administered to the upper-respiratory tract to protect against challenge with that virus in chickens. Clinical signs, microscopic lesions, virus shedding, and seroconversion were examined.

MATERIALS AND METHODS

Nucleic acid sequencing. The sequence of the H5 and PB1 genes of the LPAI Black Duck/NC/674-964/06 H5N1 virus used for challenge in this study were determined by RT-PCR amplification and cycle sequencing of the amplified product. Briefly, viral RNA was extracted from the virus (High Pure RNA Isolation Kit, Roche Diagnostics, Penzberg, Germany) and used as template in the reverse transcriptase-polymerase chain reaction (RT-PCR, Titan One Tube RT-PCR System, Roche Diagnostics). The HA1 portion of the H5 gene was amplified with primers H5HA6F and H5HA6R designed from Genbank (http://www.ncbi.nlm.nih.gov/) accession number ABP49258 and the PB1 gene was amplified with primers PB1F and PB1R designed from Genbank accession number ABP49203 (Table 1). The amplified DNA was electrophoresed on a 1% agarose gel, cut out of the gel and purified (QIAquick PCR Purification Kit, Qiagen, Valencia,138 CA). Cycle sequencing was carried out with the BigDye terminator kit (Applied Biosystems, Foster City, CA) and subsequently run on an ABI 3730 sequencer (Applied Biosystems, Foster City, CA) at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA). Sequencing primers and their locations are

presented in Table 1. The sequences were assembled and the amino acid sequence of the genes was deduced using Lasergene 8, DNASTAR, Inc. (Madison, WI, USA).

Birds and housing. One-day old specific pathogen free white leghorn chicks were obtained from Merial (Gainesville, GA), randomly divided into four different groups, 10 birds per group, and housed in positive-pressure Horsfall isolation units. The birds were vaccinated, held in the Horsfall isolators until moved to a USDA approved (permit # 103372) BSL2-Ag+ facility containing HEPA filtered positive pressure isolation units for challenge with the H5N1 LPAI virus.

Experimental design. The synthetic replikin peptide vaccine used in this study was provided by Replikins Ltd. (Boston, MA) and was based on the exact sequence of the challenge virus. For each group of 10 birds that received vaccine, the lyophilized vaccine was rehydrated in 5ml of PBS (pH 7.4), and 100 ul was delivered to the nares and 100 ul was placed in the eyes of each bird. The remaining 3 ml of rehydrated vaccine was further diluted in a total of 10ml PBS and delivered by fine spray to all 10 birds in the group. Fine spray was created with a Preval® sprayer (Precision Valve Corp., Yonkers, NY) while temporarily blocking the fresh air delivery to the isolator. Fresh air was resumed approximately 5 minutes following vaccination. The total vaccine dose including that available in the spray was approximately 20 mg per bird. At 28 days of age, the birds were moved to the BSL2-Ag+ facility and challenged by cloanal cleft inoculation (0.1 ml) with at least 1X106 embryo infectious dose50 of pathogenic Black Duck/ NC/674-964/06 H5N1 virus. To determine the titer, the virus was inoculated into 10-day old SPF embryonating chicken eggs and the infectious titer was calculated by the Reed and Muench method (24). Lesions found in embryos on the last day of a 7-day incubation were recorded and the hemagglutination (HA) test was conducted on the allantoic fluid of all surviving embryos (19). To detect virus shedding, oropharyngeal and cloacal swabs were collected in 1ml of sterile PBS (pH 7.4) from each bird at 2, 4, and 7 days post-challenge. All swab samples were stored at -80C and thawed only once for RNA extraction. The presence of virus was determined by quantitative real-time RT-PCR directly from the swab samples. Two birds from each group were killed and necropsied at 3 days post-challenge. At necropsy, tracheas, heart, lung, liver, intestines, gonad, brain, spleen, bursa of Fabricius, kidney, muscle, and nasal turbinates were fixed in 10% neutral buffered formalin and submitted for histopathology.

Histopathology. Tissue samples were routinely processed and embedded into paraffin blocks. Thin sections were cut and stained with hematoxylin and eosin and examined by light microscopy.

Serology. To verify that the birds were not exposed to AIV prior to challenge, serum was collected and evaluated for antibodies by commercial ELISA (IDEXX, Inc., Westbrook ME). Serum was also collected at 7, 14, and 21 days post-challenge and examined for AIV antibodies by commercial ELISA (IDEXX) and the HI test (19).

RNA extraction and quantitative real-time RT-PCR. Viral RNA was extracted from the swab samples using the MagMax-96 Total RNA Isolation Kit (Ambion Incorporated,

Austin, TX) and a KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA), according to the manufacturer's recommendations. The Ambion Ag Path ID One Step RT-PCR kit (Ambion Incorporated, Austin, TX) was used for nucleic acid amplification with a 25 μL reaction mixture containing the following reagents: 12.5 μL of kit-supplied 2X RT-PCR buffer, 1 μL of kit-supplied 25x RT-PCR enzyme mix and 10 μL of extracted viral RNA. Each reaction mixture utilized picomoles of matrix gene primers (forward and reverse) and probe sequences (allthree using 0.5 μL each), following the protocol of the real time RT-PCR assay developed for type A influenza virus (12). Real-time RT-PCR was carried out in a Smart Cycler thermocycler machine (Cepheid, Sunnyvale, CA) with the following conditions for the RT step (50oC for 30 min and 94oC for 15 min) and the PCR cycling protocol (94oC for 15 sec and 60oC for 20 sec for 45 cycles). Data are reported as the average cycle threshold (CT) value.

RESULTS

The Black Duck/NC/674-964/06 virus was selected for theses studies because our facilities are only approved for LPAI viruses, and we wanted to examine a relatively recent H5N1 type virus.

Nucleic acid sequencing. The sequence of the H5 and PB1 genes of the LPAI Black Duck/NC/674-964/06 virus was determined, and the sequences were submitted to GenBank (accession numbers for H5= 1237333, and PB1= 1237358). These sequences were used to synthesize the proprietary replikin peptide vaccine (Replikins, LTD.) used in this study. In general an computer algorithm was used to identify unique replicating sequences with in the proteins and synthetic peptides 7 to 50 amino acids in length were synthesized to each of the sequences to produce the vaccine. The peptides were not coupled to any proteins. The exact peptide sequences are available from Replikins, LTD.

Vaccine/Challenge study. On day 2 post-challenge, a mild depression, characterized by huddling and a reluctance to eat, was observed in some of the birds in the challenged group that was not vaccinated. None of the other birds showed overt clinical signs of disease. At necropsy (day 3 post-challenge), gross lesions were not observed in any of the birds. Virus detection data is presented in Table 2. Virus shedding was detected in the challenged birds that were not vaccinated. Virus was detected in oropharyngeal swabs from those birds on days 2, 4 and 7 post-challenge and in the cloacal swab from one bird on day 2 post-challenge. Weak and thus suspect positive samples were detected on day 2 as evidenced by the high average Ct value (38.44). No virus shedding was detected in the vaccinated challenged birds or in the control birds that were not challenged. Prior to challenge, AIV specific antibodies could not be detected in any of the birds by ELISA. Serum was collected from all of the birds at 7, 14, and 21 days post challenge, tested for HI antibodies and the data is presented in Table 3. None of the negative control birds were positive for antibodies to AIV. No HI titers were detected in vaccinated birds that were not challenged on days 7 and 21 post-challenge and a low average HI titer of 16.6 was observed in those birds on day 14 post-challenge. Birds that were vaccinated and challenged had no HI antibodies on day 7 post-challenge and only 3of 7 (average titer=

37.3) and 2 of 7 (average titer= 24.0) birds seroconverted on days 14 and 21 respectively. All of the birds that were not vaccinated and challenged seroconverted with HI antibodies being detected in 4 of 7 (average titer= 14.8), 7 of 7 (average titer= 72.0) and 3 of 7 (average titer= 26.7) on days 7, 14 and 21 respectively. Data from the ELISA test shows that 3 of 7 birds in the challenge group that was not vaccinated seroconverted (data not shown). In addition, none of the birds were positive in the vaccinated challenge group on days 14 and 21, and none of the non236
challenged birds were positive. No microscopic lesions were observed in any of the tissues from the non challenged birds. Birds that were vaccinated and challenged did not have remarkable lesions in any of the tissues. The non-vaccinated challenged group had lesions in both the respiratory and gastro-intestinal tract, which was typical and consistent with infection with LPAI viruses. All of the birds showed a mild catarrhal rhinitis, with some birds having a sloughing of the respiratory epithelium and the presence of a mild amount of catarrhal tracheitis. Mucin and edema was also observed in the lamina propria and secondary bronchus in some birds. Lesions in the gastro-intestinal tract were mostly confined to the ceca and small intestine, and consisted of focal moderate lymphocytic infiltration in the lamina propria. A mild proliferation of gut-associated lymphoid tissues (GALT) was also observed in the duodenal samples from some birds in this group.

DISCUSSION

In this study, specific pathogen free leghorn chickens were vaccinated via the upper-respiratory tract 3 times with a synthetic replikin peptide vaccine based on the HA and PB1 gene sequences from the LPAI Black Duck/NC/674-964/06 H5N1 virus then challenged with the same LPAI virus. Prior to challenge, serum tested negative for AIV antibodies using a commercial ELISA, which verified that the birds were not previously exposed to AIV. Since the commercial ELISA test measures antibodies to the NP protein, we did not expect any positive results from the vaccinated birds. At 2 days post-challenge the non-vaccinated birds that received the challenge virus were mildly depressed. No other clinical signs were observed in that group or any of the other groups, which is typical of LPAI viruses in chickens (13). We were not able to detect the challenge virus in oropharyngeal or cloacal swabs from vaccinated and challenged birds by real time RT-PCR on days 2, 4 and 7 post262challenge. We did however detect the challenge virus in 3, 2, and 1 of the non-vaccinated and challenged birds on days 2, 4 and 7 respectively, indicating that at least some of the birds in that group were infected and viral replication (viral shedding) occurred. This was not unexpected since virus detection in birds inoculated with LPAI viruses is typically not 100 percent (11). Viral shedding in that group was observed in the oropharyngeal samples except for one bird that shed virus from the gastro-intestinal tract on day 2 post challenge. Not detecting challenge virus in the vaccinated and challenged birds was surprising since sterile immunity is extremely difficult or almost impossible to achieve through vaccination (5, 8, 11, 15, 16, 20, 22). It is possible that some virus replication could have occurred in that group but if it did, the levels were below the sensitivity of the real time RT-PCR test (12). However, in a recent study examining the efficacy of a H5 gene expressed in Baculovirus to protect against a homologous virus challenge in chickens, no challenge virus was detected in the vaccinated birds by virus isolation in eggs, indicating that it may be possible to decrease

virus shedding to undetectable levels if a homologous vaccine to the challenge virus is used (10). In addition, another study found that the type of vaccine and the timing of challenge following vaccination can reduce transmission of an H7N7 virus to levels that would prevent a major outbreak (23). Antibodies to AIV were detected in vaccinated as well as challenged birds (Table 3). All of the birds in the non-vaccinated and challenged group seroconverted indicating that they were infected with the challenge virus. Only 3 of 7 birds in the vaccinated and challenge group seroconverted and the HI antibody titers were much lower than the nonvaccinated and challenged birds. Thus, it appears that some of the vaccinated birds were not infected with the challenge virus. We also detected HI antibodies in birds that were vaccinated and not challenged. This was not surprising because a portion of the synthetic replikin peptide vaccine was prepared against the HA protein. Some of the birds with AIV antibodies at 14 days post-challenge were negative at 21 days post-challenge. Since the HI antibody titers were extremely low for some of the birds at 14 days post-challenge and given the mild nature of LPAI in chickens, it is likely the antibody levels for some birds positive at 14 days post-challenge fell below the level of detection for the HI test at 21 days post-challenge (17). We did not measure mucosal immunity because detection of antibodies to the peptide vaccine or to the virus in an ELISA based test is not a guarantee that the antibodies are neutralizing or that the mucosal immunity is protective. However, demonstrating a level of protection against challenge is evidence that the vaccine indeed induced some immunity (local or otherwise). Microscopic lesions in the non-vaccinated challenged birds were typical of a LPAI virus infection in chickens, which supports our findings that those birds were infected. A lack of appreciable lesions in the vaccinated and challenged birds indicates that little or no virus replication was occurring.

Based on clinical signs, virus detection, serology, and microscopic lesions, it appears that most of the birds in the vaccinated and challenged group were protected from infection with the challenge virus and did not shed detectable levels of virus. Our data indicates that virus shedding and subsequent transmission of LPAI viruses could be blocked or at least reduced to levels that would avert an outbreak if a homologous vaccine against the challenge virus is used to vaccinate the birds. This information is extremely important because reducing virus shedding and transmission to other susceptible birds or neighboring flocks will decrease the potential for H5 LPAI viruses to become HPAI viruses. In addition, although these results are preliminary, the study demonstrates that mass vaccination (delivery to the upper-respiratory tract) could be used to protect against H5 LPAI virus if multiple applications can be given over a short period of time.

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Table 1. Primers used in this study.

Primer	Sequence	Position[A]
H5HA6FB	AAGCAGGGGTCTAAACTATCAAAA	-28 to -5
H5HA6RB	CCATGATTGCCAGTGCTAGGGA	1,640 to 1,662
HA1308F	AAGATGGGTTTTTAGATGTATGGA	1,308 to 1,332

HA1242F	TCGAAGCCGTTGGGAAAG	1,242 to 1,260
HA302F	TGGTCATACATCGTGGAAAA	302 to 322
HA177F	AAAAAGAGCACAATGGGAAA	177 to 197
HA1414R	ATCGTATAGGTTCTTGACATTTGA	1414 to 1438
HA1260	TCTTTCCCAACGGCTTCG	1260 to 1278
HA977R	CGATTTGACATACTTGGGGCACTC	977 to 1,001
HA349R	TCGTAGTCGCTGAAGTCTCCTG	349 to 371
PB1F[B]	AGCGAAAGCAGGCAAACCATT	-119 to -98
PB1R[B]	ATTCACTATTTCTGCCGTCTAA	2282 to 2304
PB543[F]	AATGGATAAAGAGGAGATGGAAA	543 to 566
PB176	AAAAGGGGAAATGGACAACAA	176 to 197
PB73F	GCCATAAGCACCACATTCCC	73 to 93
PB2236R	CATGATCTCAGCAAACTCCTC	2236 to 2257
PB1894R	TGGGTTCAGGGGGTTACAAAG	1894 to 1915
PB1391R	CTCCTGCTTGTATCCCCTCAT	1391 to 1412
PB612R	CTTTGTGTGACCATTTTCTTG	612 to 633
PB545R	TTTCCATCTCCTCTTTATCCA	545 to 566

Table 2. Virus detection[A] in oropharyngeal and cloacal samples by real time RT-PCR.

Treatment [C]	Day 2	Day 4	Day 7
Negative control	0/10	0/7	0/7
Vaccinated not challenged	0/10	0/7	0/7
Vaccinated and challenged	0/10	0/7	0/7
Not vaccinated and Challenged	3/10[B] (38.44 avg.)	2/7[C] (30.76 avg.)	1/7[C] (34.7)

[A]Number positive per total number of birds examined (Cycle threshold value, average of positive samples).

[B]Two oropharyngeal and one cloacal sample from three different birds were positive.

[C]Only oropharyngeal samples were positive.