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Original Article

Development of blocking ELISA for detection of antibodies against avian influenza virus of the H7 subtype

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Background and Purpose: The conventional method used for subtyping of antibodies against avian influenza viruses is hemagglutination inhibition (HI) test. However, the HI test is laborious and requires preparation of antigen from viable viruses that might be hazardous. The aim of this study was to develop a blocking enzyme-linked immunosorbent assay (B-ELISA) for detection of antibody of avian influenza of the H7 subtype. The B-ELISA is fast and avoids the need to culture whole viruses.

Methods: The B-ELISA was based on the reaction between a monoclonal antibody and a recombinant hemagglutinin protein purified from *Escherichia coli*. The specificity of the B-ELISA was determined by testing H7-negative field sera and the sensitivity of the B-ELISA was determined by testing sera collected from experimentally immunized chickens.

Results: The specificity of the B-ELISA was found to be 97.7% when compared with the HI test. The sensitivity was found to vary with the HI titer of sera. A sensitivity of 100% was achieved when test sera had HI titers $\geq 2^7$. The sensitivity dropped to 33% and 20% when test sera had HI titers of 2^6 and 2^5, respectively. Nearly all test sera with HI titers $\leq 2^4$ were scored negative by the B-ELISA.

Conclusion: The B-ELISA might serve as a useful tool for detection of H7-specific antibodies, with the added advantage that the recombinant hemagglutinin antigen could be produced in *E. coli* in large quantities, without handling the whole virus.

Key words: Antibodies, viral; Enzyme-linked immunosorbent assay; Influenza in birds; Sensitivity and specificity

Introduction

Avian influenza (AI) is a highly contagious disease caused by type A influenza virus, a member of the family *Orthomyxoviridae* [1]. Avian influenza virus (AIV) has many subtypes that derive from 2 surface glycoproteins — hemagglutinin (HA) and neuraminidase (NA) [2]. Sixteen HA (H1-H16) and 9 NA subtypes (N1-N9) have been identified. All sixteen HA subtypes of influenza virus are found in aquatic birds, which serve as the primordial reservoir of all influenza A viruses [3]. Among HA subtypes, only H5 and H7 are highly virulent in poultry [4]. In humans, although viruses of only H1, H2 and H3 subtypes have caused pandemics, growing evidence has shown that viruses of other subtypes could infect humans [5]. For example, the H5N1 virus that caused the outbreak of influenza in Hong Kong in 1997 could transmit from poultry to humans, and has the potential to cause high mortality in both hosts [6]. Outbreaks of the H5N1 virus between 2003 and 2007 in many Asian, European, and African countries have caused the depopulation of hundreds of millions of birds and 318 cases of human infection (60.4% of them lethal) [7]. In addition, the outbreak of H7N1 virus in Italy in 1999-2000 caused the depopulation of over 13 million birds and a significant economic loss [8,9]. Moreover, an outbreak involving another H7 virus (H7N7) in the Netherlands in 2003 also led to a dramatic economic loss; this H7N7 virus might be able to transmit from

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person to person [10,11]. These reports show that the H5 and H7 viruses pose a threat to human health and the poultry industry worldwide, and highlight the importance of adequate surveillance and outbreak preparedness for these viruses.

Conventional methods used for detection of AIV antibodies are agar gel immunodiffusion, hemagglutination inhibition (HI) test, and enzyme-linked immunosorbent assay (ELISA) [2]. Among these 3 methods, only the HI test is subtype specific. However, it is also laborious and requires preparation of antigen from viable viruses, which might be hazardous when the viruses are of the H5 or H7 subtype. Moreover, the HI test might be interfered with by steric hindrance from NA antibodies, binding to the NA protein of the virus, leading to nonspecific inhibition and misidentification of an isolate [12]. An ELISA for detection of H7 antibodies in avian sera reported by Sala et al in 2003 [13] had 99% concordance of results with the HI test. However, a whole virus was used as the antigen in this ELISA and some false-positive results were observed [13].

The aim of this study was to develop a blocking ELISA (B-ELISA) for detection of antibodies for AI of the H7 subtype. The B-ELISA employed a recombinant HA antigen purified from *Escherichia coli*, thus circumventing the need for culturing of whole viruses. Moreover, exclusive use of the HA-derived antigen in the B-ELISA eliminated the possibility of nonspecific reaction caused by NA antibodies.

Methods

Viruses and reference sera

Reference strains of influenza viruses used in this study were: A/Duck/Yilan/106/86 (H1N1), A/Shorebirds/Taiwan/35/98 (H2N3), A/Duck/Ukraine/1/63 (H3N8), A/Duck/Czechoslovakia/56 (H4N6), A/Duck/Hong Kong/820/80 (H5N3), A/Chicken/Taiwan/na3/98 (H6N1), A/Hong Kong/301/78 (H7N1), A/Turkey/Ontario/6118/68 (H8N4), A/Turkey/Wisconsin/1/66 (H9N2), A/Chick/Germany/N/49 (H10N7), A/Duck/England/56 (H11N6), A/Duck/Alberta/60/76 (H12N5), A/Gull/Maryland/704/77 (H13N6), A/Mallard/Gurjev/263/82 (H14N5), A/Shearwater/West Australia/2576/79 (H15N9).

Among these, strains of H1, H2 and H6 were Taiwanese isolates, the subtypes of which were confirmed in AI reference center at the Central Veterinary Laboratory (Weybridge), Addlestone, Surrey, UK. Other strains were obtained from Dr H Kida, School of Veterinary Medicine, Hokkaido University, Sapporo, Japan, or from Dr RG Webster, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. The H1-H15 monospecific reference sera were prepared by immunizing 4-week-old chickens with formalin-inactivated H1-H15 viruses. The HI test was performed in microtiter plates, as described previously [14].

PCR amplification and cloning of HA genes of H7 and H5 subtypes

Two AIV strains — A/Hong Kong/301/78 (H7N1) and A/Duck/Hong Kong/820/80 (H5N3) — were used as the source for isolation of viral RNA. Procedures used for RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) amplification were as described previously [15]. Two sets of primers were used to amplify the HA genes of the H7 and H5 subtypes. The names and sequences of these primers were H7 (+): 5'-TTAAGGATCCCCAC-CCCAATGTGACCAATTC-3'; H7 (–): 5'-TTAACTC-GAGTGTCCCTCCACTATGATAGCA-3'; H5 (+): 5'-TTAAGGATCCCCAGGAGACTTCACCGATT-3'; and H5 (–): 5'-TTAACTCGAGTCCACGGGGGTCT-GACATTT-3'

These primers contained restriction enzyme (*Bam*HI or *Xho*I) cutting sites at their 5'-end (underlined sequences), followed by sequences specific to HA genes of AIV. The H7 primers amplify a 648-bp fragment encoding a truncated molecule (amino acids 81-296) of the HA protein and the H5 primers amplify a 573-bp fragment encoding a truncated molecule (amino acids 105-295) of the HA protein. Sequence analysis of the 648-bp fragment amplified from the H7 virus showed that this fragment (accession number AY672090) encoded a protein showing 97.7% to 99.1% sequence identity to the HA protein of the H7N1 and H7N7 viruses isolated in Italy and the Netherlands [8,10].

The RT-PCR product was cut by *Bam*HI plus *Xho*I, purified by Geneclean III Kit (Bio 101, Vista, CA, USA), and then inserted into the expression vector pET32a (Novagen, Madison, WI, USA) as described in the user's manual (Novagen). The recombinant protein produced by the pET32a vector is a fusion protein containing a tag at its amino terminus.

Expression and purification of recombinant HA proteins

The recombinant HA proteins were expressed in *E. coli* strain BL21 (DE3) and purified by nickel

chromatography (Novagen), as described previously [16]. The concentration of purified protein was determined by Protein Assay Kit (Bio-rad, Hercules, CA, USA).

Development of monoclonal antibody

The purified recombinant HA protein of the H7 subtype was submitted to LTK Biotech Inc. (Taipei, Taiwan) for monoclonal antibody production, on a contract basis. The antibody-secreting hybridomas were screened by ELISA and Western blot for those recognizing the HA protein of the H7 subtype but not that of the H5 subtype. The monoclonal antibodies obtained were further screened by indirect immunofluorescence assay with chicken embryo fibroblast cells infected with H7 or H5 virus; monoclonal antibodies that recognized the H7 virus-infected cells but not the H5 virus-infected cells were saved for development of the B-ELISA.

Western blot

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the nitrocellulose membrane. The membrane was blocked by 3% skimmed milk and probed with reference chicken antisera or monoclonal antibodies. The immune complex was detected by alkaline phosphatase-labeled anti-chicken or anti-mouse immunoglobulin G (IgG) [Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA].

Development of the B-ELISA

ELISA plates (High binding; Costar, Cambridge, MA, USA) were coated overnight at 4°C with 100 μL of coating buffer (Kirkegaard and Perry Laboratories) containing the purified recombinant HA protein (250 ng per well). Plates were then blocked with 300 μL of blocking solution (Kirkegaard and Perry Laboratories) at room temperature for 2 h. The blocking solution was poured off and 100 μL of inactivated test sera (diluted 1:2 in blocking solution supplemented with 0.5 μg/mL of the purified tag protein) was placed in wells at room temperature for 1 h. After washing 5 times with wash solution (Kirkegaard and Perry Laboratories), 100 μL of the monoclonal antibody (tissue culture hybridoma fluids, diluted 1:256 in blocking solution) was placed in each well at room temperature for 1 h. After washing, 100 μL of peroxidase-labeled goat anti-mouse IgG conjugate (1 μg/mL; Kirkegaard and Perry Laboratories) was placed in each well at room temperature for 1 h. After washing, 100 μL of substrate-chromogen mixture (0.1 mg tetramethylbenzidine/mL, 0.1 M sodium acetate and 0.05% hydrogen peroxide) was added into each well, followed by incubation at room temperature for 10 min. The stop solution (50 μL of 2 N hydrochloric acid) was added and absorbance values at 450 nm were measured by an ELISA reader (Dynex Technologies Limited, West Sussex, UK). The percentage of blocking was calculated according to the following formula: % inhibition = 100% × (optical density [OD] of sample – OD of positive control)/(OD of negative control – OD of positive control). The positive control serum was collected from specific pathogen-free (SPF) chickens vaccinated with formalin-inactivated H7 viruses and the HI titer of this serum was 2^{10}. The negative serum was collected from unvaccinated SPF chickens and the HI titer of this serum was $<2^{1}$.

Preparation of field sera

Sera were collected from 18 flocks, designated as F1-F18; each flock was from a different farm. F1-F11 were chicken flocks, F12-F17 were duck flocks and F18 was a goose flock. The ages of birds of these flocks ranged from 21 days to 23 weeks. For flock F1, blood was collected from 5 randomly selected birds and for flocks F2-F18, blood was collected from 10 randomly selected birds.

Preparation of experimental sera

Because no H7-positive field serum was available in this country, the sensitivity of the B-ELISA was evaluated by testing sera from experimentally immunized chickens. Ten SPF chickens were randomly assigned into 2 groups that were housed separately in different Horsfall-Bauer isolators with HEPA-filtered intake and exhaust air. At the age of 6 weeks, chickens of the first group were immunized with the inactivated H7 virus, and those of the second group were immunized with the inactivated H5 virus. Serum was collected from all chickens weekly up to 8 weeks post-immunization, stored at –20°C, and then subjected to the B-ELISA and HI tests.

Results

Production and purification of recombinant HA proteins

The HA proteins of H7 and H5 subtypes were expressed as recombinant proteins in *E. coli*. The recombinant HA protein of the H7 subtype was used for development of the monoclonal antibody and the

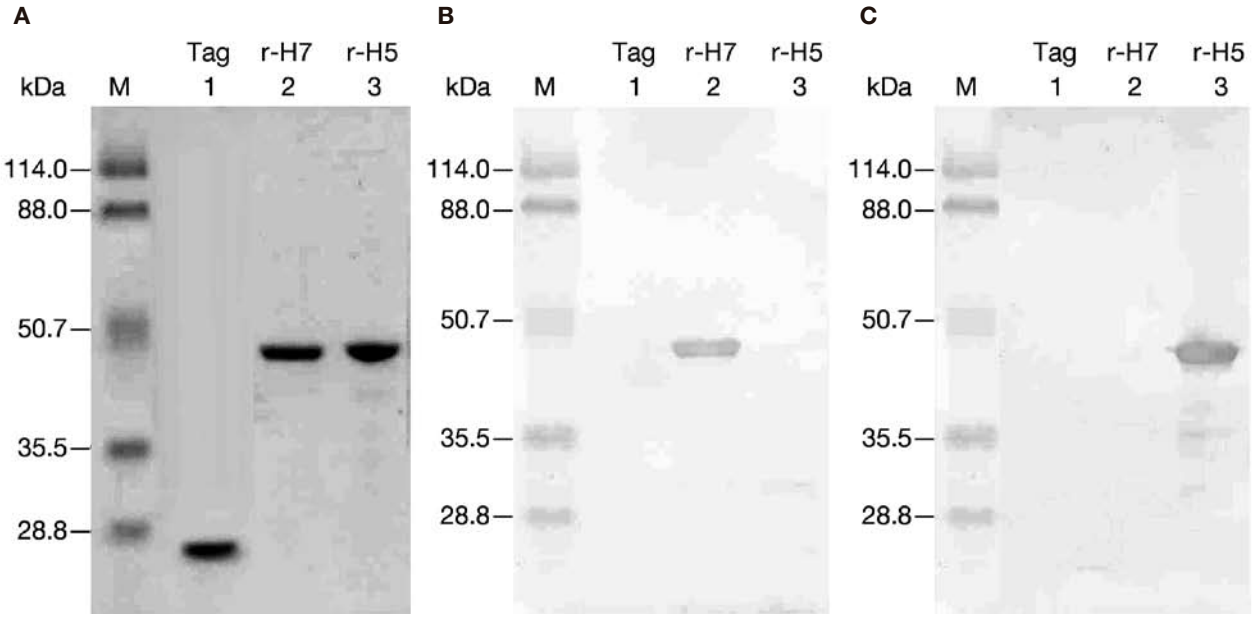

Fig. 1. (A) Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified tag protein (lane 1), recombinant H7 protein (r-H7) [lane 2] and recombinant H5 protein (r-H5) [lane 3]; (B) and (C) Immunoblot of duplicated gels probed with H7- and H5-specific reference sera, respectively. Lane M, molecular weight marker.

B-ELISA, whereas the recombinant protein of the H5 subtype was used as the negative control. Only the regions corresponding to amino acid residues 81-296 (for the H7 subtype) and residues 105-295 (for the H5 subtype) of the HA protein were expressed, because these regions were located at the HA1 subunit of the HA protein and contained the receptor binding and antigenic determinant sites [17]. The recombinant HA proteins were expressed as fusion proteins with a tag fused to their amino termini. The molecular weight of the tag protein alone was 20.8 kDa and those of the recombinant H7 protein (r-H7) and the recombinant H5 protein (r-H5) were 43.5 kDa and 42.6 kDa, respectively. Fig. 1A shows that the tag protein alone, as well as r-H7 and r-H5, could be expressed and purified from *E. coli* (Fig. 1A, lanes 1-3). Western blot analysis showed that the H7-specific reference serum could recognize r-H7 but not the tag protein or r-H5 (Fig. 1B, lanes 1-3). In contrast, H5-specific reference serum could recognize r-H5 but not the tag protein or r-H7 (Fig. 1C, lanes 1-3). These results indicate that both r-H7 and r-H5 retained their specific antigenicity.

Development of monoclonal antibody

Monoclonal antibodies directed against r-H7 were obtained from BALB/c mice immunized with purified r-H7. One of the resulting monoclonal antibodies, designated as MAb705, was selected for further characterization. Western blot analysis showed that MAb705 recognized r-H7 but not the tag protein or r-H5 (Fig. 2, lanes 1-3). Moreover, MAb705 recognized a 45-kDa protein present in the allantoic fluid containing the H7 virus but not in

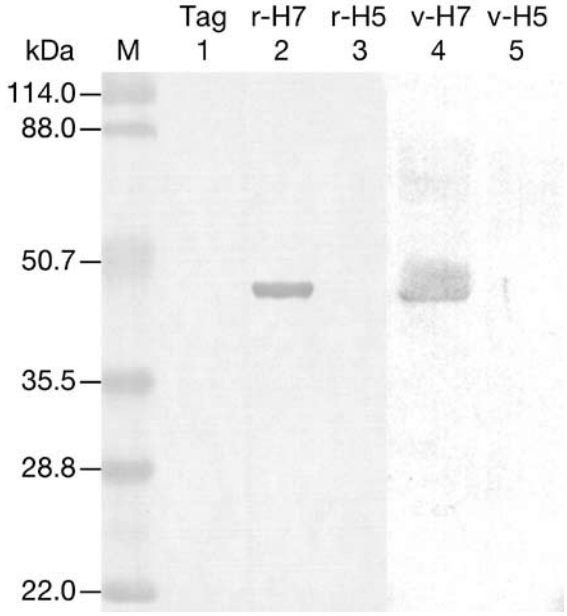

Fig. 2. Immunoblot of purified recombinant proteins and virus-containing allantoic fluid probed with the monoclonal antibody MAb705. Lane M, molecular weight marker; lane 1, purified tag protein; lane 2, recombinant H7 protein (r-H7); lane 3, recombinant H5 protein (r-H5); lane 4, allantoic fluid containing H7 virus; lane 5, allantoic fluid containing H5 virus.

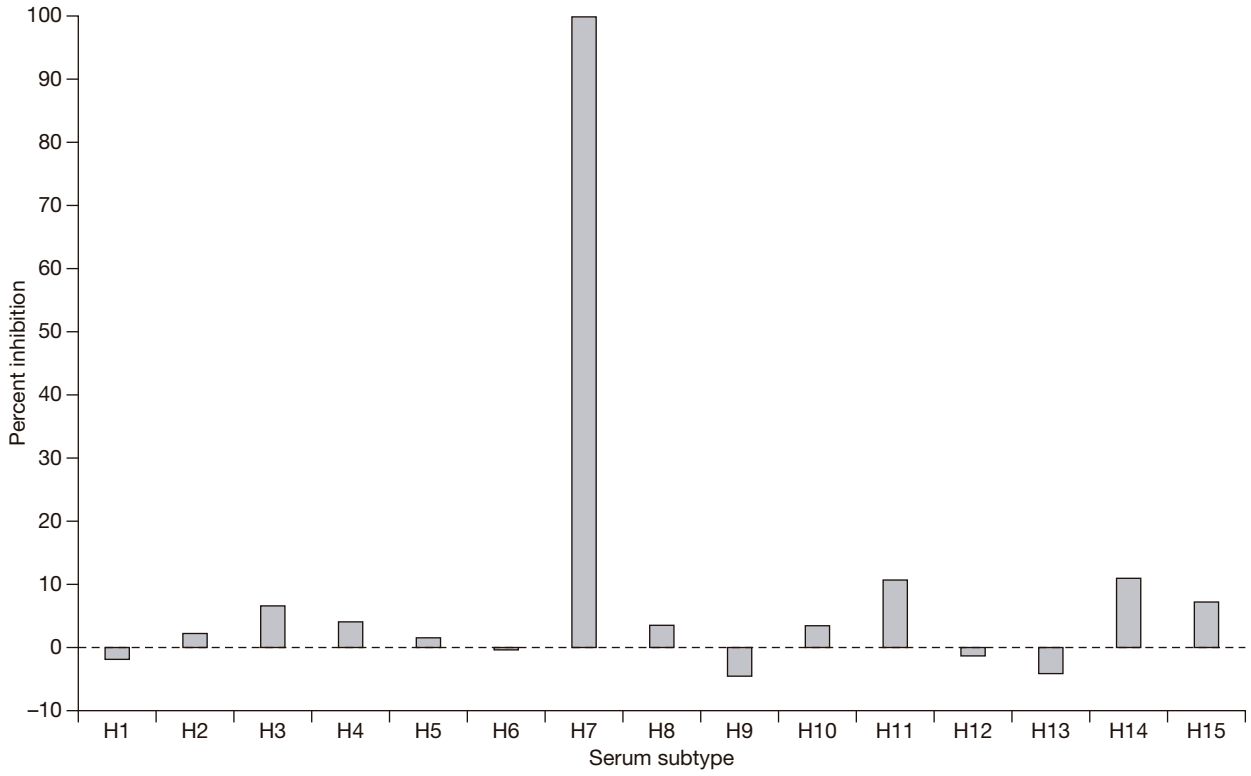

Fig. 3. Blocking enzyme-linked immunosorbent assay results with H1-H15 reference sera prepared from chickens. The subtype of the reference serum is indicated at the bottom of each bar.

that containing the H5 virus (Fig. 2, lanes 4 and 5). The size of the 45-kDa protein was consistent with that of the HA1 subunit of the HA protein [1], and this result was conceivable because MAb705 was directed against r-H7, which contained a portion of the HA1 subunit of the HA protein. Indirect immunofluorescence assay showed that MAb705 reacted with chicken embryonic fibroblast cells infected by the H7 virus, but did not react with cells infected by the H5 virus (data not shown). These results indicate that MAb705 was specific for the H7 virus; this monoclonal antibody was used for development of the B-ELISA.

Development of the B-ELISA

Optimal dilutions of the coating antigen, test sera, monoclonal antibody, and peroxidase-labeled goat anti-mouse IgG conjugate were determined by checkerboard titration. The B-ELISA developed was then tested against a panel of H1-H15 monospecific reference sera prepared from chickens. The results showed that when the H7-specific reference serum gave 100% inhibition, reference sera of other subtypes gave only 0 to 12% inhibition (Fig. 3). Thus, the B-ELISA could discriminate the H7-specific reference serum from reference sera of other subtypes.

B-ELISA results with field sera

The cut-off value and specificity of the B-ELISA were determined by testing H7-negative field sera. A total of 175 field sera collected from 18 flocks were tested. All of these sera were found to be negative for the H7-specific antibody by the HI test (HI titer, $\leq 2^2$). With the B-ELISA, these field sera gave –12% to 32% of inhibition (mean ± standard error, 5.8 ± 7.1%) [Fig. 4]. A cut-off value (20% inhibition) was established by the mean plus 2 standard errors. By using this cut-off value, 171 of the 175 field sera were scored negative by the B-ELISA (Fig. 4). Because all of these were scored negative by the HI test, the specificity of the B-ELISA was considered to be 97.7% (171/175) when compared with the HI test.

B-ELISA results with experimental sera

To evaluate the detection limit of the B-ELISA, the H7-specific reference serum (HI titer, 2^{10}) was serially diluted 2-fold and tested against the B-ELISA. By using 20% inhibition as the cut-off value, the serum with HI titer $\geq 2^5$ was scored positive by the B-ELISA (Fig. 5). This result suggested that the detection limit of the B-ELISA might correspond to the HI titer of about 2^5.

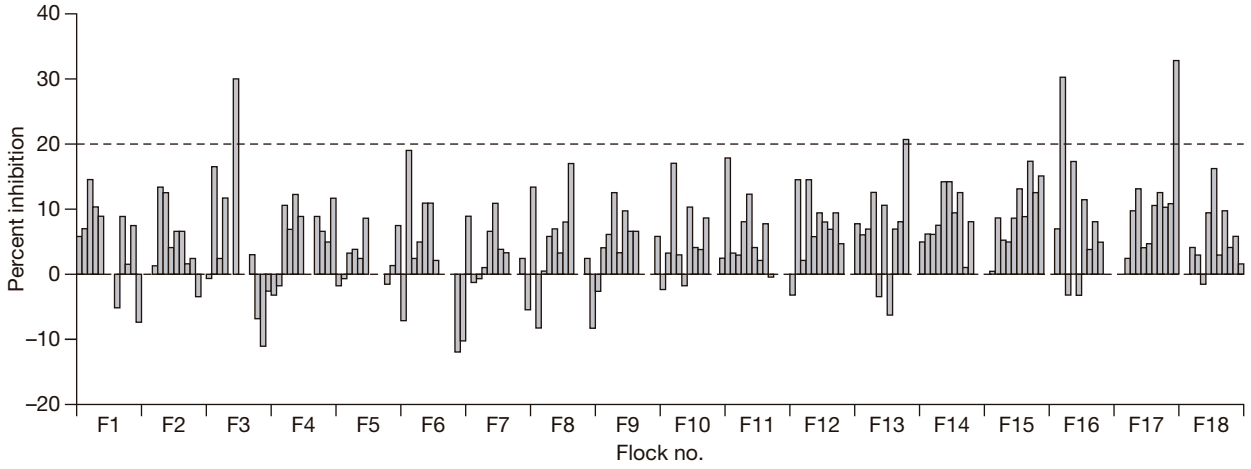

Fig. 4. Blocking enzyme-linked immunosorbent assay results with H7-negative sera collected from the field (18 flocks). Each bar is the result for a single bird. The dotted line represents the cut-off value of the mean plus 2 standard errors of the percent inhibition.

The result of the B-ELISA showed that for chickens in the first group (H7-immunized, Fig. 6A), no serum was scored positive at 1 and 2 weeks post-immunization, 2 of the 5 sera were scored positive at 3 and 4 weeks post-immunization, 4 of the 5 sera were scored positive at 5, 6 and 7 weeks post-immunization, and all sera were scored positive at 8 weeks post-immunization (Fig. 6A). When the result of the HI test was compared with that of the B-ELISA, 100% of sera (17/17) with HI titers $\geq 2^7$ were scored positive by the B-ELISA, whereas 33% of sera (2/6) with HI titer of 2^6 and 20% of sera (1/5) with HI titer of 2^5 were scored positive by the B-ELISA (Fig. 6A). All sera with HI titers $\leq 2^4$ were scored negative by the B-ELISA; the only exception was a serum collected at 3 weeks, which had an HI titer of 2^0 and was scored positive by the B-ELISA (Fig. 6A).

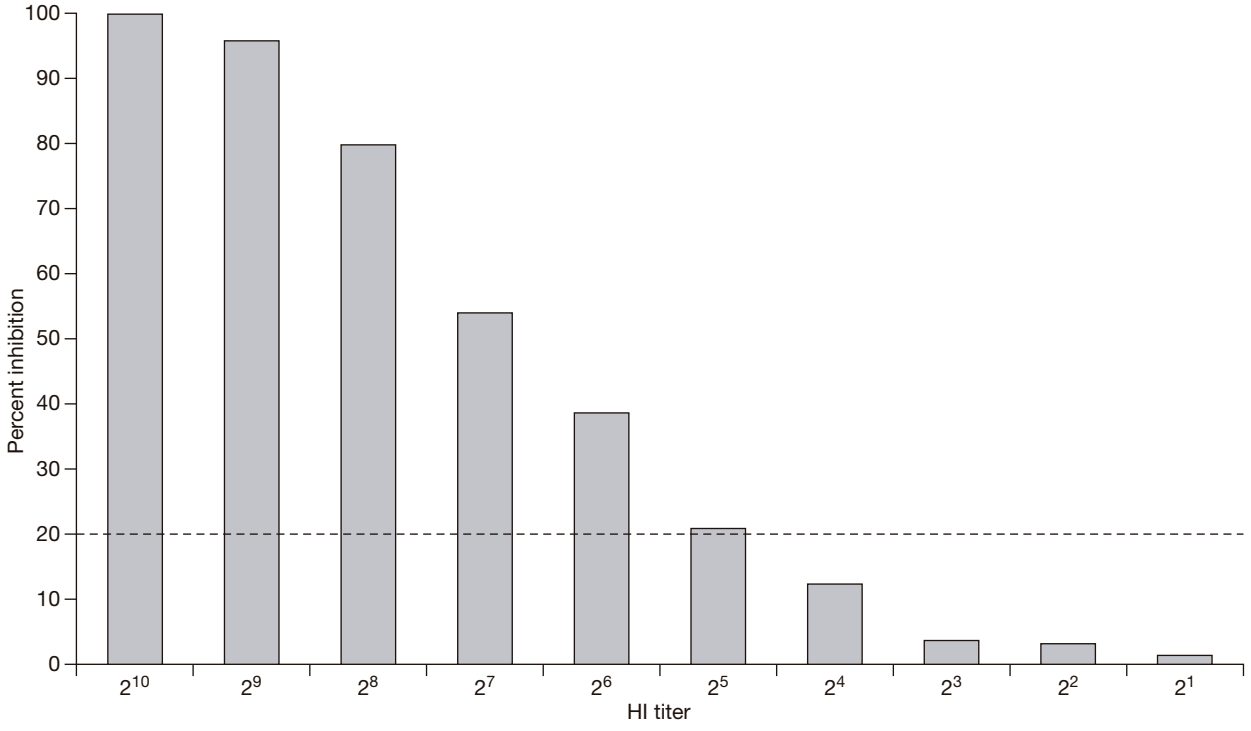

Fig. 5. Blocking enzyme-linked immunosorbent assay results with serially diluted H7-specific reference serum. The hemagglutination inhibition (HI) titer of diluted serum is shown at the bottom of each bar. Each bar is the mean of 3 experiments and the dotted line represents the cut-off value (20%).

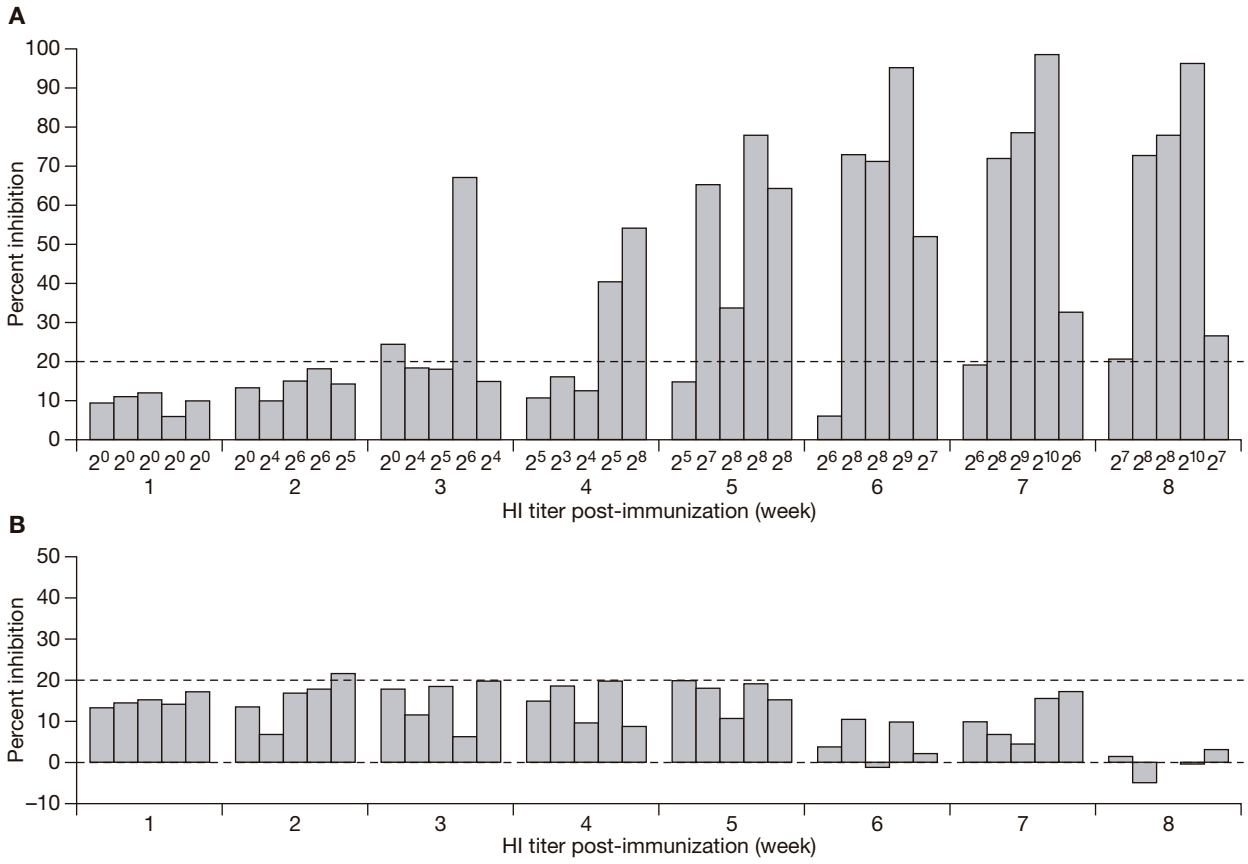

Fig. 6. Blocking enzyme-linked immunosorbent assay (B-ELISA) results with sera collected from chickens experimentally immunized with (A) inactivated H7 virus; and (B) inactivated H5 virus. Each bar is the B-ELISA result for serum collected from a single chicken. The hemagglutination inhibition (HI) titer and the time post-immunization are shown at the bottom of each bar. The dotted line represents the cut-off value (20%).

In contrast to sera collected from chickens immunized by the H7 virus, sera collected from chickens immunized by the H5 virus were found to be negative for the H7-specific antibodies, but were positive for the H5-specific antibodies by the HI test (data not shown). All sera collected from H5-immunized chickens were scored negative by the B-ELISA, except for a serum collected at 2 weeks post-immunization (Fig. 6B). This result indicated that the presence of H5-specific antibody in the serum did not affect the specificity of the B-ELISA in the detection of H7-specific antibodies.

Discussion

A B-ELISA developed for detection of H7-specific antibodies showed specificity of 97.7% when tested against H7-negative field sera. Moreover, the sensitivity of the B-ELISA was found to vary with HI titer of the test serum. All sera with HI titers $\geq 2^7$ were scored positive by the B-ELISA, whereas 33% and 20% of sera with HI titers of 2^6 and 2^5, respectively, were scored positive. Moreover, nearly all sera with HI titers $\leq 2^4$ were scored negative by the B-ELISA. Because HI titers $\geq 2^4$ were considered to be positive for the HI test, the B-ELISA appeared to be less sensitive than the HI test. However, it should be noted that the HI titer observed in this study was determined by use of virus antigen (the H7N1 virus) homologous to the virus used for immunizing chickens. It was, therefore, possible that the HI titer might have been overestimated because the N1 antibody present in the sera might increase the HI titer by steric hindrance. In this regard, the true sensitivity of the B-ELISA might be higher than observed.

The B-ELISA has several advantages over the HI test. Firstly, the HA antigen used in the B-ELISA was produced in *E. coli*, thus avoiding the need for culturing H7 virus. Secondly, only the HA protein, and not the whole virus, was used in the B-ELISA, thus eliminating the possibility of false-positive results

caused by NA antibodies. Thirdly, only a single dilution was needed, and therefore the B-ELISA might be automated for large-scale screening of avian sera. Considering these advantages, we believe that the B-ELISA might serve as a prototype ELISA, further modification of which has the potential to improve sensitivity and specificity.

The antigen used in the B-ELISA was a recombinant protein (r-H7) derived from the virus strain A/Hong Kong/301/78 (H7N1). Sequence analysis of the HA gene of this strain (accession number AY672090) showed that its HA protein exhibited 97.7% to 99.1% sequence identity to the HA proteins of the H7N1 virus found in Italy and the H7N7 virus found in the Netherlands [8,10]. This high degree of sequence homology suggests that antibodies produced in response to the HA protein of these H7N1 and H7N7 viruses might also bind to r-H7 and be scored positive by the B-ELISA. In this regard, the B-ELISA might be useful in detection of antibodies against these H7N1 and H7N7 viruses.

Acknowledgments

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