Abstract

Starting from late 2003 multiple outbreaks of highly pathogenic avian influenza type A H5N1 virus have occurred first in Southeast Asia and then in the rest of the world. There is a consensus that influenza pandemics will not fade away as illustrated by H1N1 swine flu outbreak this year. The possibility of a mutation which would cause more deadly virus spread in the human population is of particular concern. The human-to-human H5N1 transmission cases with fatal outcome have already been reported. There is unmet need for an effective vaccine to prevent bird flu outbreaks and potential human pandemic. Immunitor USA Inc is a start-up company which has developed the unique technology that enables the formulation of killed virus into an oral tablet. The vaccine consists of heat- and chemically-inactivated H5N1 virus which was expanded by a standard method in embryonated chicken eggs. The experimental lots of H5N1 oral vaccine have shown promising results in chicken challenge studies. However vaccine’s protection in terms of survival as the endpoint was partial and thus further studies are needed to identify optimally effective dose and vaccination schedule. These experiments will serve as proof-of-concept for developing an effective and safe vaccine capable of preventing and perhaps treating influenz virus infection.

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Keywords: Influenza, bird flu, highly pathogenic, safety, killed virus, mucosa, immunogen

1. Introduction

During 2003-2004 seasons, the outbreaks of the avian influenza type A H5N1 virus have occurred in 57 of Vietnam’s 64 provinces. This led Vietnam to cull over 40 million chickens and inflicted devastating losses on the local poultry farmers. In Thailand the disease has been detected in 40 of 76 provinces with 36 million birds culled as a result. In China 16 out of 31 provinces, including Tibet, were hit by bird flu. The outbreaks of smaller magnitude occurred in South Korea, Japan, Indonesia, Myanmar, Cambodia, Laos, and Malaysia. By the end of 2004, avian flu has struck 11 Asian nations. The FAO estimated that close to 120 million chickens and other fowl have either died or culled in Asia during these outbreaks. In 2005 the outbreaks have spread further reaching Russia, Mongolia, Turkey, Greece, Romania, and Croatia. More recent reports have identified the presence of bird flu in migrating wild fowl in the Middle East, Africa, North America, and Western Europe. There is a consensus that avian flu outbreaks won't be eradicated by culling of domestic birds [1]. Even if seasonal outbreaks fade away there will be still the unmet need for a vaccine to prevent future incidents of pandemic flu virus such as current H1N1 swine flu virus.

The threat of avian virus spread to human population is of particular concern. Over 380 human cases have been confirmed to date of which about two third have died [2]. Moreover, human-to-human H5N1 transmission with fatal outcome has been reported shortly after first outbreak [2,3]. Thus, the possibility of pandemic flu virus spread in global human population cannot be underestimated. According to the WHO figures, even regular seasonal influenza epidemic results in three to five million cases of severe illness and between 250,000 to 500,000 deaths each year in the industrialized world alone. Current H5N1 appears to be especially dangerous for humans as the mortality rate is 63% despite the fact that most infected patients were promptly treated with the best available influenza drugs such as Tamiflu or Relenza [2].

Immunitor USA Inc., is Maryland-based biotech company which has licensed oral vaccine technology to MDM Group Inc. Immunitor has developed unique technology that enables to formulate “killed” virus as an oral pill. Immunitor has manufactured experimental lots of H5N1 oral vaccine and evaluated their efficacy in a chicken model as a proof-of-concept for developing human pandemic flu vaccine. The vaccine which is made as an oral tablet consists of heat- and...
chemically-inactivated H5N1 2004 isolate from a farm chicken in Thailand and expanded in embryonated chicken eggs [1]. The results of preliminary studies are presented in this paper.

2. Materials and Methods

2.1. Vaccine preparation

The initial stock of virus was a wild-type isolate freshly isolated from a diseased farm chicken as kindly provided by Drs. Chaisingh and Suktinthai from the National Animal Health Institute, Kasetsart University, Thai Ministry of Agriculture [1]. As heterologous virus control we used H5N3 strain from the WHO reference stock. While this virus has shared H5 determinant it has unrelated neuraminidase subtype and was presumed to provide lesser protective effect. Viruses were grown in the allantoic cavities of 9-11-day old embryonated chicken eggs at 37°C for 24-48 hours. Allantoic fluid was harvested and used for vaccine preparation according to a proprietary process developed by us, which involves killing virus by heat and chemical means. Briefly, the procedure involves limited acid hydrolysis and heat treatment at 120°C for several hours. The process is well established and has been validated for seven types of vaccines currently manufactured by Immunitor. The complete inactivation of virus has been confirmed by passages of obtained vaccine in chicken embryos.

2.2. Challenge virus

The challenge virus was the same H5N1 strain used for vaccine preparation. The infectious dose was established by titration in freshly isolated chicken lung fibroblasts. The prepared stock was kept frozen until used for challenge experiments. Chicken were challenged with the lethal dose equivalent to 6.8x10⁶.⁵ TCID₅₀ of H5N1 given intraorally in 0.1 ml saline solution.

2.3. Challenge experiments in chicken

Adult layer chicken were given orally one vaccine tablet once per day for seven days and challenged the next day with lethal dose equivalent to 6.8x10⁶.⁵ TCID₅₀ of H5N1. Three to five chicken in each group were used for each challenge experiment. Controls included non-vaccinated group and H5N3 vaccinated chicken which were challenged with H5N1 virus. The additional control consisted of sterile injectable preparation of H5N1 vaccine made from the same stock.

2.4. Field experience

A local farmer, who have heard that our vaccine may prevent bird flu, decided to give pills to all his chicken. He gave two pills in one dose to approximately 50 adult chicken and one pill each to nine small chicks that were hatched recently.

3. Results

3.1. Challenge studies

The results from the challenge experiment reveal that all 3 unvaccinated chicken in the control group were dead within 30 hours. However, three and four out of five vaccinated chicken with H5N3 and H5N1 vaccines respectively were alive at this cut-off time (Table 1 and Figure 1). Based on these results it appeared that the protection conferred by H5N1 vaccine was 80% up to 42 hours. However the death was only delayed, not prevented, as all birds have died after 48 hours. The chickens vaccinated with control vaccine made from heterologous H5N3 virus were dead earlier. The Chi-Square analysis of surviving versus dead chicken with standard cut-off P value at ≤0.05 is in support of these observations. This non-parametric analysis of preliminary data, while limited, indicated that only H5N1 vaccine was capable of producing statistically significant result (p=0.028). Nevertheless, the protection was of short duration and clearly not optimal.

Table 1. Results from H5N1 challenge test at 30 hours post-infection

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Dead</th>
<th>Alive</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sick</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>NA*</td>
</tr>
<tr>
<td>H5N3 vaccine</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0.074</td>
</tr>
<tr>
<td>H5N1 vaccine</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>0.028</td>
</tr>
</tbody>
</table>

*Not available since P value is assessed by χ² against control
As an additional control, to support the efficacy of vaccine, we have tested injectable preparation which consisted of the same per unit of weight vaccine dose but dissolved in a sterile saline solution. Chicken were primed with one dose of vaccine by i.m. injection at day one, followed by second priming injection 2 weeks later and then challenged by a lethal oral dose equivalent to 6.8x10^{6.5} TCID_{50} of H5N1 after one week of rest period. The results of this experiment are shown in Table 2 and Fig. 2. Although one chicken became sick and died at 3 days post-challenge, the remaining four chicken have survived and were still healthy and alive 20 days after the challenge, at which timepoint they were sacrificed. No control vaccination with injectable H5N3 vaccine was performed since we did not anticipate positive results with injectable vaccine. No immunological or virus shedding studies were performed on vaccinated chicken.

Table 2. Results from vaccination with injectable H5N1 vaccine

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Dead</th>
<th>Alive</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>NA*</td>
</tr>
<tr>
<td>H5N1</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0.005</td>
</tr>
</tbody>
</table>

3.2. Field experience

While this experience is serendipitous by nature it is intriguing in the context of above results. About one week after all farm chicken had been vaccinated there was as a flu outbreak at the surrounding farms that had decimated the entire chicken population in the neighborhood. As chicken were bred in a free-range style the disease has spread to the farmer’s birds. Within three days all adult chicken and 5 out of 9 chicks were dead (Table 3 and Figure 3). While no protection was observed in adult chicken the protective effect in young chicks appears to be highly significant (p<0.00001). Although these results are anecdotal, this experience suggests that a single oral dose of vaccine may be effective in a field situation and that the efficacy might be dose- and/or age-related.
Table 3. Survival of farm chicken fed with H5N1 pills prior to outbreak

<table>
<thead>
<tr>
<th>Farm chicken (vaccine dose)</th>
<th>Dead</th>
<th>Alive</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (2 pills)</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>NA</td>
</tr>
<tr>
<td>Chicks (1 pill)</td>
<td>5</td>
<td>4</td>
<td>9</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>

Fig. 3 Survival of orally vaccinated chicken (■) and young chicks (●) at the farm that experienced bird flu outbreak.

4. Discussion

We were able to produce the first lot of vaccine within one week from the moment virus was inoculated into embryonated eggs. The first lot of vaccine was then tested for protection against H5N1 challenge in chicken. The results indicate that H5N1 vaccine was capable of delaying death (p=0.028) even though after 48 hours all vaccinated chicken were dead. As a control we used vaccine from unrelated H5N3 virus. While this virus has shared H5 determinant it has unrelated neuraminidase subtype and was presumed to provide lesser protective effect. As expected vaccine made from unrelated strain was statistically less effective (p=0.074) in delaying death from H5N1 heterologous challenge [4]. This appears to be due to the fact that in addition to H5, a glycosylation site within N1 determinant contributes to the high virulence of H5N1 [5]. Unfortunately no further challenge experiments were carried out to further confirm the significance of these findings. Nevertheless, the positive data from injectable vaccine experiment which demonstrated 80% survival suggest that our vaccine has a prophylactic potential when oral formulation and/or vaccination schedule are optimized.

The results from field experiment appear to support this conjecture. All adult chicken were dead but 44% of young chicks have survived. We do not know the reason for this discrepancy. One explanation lies in almost one log difference between received vaccine doses and size of recipients. Adult chicken who received a total of 2 pills weigh about 2-3 kg each, but chicks weighed only 60-100g at the time when they were given a single pill. Another conceivable explanation is that unlike adult birds the chicks may have smaller number of gastroliths or pebbles in their gizzards and it is thus possible that the pill has been not ground-up and annihilated by gastric juices. Thus, the pill may have reached the gut in intact form – the site where the vaccine’s antigens needs to be absorbed and the mucosal immune response to antigens occurs.

While protection observed in chicks is incomplete, i.e., 44%, the difference in survival compared to adult chicken was highly significant (<0.00001). The observed rate of protection was hardly surprising - so far the complete protection has been seldom observed in experimental avian flu vaccine trials reported by others [6-8]. For example only 50% protection was observed by Swayne et al., [9] with their H5N1 vaccine. The cross-protective capacity of the same vaccine can vary widely from 54.5% to 100% depending on the choice of challenge virus [10]. Also the post-vaccination timing for challenge appears to be critical. In the field study at two Hong Kong farms H5N1 infection was most likely to cause death in recently vaccinated chickens up to 18 days post-vaccination, but no deaths were observed after that period [11]. Indeed 100% protection was observed when chickens were challenged one month after vaccination [12]. Similar findings were reported by another group from Harbin, which indicated that hemagglutinin-inhibition antibody became detectable one week after vaccination and reached a peak at six weeks post-vaccination [13]. Finally, the age of vaccinated chicken appears to play an important role in protection as has been reported by Schultz-Cherry et al., [14]. Indeed, their study has shown that H5N1 vaccine appears to be 100% effective in 2-week old chicken but partially protective in ovo and in one-
day-old chicks. We are thus confident that the level of protection conferred by our vaccine can be improved when factors such as age, timing of challenge and vaccine doses are taken into consideration in future studies.

It is clear that growing highly pathogenic virus can be a challenge for scaled-up vaccine production. Beside low yield of virus due to pathogenic effect on chicken embryos, there is an inherent biohazard issue associated with handling the virus. For these reasons the virus stock for candidate vaccine needs to be selected from low pathogenic strains or recombinant viruses obtained by reverse genetics or other means must be used.

Today the eminent danger of H5N1 pandemic is faded away but this experience has helped us to successfully make tableted vaccine (HAP-V) shortly after H1N1 swine flu outbreak has been reported in Mexico. However, our company is small and has no competitive advantage or established reputation to penetrate the prophylactic flu vaccine market. We have thus been entertaining the idea whether flu vaccine can be employed as a therapeutic strategy for acute and rapidly evolving infection like influenza. This possibility is backed by reports in the Russian medical literature which indicate that influenza vaccine when delivered by mucosal route can speed up the recovery process from flu symptoms, i.e., act as a therapeutic modality after infection has already been established [15]. In view of Russian data our vaccine needs to be tested as a therapy - a property highly relevant in a situation when unvaccinated individuals are exposed to pandemic virus and start to display symptoms of the disease. Even if half of people are saved by such an intervention it can be a significant achievement especially considering the fact that less than a half of H5N1-infected individuals appear to survive despite treatment with Tamiflu or Relenza.

In event there is a pandemic with highly pathogenic flu virus, like H5N1, our vaccine may become extremely valuable since as an oral pill it will be easier to distribute to a large number of people within very short period of time. In fact, the article published by Agafonov et al., [16] is highly illustrative of this advantage. During accidental smallpox outbreak in Moscow a vaccination team, consisting of 2-3 persons, was capable of vaccinating within one hour 1,456 persons by oral method, 891 by spray method, and only 27 people by injection. Thus, oral delivery is much faster than conventional methods – a factor very critical in emergency situations. Furthermore, oral delivery does not require medical training and substantial distribution-related expenses as it would be necessary for parenteral delivery means. Most people will certainly prefer taking the pill rather than subjecting themselves to needle injection. However, making vaccine as a pill has been historically a very challenging task [17]. Our technology represents the long-awaited breakthrough and is supported by data from clinical trials of our therapeutic vaccines for HIV and viral hepatitis B and C infections [18,19]. Furthermore, our recent clinical trial of therapeutic vaccine designed to treat chronic inflammatory diseases, such as obesity and atherosclerosis, provides additional support to the advantage of oral delivery [unpublished].

In conclusion, our results suggest that oral formulation of killed flu virus holds a promise as potentially effective prophylactic and therapeutic vaccine. However, it is clear that several factors are at play as revealed by this study. Further experiments need to be carried out to test variables such as feeding schedule, dose, timing of challenge, antibody and cell-mediated immune responses, in order to identify the optimal vaccine formulation.

5. Acknowledgements

We express our profound gratitude to Drs. Chaisingh, Tawesak, Damrongwatanapokin, and Sukinthai of the National Animal Health Institute, Kasetsart University, the Thai Ministry of Agriculture for providing unwavering support and material contribution to the initial stages of this work. The untiring enthusiasm of Dr. Orapun Metadilogkul in starting this project is appreciated very much.

6. References


