Avian Pathology (1999) **28**, 245–255

Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines

D. E. Swayne*, J. R. Beck, M. Garcia† and H. D. Stone

USDA, Agriculture Research Service, Southeast Poultry Research Laboratory, 934 College Station Road, Athens, Georgia 30605, USA

The influence of vaccine strain and antigen mass on the ability of inactivated avian influenza (AI) viruses to protect chicks from a lethal, highly pathogenic (HP) AI virus challenge was studied. Groups of 4-week-old chickens were immunized with inactivated vaccines containing one of 10 haemagglutinin subtype H5 AI viruses, one heterologous H7 AI virus or normal allantoic fluid (sham), and challenged 3 weeks later by intra-nasal inoculation with a HP H5 chicken-origin AI virus. All 10 H5 vaccines provided good protection from clinical signs and death, and produced positive serological reactions on agar gel immunodiffusion and haemagglutination inhibition tests. In experiment 1, challenge virus was recovered from the oropharynx of 80% of chickens in the H5 vaccine group. In five H5 vaccine groups, challenge virus was not recovered from the cloaca of chickens. In the other five H5 vaccine groups, the number of chickens with detection of challenge virus from the cloaca was lower than in the sham group ($P < 0.05$). Reductions in the quantity of challenge virus shed from the cloaca and oropharynx were also evident in some H5 vaccinate groups when compared to the sham group. However, there was no positive correlation between the sequence identity of the haemagglutinin gene from the vaccine strain and challenge virus, and the ability to reduce the quantity of challenge virus shed from the cloaca or oropharynx.

As the quantity of AI antigen in the vaccines increased, all parameters of protection improved and were virus strain dependent. A/turkey/Wisconsin/68 (H5N9) was the best vaccine candidate of the H5 strains tested ($PD_{50} = 0.006$ μg AI antigen). These data demonstrate that chickens vaccinated with inactivated H5 whole virus AI vaccines were protected from clinical signs and death, but usage of vaccine generally did not prevent infection by the challenge virus, as indicated by recovery of virus from the oropharynx. Vaccine use reduced cloacal detection rates, and quantity of virus shed from the cloaca and oropharynx in some vaccine groups, which would potentially reduce environmental contamination and disease transmission in the field.

Introduction

Avian influenza (AI) is a disease of poultry that has occurred worldwide over the past 100 years (Easterday *et al.*, 1997). Two clinical forms are seen in the field: a mild disease affecting the respiratory, reproductive and/or urinary tracts, and a severe systemic disease, causing high morbidity and mortality. As a regulatory requirement, AI viruses have been tested in poultry for the ability to produce clinical disease and death. AI viruses are classified as highly pathogenic (HP), mildly pathogenic (MP) and non-pathogenic (NP) based on the mortality rates of *in vivo* tests and the results of *in vitro* tests (Senne *et al.*, 1986; US Animal Health Association (USAHA), 1994). The MP and NP AI viruses are usually associated with mild clinical disease in the field. By contrast, the HP form or 'fowl plague' affects international trade and is categorized by Office International des Epizooties (OIE) as a List A disease (OIE Code Commission, 1992). Efficient and cost-effective methods to eliminate or prevent HP AI in poultry are essential to develop, and maintain viable export markets for poultry and poultry products, as well as a domestic market. Historically, HP AI outbreaks have been controlled by costly eradication programmes focusing on quarantine and slaughter (Easterday *et al.*, 1997). However, the current political climate places more responsibility and financial obligations

* To whom correspondence should be addressed. Tel: + 1 706 546 3433, Fax: + 1 706 546 3161, E-mail: dswayne@arches.uga.edu
† Present address: Department of Avian Medicine, University of Georgia, Athens, GA 30605, USA.
Received 10 August 1998, Accepted 17 December 1998.

ISSN 0307-9457 (print)/ISSN 1465-3338 (online)/99/030245-11

Table 1. *Avian influenza virus used for vaccine preparations or challenge exposure*

	Abbreviation	Subtype	Isolation date (month/year)	HA1 Sequence identity with Q1/95 (%)		Vaccine viruses tested by experiment number	
				Nucleotides	Amino acids[a]	1	2
A/turkey/Wisconsin 68	TW/68	H5N9	11/68	89.1	96.8	×	×
A/turkey/Oregon/71	TO/71	H7N3	3/71	61.2	79.6	×	×
A/turkey/Minnesota/3689-1551/81	TM/81	H5N2	10/81	92.1	97.8		×
A/mallard/Ohio/556/87	MO/87	H5N9	11/87	94.1	97.7	×	
A/chicken/Mexico/31381-7/94	M10/93	H5N2	10/93	96.5	98.8	×	
A/chicken/Mexico/26654-1374/94[b]	M5/94	H5N2	5/94	96.7	98.2	×	×
A/turkey/Minnesota/10734-5/95	TM/95	H5N2	11/94	94.0	97.6	×	
A/chicken/Jalisco/14589-660/94	J12/94	H5N2	12/94	98.5	99.3	×	
A/chicken/Queretaro/14588-19/95	Q1/95	H5N2	1/95	100	100	×	
A/chicken/Veracruz/28159-398/95	V1/95	H5N2	1/95	98.2	99.3	×	
A/chicken/Puebla/28159-474/95	P3/95	H5N2	3/95	95.3	97.7	×	×
A/chicken/Chiapas/28159-488/95	C4/95	H5N2	4/95	96.9	98.9	×	

[a] Based on number of identical amino acids/total number of amino acids

[b] AI virus strain used in the inactivated vaccine of the Mexican AI control programme.

on the poultry industry to find and finance more cost-effective solutions to disease control.

Sporadic outbreaks of MP AI in turkeys have occurred in Minnesota during the autumn months and inactivated vaccines have been used in control efforts (Halvorson *et al.*, 1987). Antibodies against the AI viral haemagglutinin and/or neuraminidase surface proteins are protective (Brugh *et al.*, 1979; Brugh & Stone, 1987), but the protection is subtype specific, i.e. an H5 vaccine protects against only H5 AI viruses, but not against the other 14 haemagglutinin subtypes. In the past, the Minnesota Turkey Industry has achieved successful control of sporadic AI outbreaks by utilizing vaccines as one tool in a comprehensive control programme, but vaccination is not the sole determinant in eradication and control efforts (Halvorson, 1995). From 1979 to 1997, 22,385,000 doses of killed vaccine were used in Minnesota turkeys (Halvorson, 1998). However, the use of H5 and H7 AI vaccines has been prohibited in the United States (US) for control of MP AI since the 1990s, and have never been allowed for use in control of HP AI. Recently, the role of AI vaccines as a tool in eradication efforts has been re-examined and an inactivated autogenous H7N3 AI vaccine was used in 238 turkey flocks during a MP H7 AI outbreak in Utah during 1995 (Pomeroy, 1995).

Internationally, an outbreak of MP H5N2 AI in commercial poultry of Mexico beginning in 1993 with subsequent emergence of HP H5N2 AI viruses in the Pueblo and Queretaro states of Mexico in December 1994 and January 1995, and an outbreak of HP H7N3 AI viruses in Pakistan in 1994 created renewed interest in the role of vaccination in the eradication of HP AI (Salem, 1995; Naeem, 1998; Villareal & Flores, 1998). Both Mexico and Pakistan have used whole-virus inactivated vaccines as part of the control strategy. However, in Mexico, antibody titres in vaccinated chickens were inconsistent, ranging from none to high titres (Salem, 1995). This confirmed the need to standardize and optimize vaccines to achieve consistent antibody production and maximal protection from field challenge (Brugh *et al.*, 1979; Wood *et al.*, 1985). Inactivated vaccines and recombinant fowlpox virus vaccines have been shown to protect against clinical signs and death, and reduce virus detection rates in and shedding from enteric and respiratory tracts following HP AI virus challenge (Brugh *et al.*, 1979; McNulty *et al.*, 1986; Stone, 1987; Taylor *et al.*, 1988; Beard *et al.*, 1991; Swayne & Stone, 1996; Swayne *et al.*, 1997).

The purpose of this study was to determine the effect of vaccine virus strain and viral antigen mass on the efficacy of H5 AI vaccines to protect against morbidity, mortality and virus shedding following challenge by a recently isolated HP H5 AI virus.

Materials and Methods

Viruses

AI viruses tested as potential inactivated vaccines are listed in Table 1. The H5 viruses were isolated over a period of 27 years and the HA1 segment of the haemagglutinin gene of vaccine viruses had nucleic acid and amino acid sequence differences of 0 to 10.9% and 0 to 3.2%, respectively, when compared to the HP Mexican-origin challenge virus Q1/95 (Figure 1 and Table 1). An H7 AI virus was included as a heterologous haemagglutinin control vaccine. In chicken intravenous pathogenicity tests, all H5 and H7 subtype viruses exhibited low lethality, except Q1/95 which was a highly lethal challenge virus classified as HP.

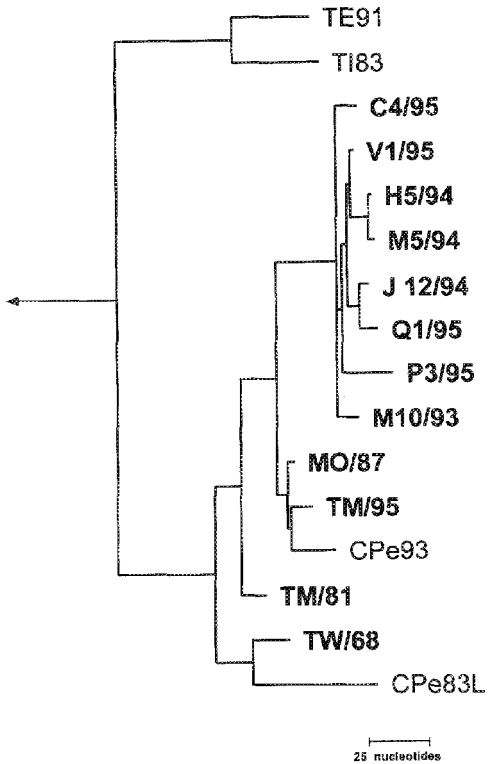

Figure 1. *Midpoint rooted phylogenetic tree for 16 H5 viruses of complete nucleotide coding sequence of the HA_1 subunit and the N-terminal fusion peptide sequence. Phylogenetic tree was constructed utilizing Phylogenetic Analysis Parsimony software (Bot et al., 1996).* Abbreviations for viruses used in this study are bolded and defined in Table 1. Additional isolates included in this comparison were TE91 = *A/turkey/England/50–92/91 (H5N1), TI83 = A/turkey/Ireland/83 (H5N8), CPe93 = A/chicken/Pennsylvania/13609/93 (H5N2) and CPe83L = A/chicken/Pennsylvania/21525/83 (H5N2). Q1/95 is the highly pathogenic challenge virus.*

Vaccines

Vaccine viruses were grown in 10-day-old specific pathogen-free (SPF) embryonating chicken eggs and the infective chorio-allantoic fluid (CAF) was pooled for each AI virus isolate in each experiment. Infectious titres were determined prior to inactivation, and haemagglutination (HA) titres and viral protein content was determined after inactivation. Inactivation was confirmed by chicken embryo inoculation (Swayne *et al.*, 1998b). For experiment 1, undiluted vaccine virus was inactivated with 3 mM 2-bromoethylamine hydrobromide (BEI; King, 1991). Pre-inactivation infectivity titres, HA titres and viral antigen content of the aqueous phase are listed in Table 2. For experiment 2, each of four viruses were inactivated with 3 mM BEI and their concentrations were adjusted with normal allantoic fluid to yield a total viral protein content of 33 mg/0.1 ml. Based on pre-inactivation titres, the infectious virus content after adjustment of TW/68, TM/81, M5/94 and P3/95 was $10^{9.1}$, $10^{7.9}$, $10^{8.9}$ and $10^{9.7}$ mean embryo lethal doses (ELD_{50})/ml, respectively, and the HA titres were 1/512, 1/108, 1/85 and 1/155, respectively.

Antigen content was determined by radial immunodiffusion (RID; Wood *et al.*, 1986). Specifically, a portion of each of the vaccine virus stocks were concentrated 100 fold by ultra-centrifugation at 82,700 *g* for 1 h and the pellet was resuspended in phosphate-buffered saline. Ten per cent detergent was added to each sample and allowed to set at room temperature for 30 min. Samples were added to an immunodiffusion plate (1.5% agarose, 0.1% sodium azide and 7.5% AI polyclonal serum (H4N8) in phosphate-buffered saline) and stored in a humidified chamber. Results were recorded from 24 h to 7 days, together with a known diluted series of quantified viral protein. Based on these results, all virus stocks used were diluted in normal allantoic fluid to yield the same antigen mass.

For both experiments, 1 vol. of aqueous vaccine virus antigen or non-infective CAF was emulsified mechanically in 4 vols of oil phase as described (Stone *et al.*, 1983; Stone, 1987). The oil phase was composed of 36 parts Drakeol 6 VR (Penreco, Karns City, PA), 3 parts Arlacel 80 (Sorbitan mono-oleate, ICI Americas, Washington DC) and 1 part Tween 80 (Polysorbate 80, ICI Americas, Washington, DC). For experiment 1, each chicken received a single injection of 0.5 ml of vaccine administered subcutaneously (s.c.) in the base (nape) of the neck. For experiment 2, each chicken received a subcutaneous injection of either 0.5, 5, 50 or 500 μl of vaccine representing 0.005, 0.05, 0.5 and 5 μg of viral protein, respectively. The 0.5- and 5-μl doses were administered via a micro-injection syringe (Unimetric Micro Syringe, Bradford Scientific, Epping, NH 03042).

Animals and housing

Four-week-old SPF white leghorn (WL) chickens were obtained from flocks maintained at Southeast Poultry Research Laboratory (SEPRL). Chickens were reared and vaccinated in standard brooder batteries in an isolation building dedicated to non-infectious studies. For the HP AI virus challenge, chickens were placed in negative-pressure stainless steel isolation cabinets ventilated with HEPA-filtered air and provided with continuous lighting. All challenge experiments were carried out in a USDA certified biosafety level 3 agriculture (BSL-3 AG) facility at SEPRL. Water and feed were provided *ad libitum*.

Experiment 1. Protection by eleven AI virus vaccine strains

Eleven different AI virus isolates and non-infectious CAF from uninfected embryos were used in the test vaccines. Specific AI viruses were selected for use based on geographical origin and date of isolation, and differences in haemagglutinin sequences, as compared to challenge virus Q1/95 (Figure 1 and Table 1). Groups of ten 4-week-old chickens were immunized s.c. with each of the 12 candidate vaccines. At 3 weeks post-vaccination (p.v.), chickens were challenged by intra-nasal (i.n.) inoculation with $10^{7.7}$ ELD_{50} of HP Q1/95 AI virus. Clinical response was recorded for 14 days post-challenge (p.c.). The percentage protection from clinical signs or death for the vaccines was calculated by the following formulas, first, for clinical signs:

$$\text{Protection} = \frac{\%\text{Clinical signs in sham group} - \%\text{Clinical signs in vaccine group}}{\%\text{Clinical signs in sham group}} \times 100$$

and for mortality:

$$\text{Protection} = \frac{\%\text{Mortality in sham group} - \%\text{Mortality in vaccine group}}{\%\text{Mortality in sham group}} \times 100$$

Serum was collected from each bird on the day of challenge and 14 days thereafter. Oropharyngeal and cloacal swabs were taken at day 3 p.c. for attempts at virus isolation and titration in 10-day embryonating SPF chicken eggs (Swayne *et al.*, 1998b). Briefly, the walls of the oropharynx and cloaca were vigorously swabbed with a cotton tipped applicator (Cotton-Tipped Applicators, Baxter, Deerfield, IL 60015–4633), placed in 1.5 ml of BHI medium (Brain Heart Infusion, Difco, Detroit, MI 48232–7059) containing antibiotics [gentamycin 100 mg/ml (gentamicin solution, Sigma Chemical, St Louis, MO 63178), penicillin 1000 IU/ml (penicillin G sodium salt, Sigma) and amphoteracin B 5 mg/ml (Amphoteracin B solubilized, Sigma)], and frozen at −70°C. The vials were quick thawed at 25°C, vortexed and allowed to set for 30 min at 25°C. Three eggs were inoculated via the

Table 2. *Experiment 1. HA titres, pre-inactivation infectivity titres and viral protein contents of aqueous antigen used for vaccine preparations*

Virus	HA titre	Infectious titre before inactivation ($Log_{10}EID_{50}$/0.1 ml)	Viral protein (μg/0.1 ml)
TW/68	512	8.5	33
TO/71	2048	8.7	40
MO/87	64	7.9	33.5
M10/93	48	7.7	29
M5/94	128	8.3	39
TM/95	96	7.9	<27.5
J12/94	48	7.7	<27.5
Q1/95	64	7.3	28
V1/95	256	8.9	31.5
P3/95	256	8.7	39
C4/95	48	7.3	<27.5

allantoic sac with 0.2 ml of the clarified media. Titrations were made using 10-fold dilutions of the clarified media in BHI with antibiotics. Titres were expressed as ELD_{50}/ml of swab fluid.

Experiment 2. Mean protective dose for four AI virus vaccine strains

Four AI virus isolates were tested in a quantitative potency test to determine the effect of viral antigen mass on protection; i.e. mean protective dose (PD_{50}). Groups of seven 4-week-old chickens were immunized s.c. with 0.5, 5, 50 and 500 μl of each of the four virus vaccines, and 14 chickens were immunized s.c. with 500 μl of normal allantoic fluid vaccine. At 3 weeks p.v., chickens were challenged i.n. with $10^{7.1}$ ELD_{50} of HP Q1/95 AI virus. Clinical responses were recorded daily for 14 days p.c. The percentage protection was calculated as above. Serum was collected for each bird on the day of challenge and 14 days thereafter. Oropharyngeal and cloacal swabs were taken at day 3 p.c. for attempts at virus isolation and titrations in 10-day embryonated SPF chicken eggs as described above. The PD_{50} against death was calculated by the method of Reed & Muench (1938).

Serology

Serological testing was accomplished using agar gel immunodiffusion (AGID) test for type A influenza and haemagglutinin-inhibition (HI) test for H5 subtype detection as described (Beard, 1970; Swayne *et al.*, 1998b). For the AGID test, precipitating antibodies were detected using A/turkey/Wisconsin/66 (H9N2) as the test antigen (Beard, 1970). For the HI test, titres were determined using serial two-fold dilutions of test sera, 4 HA units of H5 antigen and 0.5% suspension of chicken erythrocytes per test well. For experiment 1, TW/68, M5/94 and Q1/95 H5 antigens were used to test vaccine groups. For experiment 2, only Q1/95 H5 test antigen was used. Positive titres were interpreted as inhibition of haemagglutination at a dilution of sera of 1:10 or greater.

Statistical analysis

Frequency of morbidity, mortality, virus isolation and serologically positive test results were analysed for significance ($P < 0.05$) by Fisher's exact test on PC-based software (Statgraphics, Manugistics, Inc., Rockville, MD). Virus isolation titres were tested for normal distribution. Normally distributed data sets were further tested by parametric one-way ANOVA or two-way analysis of variance (TWANOVA). Those ANOVA data sets with significant differences were further analysed by Student—Neuman—Keuls multiple comparison test. Data sets not normally distributed were analysed by non-parametric analysis of variance test (Kruskal—Wallis) and, for significantly different groups ($P < 0.05$), Dunn's multiple comparison test was performed. Normality, ANOVA, Kruskal—Wallis, Student—Neuman—Keuls and Dunn's tests were performed on PC-based software (SigmaStat, Jandel Scientific, San Rafael, CA).

The minimum virus titre detected by virus isolation procedures in this study was $10^{1.0}$ ELD_{50}/ml. Thus, for statistical purposes, all oropharyngeal and cloacal swabs from which virus was not isolated were given a numeric value of $10^{0.9}$ ELD_{50}/ml which represents the lowest detectable level of virus if the virus isolation procedure were modified to use four instead of three embryonating chicken eggs per sample.

Pearson product moment correlation (r) was used to test for associations between HA titre, infectious titre and antigen mass of vaccines. Spearman rank correlation (r_s) was used to test association between sequence homology of vaccine and challenge virus haemagglutinin, and reductions in titres of challenge virus shed from cloaca and oropharynx. Pearson product moment correlation and Spearman rank correlation were performed on PC-based software (SigmaStat, Jandel Scientific, San Rafael, CA).

Results

Experiment 1

Three methods were used to quantify vaccine antigen mass; HA titre, pre-inactivation infectivity titre in embryonating chicken eggs and AI viral protein content (RID; Table 2). There was poor correlation between AI viral protein content and HA titre ($r = 0.524$, $P = 0.18$) and AI viral protein content and infectious titre ($r = 0.63$, $P = 0.10$). There was good correlation between HA and infectious titres ($r = 0.81$, $P = 0.003$).

At 3 weeks p.v., all chickens immunized with the 11 different vaccine viruses had similar pre-challenge seroconversion rates using the influenza type A specific AGID test (Table 3). By contrast, pre- and post-challenge subtype specific HI positivity rates and geometric mean titres (GMTs) for immunized chickens varied between the 11 different vaccine viruses and the three different HI test antigens (Figure 2). Both pre- and post-challenge HI titres tended to be higher when the test antigens and vaccine viruses were closely related phylogenetically (Figures 1 and 2). For example, the highest pre-challenge HI titres using TW/68 as HI

Table 3. *Experiment 1. Protection from clinical signs and death, and positive AGID pre-challenge serological responses for WL chickens immunized at 4 weeks of age with inactivated AIV vaccines and challenged at 7 weeks of age with HP Q1/95*

Vaccine virus		Pre-challenge positive by AGID[A]	Challenge response	
Strain	Subtype		Number with no clinical signs	Number of survivors
Sham	—	0[a]	0[a]	1[a]
TW/68	H5N9	9[b]	9(90)[B]	9(88)[B]
TO/71	H7N3	10[b]	0[a](0)	1[a](0)
MO/87	H5N9	9[bC]	9[b](100)	9[b](100)
M10/93	H5N2	10[b]	10[b](100)	10[b](100)
M5/94	H5N2	9[b]	9[b](90)	9[b](88)
TM/95	H5N2	10[b]	10[b](100)	10[b](100)
J12/94	H5N2	10[b]	10[b](100)	10[b](100)
Q1/95	H5N2	10[b]	10[b](100)	10[b](100)
V1/95	H5N2	9[b]	9[b](90)	9[b](88)
P3/95	H5N2	8[b]	9[b](90)	9[b](88)
C4/95	H5N2	8[b]	10[b](100)	10[b](100)

[A] Number of positive/10 tested. Different lower case superscripts denote significant difference between treatments groups; Fisher's Exact test, $P < 0.05$.
[B] Percentage protection in parentheses; see Materials and Methods for formula.
[C] Nine birds in this group.

test antigen were obtained in chickens vaccinated with US-origin H5 vaccine viruses TW/68 and TM/95 (1:299 and 1:171, respectively), while highest pre-challenge HI titres using M5/95 or Q1/95 antigen were obtained with M5/94 (1:80 and 1:121, respectively) and V1/95 vaccine viruses (1:61 and 1:80, respectively). However, some Mexican-origin vaccine viruses produced low HI titres irrespective of HI test antigen used. At week 2 post-challenge, individual GMTs were 2 or 3 $\log_2$ higher than the respective pre-challenge titres (Figure 2). Chickens immunized with heterologous AI vaccine TO/71 (H7N3) lacked HI titres to the H5 test antigens on pre-challenge, but the one surviving chicken at day 14 p.c. had a positive HI titre against H5 test antigens (Figure 2).

Chickens in all H5 vaccine groups had similar protection against clinical signs and death following challenge by HP QI/95 (88 to 100%; Table 3), and the four chickens that died lacked AGID and HI antibodies at the time of challenge. All chickens vaccinated with normal allantoic fluid (sham group) or heterologous H7 TO/71 vaccines developed clinical signs and only 10% survived after challenge (Table 3). All survivors had antibodies measured by AGID and HI tests.

Challenge virus was frequently recovered from the oropharyngeal cavity of chickens from all groups on day 3 p.c., but M5/94 and J12/94 groups had significantly lower rates ($P < 0.05$) of virus detection than the other groups (Table 4). However, when compared to the sham-vaccinated group, the titre of virus recovered was also significantly lower ($P < 0.05$) for the three US-origin H5 vaccine groups and all Mexican-origin H5 vaccine groups except C4/95. By contrast, virus detection in the intestinal tract (cloacal swabs) was infrequent for chickens from all H5 vaccine groups and significantly less frequent ($P < 0.05$) than virus detection from the sham and H7 TO/71 groups. The titres of virus recovered from the cloaca were low for all groups, but when compared to titres from the sham group, they were significantly lower for all US-origin H5 groups, and M5/94, J12/94, Q1/95, V1/95 and P3/95 Mexican-origin H5 groups. There was no correlation between haemagglutinin sequence homology of H5 vaccine strains as compared with challenge virus, and reduction in titres of recovered virus from oropharynx ($r_s = -0.166$, $P = 0.10$) or cloaca ($r_s = 0.004$, $P = 0.97$).

Experiment 2

Sera from most chickens vaccinated with the two highest doses (0.5 and 5 μg) of any of the four virus vaccines were positive by AGID (86%, 48/56) and HI (91%, 51/56) test at 3 weeks p.v., whereas sera from chickens vaccinated with the two lowest doses (0.005 and 0.05 μg) were infrequently positive by AGID (3%, 2/56) and HI (5%, 3/56) tests (Table 5 and Figure 3). The GMTs, as determined using Q1/95 test antigen, were highest for the TW/68-vaccinated group at both the 0.5- and 5-μg vaccine doses (Figure 3). At week two p.c., all surviving chickens were serologically positive by AGID and HI tests. Post-challenge GMTs were 1 to 3 $\log_2$ higher than the pre-challenge

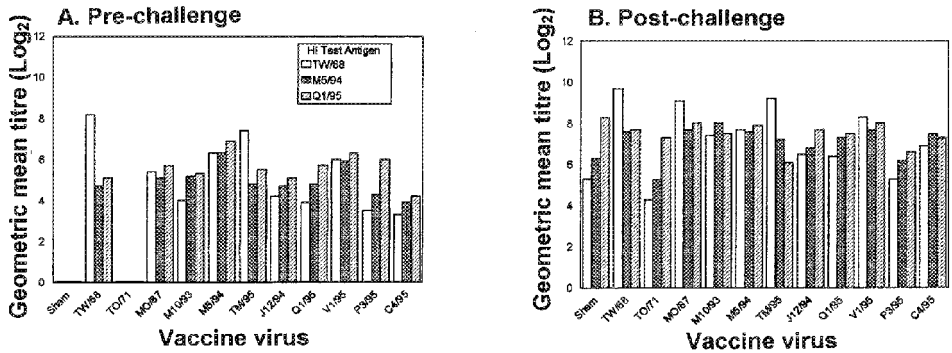

Figure 2. *Experiment 1. HI titres of seropositive chickens 3 and 5 weeks p.v. (A) Pre-challenge. (B) Two weeks p.c. HI titres given as GMTs: 1 = 1:2, 2 = 1:4, 3 = 1:8, etc.*

GMTs and were similar in magnitude among the different vaccine groups and antigen doses.

Significant protection (69 to 100%) against clinical signs, and death was seen in the groups vaccinated with 0.05, 0.5 and 5 μg of vaccine for TW/68, M5/94 and P3/95 vaccine viruses, and in the 0.5 and 5 μg doses for TM/81 vaccine virus (Table 5). All chickens with detectable HI or AGP antibody titres were protected against death. However, some chickens that failed to give positive AGID (28/62, 45%) or HI (24/58, 41%) serological results were protected, especially at the 0.05 μg vaccine dose for TW/68, M5/94 and P3/95 (Figure 3 and Table 5). None of the chickens in the sham-vaccinated group were protected against clinical signs and only one survived challenge.

Challenge virus was recovered from the oropharyngeal swabs of 99% of the birds (*Table 6*). Compared to the sham-vaccinated group, significantly lower quantities of virus were recovered in the TM/81- and M5/94-vaccinated groups at the 0.05-μg dose, in all four vaccine virus groups at the 0.5-μg dose, and in the TM/81, M5/94 and P3/95 groups at the 5-μg dose (Table 6). For the intestinal tract (cloacal swabs), virus detection rates and titres at the two lower vaccine doses were not significantly different among the sham-vaccinated and the four vaccine groups, except for the M5/94 group at 0.05-μg dose (Table 6). As compared to the sham-vaccinated group, virus detection rates were significantly lower at the 0.5-μg vaccine dose for TW/68, M5/95 and P3/95 groups, and titres of recovered virus were significantly lower for TW/68 and P3/95. For the 5-μg antigen dose, a significant reduction ($P < 0.05$) in virus detection rates was evident for all vaccine groups as compared to the sham-vaccinated group, and virus titres were significantly lower for TW/68, M5/95 and P3/95. There was no correlation between haemagglutinin sequence homologies of four H5 vaccine strains relative to the challenge virus, and reduction in titres of recovered virus from oropharynx ($r_s = -0.111$, $P = 0.17$) or cloaca ($r_s = 0.0561$, $P = 0.49$).

For the quantitative potency test, there was a 26-fold variation in the PD_{50} values based on mortality obtained for the four strains studied (Table 6). Based on mortality, the PD_{50} values were 0.006, 0.156, 0.016 and 0.030 μg of viral protein for TW/68, TM/81, M5/94 and P3/95,

Table 4. *Experiment 1. Attempted virus isolation from cloacal and oropharyngeal swabs on 3 days p.c. with HP Q1/95 of WL chickens immunized with killed AIV vaccines at 4 weeks of age and challenged at 7 weeks of age*

Vaccine virus	Virus recovery from: Oropharyngeal swabs	Virus recovery from: Cloacal swabs
Sham	$10^{Aa}(5.3^{B})^{a}$	$7^{a}(1.69)^{a}$
TW/68	$9^{a}(3.6)^{bcd}$	$1^{b}(0.96)^{bc}$
TO/71	$10^{a}(4.8)^{ac}$	$5^{a}(1.26)^{ac}$
MO/87	$8^{Ca}(3.5)^{bcd}$	$0^{b}(NI^{D})^{bc}$
M10/93	$8^{a}(3.0)^{bcd}$	$2^{b}(0.97)^{ac}$
M5/94	$4^{b}(1.8)^{b}$	$0^{b}(NI)^{bc}$
TM/95	$10^{a}(3.4)^{bcd}$	$0^{b}(NI)^{bc}$
J12/94	$6^{b}(2.6)^{bd}$	$0^{b}(NI)^{bc}$
Q1/95	$8^{a}(2.7)^{bd}$	$0^{b}(NI)^{bc}$
V1/95	$7^{a}(2.3)^{b}$	$1^{b}(0.96)^{bc}$
P3/95	$9^{a}(3.3)^{bcd}$	$1^{b}(0.91)^{bc}$
C4/95	$10^{a}(4.5)^{acd}$	$2^{b}(0.97)^{ac}$

[A] Virus recovered/ 10 examined. Different lower case superscripts denote significant differences; Fisher's Exact test, $P < 0.05$.

[B] Mean of the $\log_{10}$ titre/ml of swab material. Parametric ANOVA and non-parametric ANOVA (Kruskal–Wallis) identified differences in titres for oropharyngeal ($P < 0.001$) and cloacal ($P < 0.001$) swabs, respectively. Different lower case superscripts denote significance ($P < 0.05$) between groups using the multirange tests of Student–Newman–Keuls and Dunn, respectively.

[C] Nine birds in group.

[D] No virus isolated

Table 5. *Experiment 2. Morbidity, mortality and AGID serological tests for WL chickens immunized at 4 weeks of age with various doses of inactivated AIV vaccines and challenged at 7 weeks of age with HP Q1/95 avian influenza virus*

Vaccine strain	Dose (μg)	Pre-challenge AGID serology[A] (no. positive/total)	Anamnestic response[B] (no. positive/total)	Challenge response	
				Number with no clinical signs[C]	Number of survivors[C,D]
Sham	0	0/14[aE]	14/14[aE]	0/14[aE]	1/14[aE]
TW/68	0.005	0[a]	7[a]	2[a](29)[F]	3[a](38)[F]
	0.05	0[a]	7[a]	7[b](100)	7[b](100)
	0.5	5[b]	5[a]	7[b](100)	7[b](100)
	5	7[b]	4[a]	7[b](100)	7[b](100)
TM/81	0.005	0[a]	7[a]	0[a](0)	0[a](0)
	0.05	1[a]	7[a]	2[a](29)	2[a](23)
	0.5	4[b]	7[a]	6[b](86)	6[b](84)
	5	6[b]	4[a]	6[b](86)	6[b](84)
M5/94	0.005	0[a]	7[a]	0[a](0)	1[a](8)
	0.05	0[a]	7[a]	6[b](86)	6[b](85)
	0.5	6[b]	7[a]	7[b](100)	7[b](100)
	5	7[b]	5[a]	7[b](100)	7[b](100)
P3/95	0.005	0[a]	7[a]	0[a](0)	0[a](0)
	0.05	1[a]	7[a]	5[b](71)	5[b](69)
	0.5	6[b]	5[a]	7[b](100)	7[b](100)
	5	7[b]	2[b]	7[b](100)	7[b](100)

[A] No. positive/seven in group at 3 weeks p.v. Chickens challenged with HP Q1/95 at 3 weeks p.v. and sera from all survivors 2 weeks later were positive by AGID test.
[B] Positive response = number that died plus number with two-fold increase in HI titres/seven in group.
[C] Seven in group.
[D] Chickens that died lacked AGID antibodies at time of challenge.
[E] Different lower case superscripts denote significance difference between treatment and sham-immunized groups, Fisher's Exact Test, $P < 0.05$.
[F] Percentage protection, see formula in Materials and Methods.

respectively. Based on morbidity, the PD_{50} values were 0.008, 0.156, 0.022 and 0.030 μg of viral protein for TW/68, TM/81, M5/94 and P3/95, respectively.

Discussion

All the H5 AI-inactivated vaccines protected chickens from clinical signs and death, following challenge by HP Q1/95 H5N2 chicken-origin influenza virus. These viruses represent a diverse group of North American AI viruses isolated over a 27-year period, and separated by geographical distances of 3500 km. They had 96.8 to 100% amino acid sequence homology with the challenge virus in the HA1 segment of the haemagglutinin. Furthermore, the TW/68 vaccine used for this study also protected chickens from clinical signs and death following challenge by a HP Asian AI virus, A/Hong Kong/156/97 (H5N1), which was isolated from a 5-year-old boy (unpublished data). These data show that molecular and antigenic similarities between the individual H5 avian influenza strains studied were sufficient to elicit solid cross-protection against a recently emergent HP North American chicken-origin H5N2 virus and the HP Hong Kong H5N1 AI virus isolated from a human. Such findings suggest that frequent changing of AI virus vaccine strains are unlikely to be necessary for AI vaccines incorporating adjuvants to be effective.

In the present and previous studies (Wood *et al.*, 1985), the quantity of viral antigen in the vaccine was important in protecting from morbidity and mortality. In a previous study (Wood *et al.*, 1985), the PD_{50}, based on mortality, was between 0.1 and 0.3 μg of viral antigen per dose, while in the present study there was a 26-fold range (0.006 to 0.156) in PD_{50} for the four vaccine strains. This indicates that antigen mass affects the efficacy of inactivated AI vaccines, but vaccine strain selection also has a major role in determining the necessary antigen content in the vaccine.

The haemagglutinin is the major influenza protein that elicits a protective immune response and antibodies directed against it are readily detected and quantified serologically by the subtype specific HI test (Brugh *et al.*, 1979; Swayne *et al.*, 1998b). In addition, natural infection or immunization with whole virus vaccines elicit type A specific antibody response against the nucleoprotein and matrix antigens as detected by the AGID test (Brugh *et*

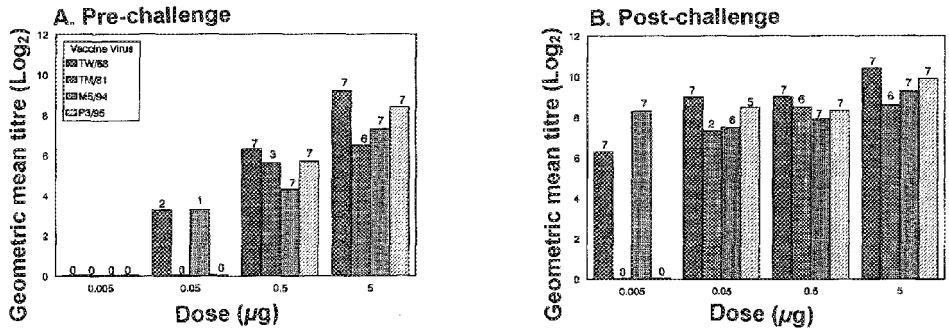

Figure 3. *Experiment 2. HI titres of seropositive chickens 3 and 5 weeks p.v. (A) Pre-challenge. (B) Two weeks p.c. HI titres given as GMTs: 1 = 1:2, 2 = 1:4, 3 = 1:8, etc. The numerical values over the bars represent the number of positive samples out of seven tested/group.*

al., 1979; Swayne *et al.*, 1998b). However, antibodies against nucleoprotein and matrix antigens do not protect from clinical signs and death (Brugh *et al.*, 1979; Webster *et al.*, 1991). In the current study, all inactivated H5 vaccines in experiment 1, and the 0.5- and 5-μg doses of H5 vaccines in experiment two produced good seroconversion, as evident by positive AGID and/or HI tests. The presence of positive AGID and HI serological reactions were good predictors of protection from clinical signs and death following lethal challenge by HP Q1/95. Furthermore, such protection was independent of the magnitude of the positive HI serological response, but the magnitude may have an effect on the longevity of the protective immune response. In contrast, with the low vaccine doses used in experiment two, 45 and 41% of AGID and HI serologically negative chickens, respectively, survived a lethal challenge.

Inactivated whole influenza virus vaccines and recombinant pox vaccines with AI virus haemagglutinin gene inserts have produced uniform protection of chickens from clinical signs and death following challenge by HP AI viruses of homologous haemagglutinin subtype (Stone, 1987; Taylor *et al.*, 1988; Barun *et al.*, 1988; Webster *et al.*, 1991; Beard *et al.*, 1991; Swayne *et al.*, 1997). However, such protection did not equate to total prevention of AI virus infection in vaccinated chickens. When compared to non-vaccinated or sham-vaccinated control groups, some influenza vaccines reduced the detection rates of chickens shedding challenge virus and lower titres of challenge virus were shed from infected chickens. A few studies reported lack of detection of challenge virus in some groups of vaccinated chickens (Wood *et al.*, 1985; Stone, 1987; Webster *et al.*, 1991, 1996; Swayne *et al.*, 1997).

The current study and/or previous studies (Brugh *et al.*, 1979; Wood *et al.*, 1985; Stone, 1987) have demonstrated several factors that contributed to variation in the ability of inactivated AI vaccines to protect from clinical signs and death, and to reduce the detection rates and the quantity of challenge virus shed from vaccinated chickens. These factors that affect protection include respiratory verses intestinal samples for evaluation, dose of vaccine (antigen mass), vaccine emulsion type and method of emulsification, age of chickens at vaccination, and time between vaccination and challenge. In the current study, reductions in detection rates and the amount of challenge virus shed were most consistently demonstrated with cloacal, instead of oropharyngeal samples. Typically, titres of challenge virus shed were $10^{1.3}$ EID_{50}/g lower for cloacal than oropharyngeal samples, but the volume of faecal material was greater than respiratory secretions, making differences in total virus output less apparent. At day 3 p.c., some chickens given the higher vaccine antigen doses lacked anamnestic responses and no virus was isolated from the cloaca, but virus could be isolated from the oropharynx. This indicates that caution should be exerted in stating that vaccines can prevent AI virus infection when given at high doses. AI virus replication in the host is dynamic and affected by multiple factors. Claiming a lack of infection based on a single isolation attempt from only one site or the lack of anamnestic serological response, may not represent the true infection status of the host.

Previous studies have shown improved protection by increasing vaccine antigen dose (Brugh *et al.*, 1979; Wood *et al.*, 1985). Furthermore, the laboratory method used to measure vaccine antigen mass may influence interpretation of results and ultimate formulation of vaccines (Brugh *et al.*, 1979; Wood *et al.*, 1985). Measurement of viral antigen by RID test is an effective and accurate method. However, indirect methods, such as the HA titre and infectious titre, are more frequently used as standards. In the current study, correlation was poor between RID test, and either the HA or pre-inactivation infectivity titres. However, when AI antigen mass in the vaccine is high, as in

Table 6. *Experiment 2. Attempted virus isolations at 3 days p.c. with HP Q1/95 from WL chickens immunized with killed AIV vaccines at 4 weeks of age and challenged at 7 weeks of age*

Vaccine Virus	Viral antigen (μg/dose) 0	0.005	0.05	0.5	5
Oropharyngeal swabs					
Sham	$12/12^{aA}(5.8)^{aB}$	—	—	—	—
TW/68	—	$7/7^{a}(4.9)^{a}$	$7/7^{a}(5.2)^{ab}$	$7/7^{a}(4.4)^{a}$	$7/7^{a}(5.2)^{ab}$
TM/81	—	$7/7^{a}(6.7)^{a}$	$6/6^{a}(4.9)^{b}$	$7/7^{a}(4.3)^{a}$	$7/7^{a}(4.1)^{b}$
M5/94	—	$6/6^{a}(4.7)^{a}$	$7/7^{a}(4.8)^{b}$	$7/7^{a}(3.7)^{a}$	$6/7^{a}(3.8)^{b}$
P3/95	—	$5/5^{a}(5.6)^{a}$	$7/7^{a}(5.5)^{ab}$	$7/7^{a}(4.6)^{a}$	$7/7^{a}(3.8)^{b}$
Cloacal swabs					
Sham	$8/12^{a}(1.3)^{a}$	—	—	—	—
TM/68	—	$3/7^{a}(0.93)^{a}$	$2/7^{a}(1.70)^{a}$	$0/7^{b}(NI)^{b}$	$0/7^{b}(NI)^{b}$
TM/81	—	$4/7^{a}(1.35)^{a}$	$4/6^{a}(1.62)^{a}$	$3/7^{a}(1.04)^{ab}$	$1/7^{b}(0.91)^{ab}$
M5/94	—	$5/6^{a}(1.30)^{a}$	$0/7^{b}(NI)^{b}$	$1/7^{b}(0.91)^{ab}$	$0/7^{b}(0.90)^{b}$
P3/95	—	$2/5^{a}(1.09)^{a}$	$2/7^{a}(1.41)^{a}$	$0/7^{b}(NI)^{b}$	$0/7^{b}(NI)^{b}$

[A] Virus isolations/number attempted. Different lower case superscripts denote significant difference between treatment and sham-immunized groups; Fisher's Exact test, $P < 0.05$.
[B] Mean of the $\log_{10}$ titres per 1 ml of swab material. Samples with no virus reisolation (NI) were given a numerical value below the detectible limits of the isolation methods (0.90) for statistical purposes. TWANOVA identified differences in titres for oropharyngeal [vaccine virus ($P = 0.013$) and dose of vaccine ($P < 0.001$), respectively] and cloacal swabs [dose of vaccine ($P < 0.001$)]. Different lower case superscripts denote significance between treatment groups within a vaccine dose; using Student–Neuman–Kuels, $P < 0.05$.

experiment 1, the method of measurement had minimal effect on effectiveness. However, if the goal is to lower the cost of production, while maintaining maximal effectiveness, accurate measurement and standardization of antigen content is critical.

Different strains of vaccine viruses produced variations in HI serological response and levels of protection from Q1/95 challenge. However, differences in the total amino acid sequence of the HA1 protein of vaccines and challenge virus in experiment one did not correlate with reductions in challenge virus titres shed from oropharynx or cloaca. For example, TW/68 (96.8% homology) and Q1/95 (100% homology) vaccines did not reduce the challenge virus detection rates from the oropharynx as compared to sham control group, but M5/94 vaccination (98.2% homology) did. In the first experiment, such variation could be attributed to differences in dose of vaccine administered. However, in experiment 2, vaccine dose was standardized, but differences in HA1 homology of vaccine and challenge virus did not correlate with reductions in challenge virus titres shed from oropharynx or cloaca. For example, at the 0.05-μg vaccine dose, M5/94 (98.2% homology) and TM/81 (97.8% homology) were more effective in lowering the titres of virus shed from the oropharynx than P3/95 (97.9% homology) and TW/68 (96.8% homology), but at 0.5-μg vaccine dose, TW/68 (96.8% homology) significantly reduced shedding from the cloaca, while TM/81 (97.8% homology) did not. Furthermore, 100% homology between the haemagglutinin of vaccine and challenge virus (Q1/95, experiment 1), did not result in the prevention of infection by the challenge virus and shedding from the oropharynx as has been reported by others (Wood *et al.*, 1985; Taylor *et al.*, 1988; Webster *et al.*, 1991), but these latter studies used lower challenge doses of HP AI viruses than the current experiment. No protection was provided by heterologous H7 vaccine (79.6% homology), since no difference in clinical signs and death rates, virus detection rates or titres of shed virus were observed compared to sham-vaccinated group.

Several other factors affect virus selection for inactivated vaccine preparations. The optimal virus strain for a commercial vaccine must replicate to high titre in embryonating eggs, have a low PD_{50} and be a stable, mildly pathogenic phenotype (Swayne & Stone, 1996). The latter is important for the vaccine manufacturer because a MP H5 or H7 virus with a demonstrated potential to mutate to a HP form should not be propagated and handled in a biosafety level 2 (BSL-2) facility as used for poultry vaccine production in the US. The TW/68 strain meets all three criteria as evidenced by high titres in eggs, low PD_{50} (0.006 μg viral protein) and having a stable MP phenotype even after manipulation in a laboratory model system that has successfully derived emergent HP H5

viruses from MP H5 viruses (Swayne *et al.*, 1998a).

Prevention and control programmes for avian influenza need a comprehensive strategy utilizing enhanced biosecurity, increased surveillance, proper diagnostic methods, education of poultry farmers and other personnel, and safe methods for eliminating infected birds (Halvorson, 1995; Swayne *et al.*, 1997). Vaccination should only be considered as a single tool in a comprehensive AI control strategy. A decision to use vaccines will depend on the pathogenicity of the field virus, haemagglutinin subtype of the field virus and vaccine, the surveillance programme, geographic distribution of the infection, impact of vaccine use on trade and national regulatory requirements.

Acknowledgements

The authors would like to thank Drs M. Brugh and C. Beard for suggestions on manuscript construction, and Dr Victor Chew, Gainesville, FL, USA, for statistical consultation. Portions of this study were presented at the 133rd Annual Meeting of the American Veterinary Medical Association, Louisville, KY, USA, July 20–24, 1996. Funding was provided by USDA, ARS CRIS project 6612–32000–016.

References

Barun, K., Shaw, M.W., Rota, P.A., Harmon, M.W., Esposito, J.J., Rott, R., Cox, N.J., Kendal, A.P. & De-Barun, K. (1988). Protection against virulent H5 avian influenza virus infection in chickens by an inactivated vaccine produced with recombinant vaccinia virus. *Vaccine, 6,* 257–261.

Beard, C.W. (1970). Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. *Bulletin of the World Health Organization, 42,* 779–785.

Beard, C.W., Schnitzlein, W.M. & Tripathy, D.N. (1991). Protection of chickens against highly pathogenic avian influenza virus (H5N2) by recombinant fowlpox viruses. *Avian Diseases, 35,* 356–359.

Bot, A., Reichlin, A., Isobe, H., Bot, S., Schulman, J., Yokoyama, W.M. & Bona, C.A. (1996). Cellular mechanisms involved in protection and recovery from influenza virus infection in immunodeficient mice. *Journal of Virology, 70,* 5668–5672.

Brugh, M., Beard, C.W. & Stone, H.D. (1979). Immunization of chickens and turkeys against avian influenza with monovalent and polyvalent oil emulsion vaccines. *American Journal of Veterinary Research, 40,* 165–169.

Brugh, M. & Stone, H.D. (1987). Immunization of chickens against influenza with hemagglutinin-specific (H5) oil emulsion vaccine, in: C. W. Beard & B. C. Easterday (Eds), *Proceedings of the 2nd International Symposium on Avian Influenza*, pp. 283–292. (Richmond, US Animal Health Association).

Easterday, B.C., Hinshaw, V.S. & Halvorson, D.A. (1997). Influenza. In: B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald & Y. M. Saif (Eds), *Diseases of Poultry* (10th edn, pp. 583–605). Ames: Iowa, Iowa State University Press.

Halvorson, D.A. (1995). Avian influenza control in Minnesota. *Poultry Digest, 54,* 12–19.

Halvorson, D.A. (1998). Epidemiology and control of avian influenza in Minnesota. In: *Proceedings of the 47th New England Poultry Health Conference* (pp. 5–11). Portsmouth: New England Poultry Association).

Halvorson, D.A., Karunakaran, D., Abraham, A.S., Newman, J.A., Sivanandan, V. & Poss, P.E. (1987). Efficacy of vaccine in the control of avian influenza. In: C. W. Beard & B. C. Easterday (Eds), *Proceedings of the 2nd International Symposium on Avian Influenza* (pp. 264–270). Richmond: US Animal Health Association.

King, D.J. (1991). Evaluation of different methods of inactivation of Newcastle disease virus and avian influenza virus in egg fluids and serum. *Avian Diseases, 35,* 505–514.

McNulty, M.S., Allan, G.M. & Adair, B.M. (1986). Efficacy of avian influenza neuraminidase-specific vaccines in chickens. *Avian Pathology, 15,* 107–115.

Naeem, K. (1998). The avian influenza H7N3 outbreak in South Central Asia. In: D. E. Swayne & R. D. Slemons (Eds), *Proceedings of the 4th International Symposium on Avian Influenza* (4th edn, pp. 31–35). Kennett Square: American Association of Avian Pathologists.

OIE Code Commission. (1992). *International Animal Health Code* (6th edn). Paris: Office International des Epizooties.

Pomeroy, B.S. (1995). Report of the subcommittee on avian influenza. In: *Proceedings of the 99th Annual Meeting of the United States Animal Health Association* (pp. 569–578). Reno, NV: US Animal Health Association.

Reed, L.J. & Muench, H. (1938). A simple method for estimating fifty per cent endpoints. *American Journal of Hygiene, 27,* 493–497.

Salem, M. (1995). Avian influenza in Mexico. In: *Proceedings of the 30th National Meeting on Poultry Health and Processing* (30th edn, pp. 99–105). Ocean City: Delmarva Poultry Industry, Inc.).

Senne, D.A., Pearson, J.E., Kawaoka, Y., Carbrey, E.A. & Webster, R.G. (1986). Alternative methods for evaluation of pathogenicity of chicken Pennsylvania H5N2 viruses. In: B. C. Easterday (Ed.), *Proceedings of the 2nd International Symposium on Avian Influenza* (pp. 246–257). Richmond, VA: US Animal Health Association.

Stone, H.D. (1987). Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. *Avian Diseases, 31,* 483–490.

Stone, H.D., Brugh, M. & Beard, C.W. (1983). Influence of formulation on the efficacy of experimental oil-emulsion Newcastle disease vaccines. *Avian Diseases, 27,* 688–697.

Swayne, D.E. & Stone, H.D. (1996). Evaluation of inactivated H5 avian influenza virus and fowlpox recombinant vaccines and diagnostic test reagents: Implications in avian influenza control and prevention strategies. In: *Proceedings of the 45th Western Poultry Disease Conference* (pp. 35–38). Cancun: ANECA.

Swayne, D.E., Beck, J.R. & Mickle, T.R. (1997). Efficacy of recombinant fowlpox vaccine in protecting chickens against highly pathogenic Mexican-origin H5N2 avian influenza virus. *Avian Diseases, 41,* 910–922.

Swayne, D.E., Beck, J.R., Garcia, M., Perdue, M.L. & Brugh, M. (1998a). Pathogenicity shifts in experimental avian influenza virus infections in chickens., in: D. E. Swayne & R. D. Slemons (Eds) *Proceedings of the 4th International Symposium on Avian Influenza* (pp. 171–181). Richmond, VA: US Animal Health Association.

Swayne, D.E., Senne, D.A. & Beard, C.W. (1998b). Influenza, In: D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson & W. M. Reed (Eds), *Isolation and Identification of Avian Pathogens* (4th edn, pp. 150–155). Kennett Square: American Association of Avian Pathologist.

Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R.G. & Paoletti, E. (1988). Protective immunity against avian influenza induced by a fowlpox virus recombinant. *Vaccine, 6,* 504–508.

USAHA (1994). Report of the Committee on Transmissible Diseases of Poultry and Other Avian Species. Criteria for determining that an AI virus isolation causing an outbreak must be considered for eradication In: *Proceedings of the 98th Annual Meeting US Animal Health Association* (p. 522). Grand Rapids: US Animal Health Association.

Villareal, C.L. & Flores, A.O. (1998). The Mexican avian influenza (H5N2) outbreak, in: D. E. Swayne & R. D. Slemons (Eds), *Proceedings of the 4th International Symposium on Avian Influenza* (4th edn, pp. 18–22). Richmond, VA: US Animal Health Association.

Webster, R.G., Kawaoka, Y., Taylor, J., Weinberg, R. & Paoletti, E. (1991). Efficacy of nucleoprotein and haemagglutinin antigens expressed in fowlpox virus as vaccine for influenza in chickens. *Vaccine, 9,* 303–308.

Webster, R.G., Taylor, J., Pearson, J., Rivera, E. & Paoletti, E. (1996). Immunity to Mexican H5N2 avian influenza viruses induced by a fowlpox-H5 recombinant. *Avian Diseases, 40,* 461–465.

Wood, J.M., Kawaoka, Y., Newberry, L.A., Bordwell, E. & Webster,