Comparison of Commercial Enzyme-Linked Immunosorbent Assay Kits with Agar Gel Precipitation and Hemagglutination-Inhibition Tests for Detecting Antibodies to Avian Influenza Viruses

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ABSTRACT. We evaluated the utility of 5 commercial enzyme-linked immunosorbent assay (ELISA) kits for detecting antibodies to avian influenza viruses. The sensitivities and specificities of the ELISA kits were compared with those of the agar gel precipitation (AGP) and hemagglutination-inhibition (HI) tests. The results suggest that some ELISA kits might not be suitable for monitoring during the early stages of avian influenza virus infections. Therefore, ELISA kits should only be used in conjunction with a profound knowledge about monitoring of avian influenza.

KEY WORDS: AGP, Avian influenza, ELISA, HI.


During the 2003–2004 winter season in the northern hemisphere, highly pathogenic (HP) avian influenza (AI), caused by the H5N1 virus subtype, caused significant damage to the poultry industry in many Asian countries, including Japan [1]. Since then, H5N1 HPAI has emerged intermittently throughout Asia, and the threat is expected to continue unless eradication of the H5N1 HPAI virus from poultry farms can be achieved [3, 9, 10, 12, 13]. Wild aquatic birds may play a critical role in transferring the H5N1 virus to different areas along their migration routes [3]. Therefore, a program of ongoing poultry monitoring and the development of rapid, effective diagnostic methods are essential to minimize the damage caused by an AI infection.

Monitoring for the presence of AI at poultry farms is carried out to rapidly determine the presence of an infection and prevent it from spreading. Serological tests have an advantage for long-term detection of infection and are especially useful for low pathogenic (LP) AI virus infections. In contrast to HPAI, LPAI occurs occasionally on poultry farms worldwide, with no or mild clinical signs in terrestrial birds [1], and can be detected by serological monitoring. In Japan, the agar gel precipitation (AGP) test is used for serological monitoring of AI at designated poultry farms in combination with a virus isolation test using embryonated chicken eggs. Hemagglutination-inhibition (HI) tests can also be used for serological monitoring to detect the antibodies after virus isolation. However, considering the need for an enhanced serological monitoring capability, a more rapid and sensitive serological test is required, because it takes 48 hr to process the results of the AGP test [2, 6]. Enzyme-linked immunosorbent assays (ELISA) for detecting antibodies against avian influenza A virus are reported to be both sensitive and rapid, and several commercial kits are available. However, the utility of these kits has not been evaluated adequately [4]. In this study, we evaluated the utility of 5 commercial ELISA kits and compared their performance to that of the AGP and HI tests.

A total of 22, 16-week-old, specific-pathogen-free (SPF), white Leghorn chickens (Line M) were purchased from Nis-seiken Co., Ltd. (Tokyo, Japan). During the study period, all chickens were individually housed in metal cages and provided with mixed feed and tap water. The study was approved by the Animal Care and Use Committee of the Research Institute for Animal Science in Biochemistry & Toxicology.

In Group 1, 9 chickens, assigned randomly, were inoculated in the leg muscle with 0.5 ml of an H5N1 AI inactivated oil-adjuvant trial vaccine prepared from A/duck/Hokkaido/Vac-1/04 (H5N1). In Group 2, 8 chickens were similarly inoculated with the same amount of an H7N7 AI inactivated oil-adjuvant trial vaccine prepared from A/duck/Hokkaido/Vac-2/04 (H7N7). Group 3 was the non-treated control group and consisted of 5 non-manipulated chickens. Blood was sampled from all of the chickens on postvaccination days 0, 7, 14, 21 and 28. Another set of sera was obtained from 5, 4-week-old SPF chickens inoculated with 10^6.0 50% embryo infectious dose of A/chicken/Ibaraki/1/2005
Five commercial ELISA kits (designated A–E) were evaluated for their ability to detect antibodies to the AI viral nucleoprotein (NP), which was coated onto all of the ELISA test kit plates. The tests were performed, and the results calculated according to the manufacturer’s instructions. The AGP test was conducted according to the procedure in the OIE (World Organisation for Animal Health) manual [2, 6]. The AGP antigens were prepared as follows: type A influenza (H3 subtype) viruses were inoculated into 10-day-old chicken embryos, and then the chorioallantoic membranes were collected and emulsified in phosphate buffered saline (PBS). After 3 freeze-thaw cycles, the emulsion was centrifuged at 1,000 × g, and the supernatant was collected. To inactivate the influenza virus, formalin was added to the supernatant to a final concentration of 0.1%.

The HI test was also performed according to the procedure in the OIE manual [6, 8]; 1:20 dilutions of sera were tested. A panel of 37 reference antisera were prepared by repeated inoculation into 4-week-old SPF chickens with each HA subtype (H1 to H16) of purified, inactivated AI virus antigens in Freund’s complete adjuvant.

The sensitivity of each ELISA kit was determined by its ability to detect the NP antibody in sera from chickens vaccinated with the inactivated oil vaccines of H5N1 and H7N7 subtypes. The presence of anti-NP antibodies in the sera was detected by all of the ELISA kits. The antibody positive rate (%) at each sampling point, by each method, is shown in Fig. 1a for the H5N1 subtype vaccine and in Fig. 1b for the H7N7 subtype vaccine. Each ELISA kit demonstrated higher positive rates than did the AGP test for both inactivated vaccines (H5N1, H7N7); more than half of the sera were ELISA positive by 14–28 days post inoculation (dpi). The most sensitive ELISA kit was kit A, with more than 80% of the sera testing positive by 14 dpi for both the H5N1 and H7N7 subtype vaccines. Approximately 30% and 60% of sera collected 14 and 28 dpi, respectively, were positive by AGP. Kinetic curves of the HI antibody positive rates varied between the 2 subtype vaccines. At 14 dpi, none of the serum samples were positive for the H5N1 subtype vaccine, although 80% were positive for the H7N7 subtype vaccine. However, by 21 and 28 dpi, most samples were HI positive for both subtype vaccines, and the HI positive rates were consistently higher than those for the AGP test.

Next, the sensitivity of the ELISA kits using sera obtained from chickens infected with live H5N2 virus was compared. ELISA kits A, B and C were used in this experiment. Kits D and E were not evaluated, because, in this study, these kits generally showed the lowest sensitivity of the 5 kits for both inactivated vaccinations (H5N1, H7N7). As shown in Fig. 1c, ELISA kit A could detect antibodies soon after infection, and all of the samples tested positive from 7–42 dpi, whereas the NP antibody positive rates for ELISA kits B and C were 40% and 60% from 21–42 dpi, respectively.

The AGP test detected antibodies in 40% of the samples by 7 dpi, rising to 100% by 14 dpi; the positive rate gradually
The relative specificity of each ELISA kit was determined using all of the sera collected at every sampling point in each inactivated vaccination (H5N1, H7N7) and H5N2 live virus inoculation according to the following formula: the number of samples positive in both the ELISA kit and the AGP (HI) test divided by the number of samples positive in the AGP (HI) tests × 100. As shown in Table 1, the relative sensitivity of the 5 ELISA kits was high (86 to 100%) when compared with the AGP test using sera from chickens inoculated with the inactivated vaccines. However, when sera from chickens infected with the H5N2 virus were used, the relative sensitivities of kits B and C were considerably lower (33% and 67%, respectively). When the ELISA kits were compared with the HI test using sera from chickens inoculated with inactivated vaccines, the relative sensitivities of kits B, D and E were low (58 to 89%). The relative sensitivities of kits B and C were especially low (23% and 53%, respectively) when tested using sera from the birds infected with the live H5N2 virus. On the other hand, the relative sensitivity of kit A was 100% for all 3 serum groups.

The relative specificity of each ELISA kit was also determined, using the test results described above, by the following formula: the number of samples negative in both the ELISA kit and AGP (HI) test divided by the number of samples negative in the AGP (HI) tests × 100 (Table 1). When compared against the AGP test, the relative specificities of kit A were 61%, 56% and 36% for the 3 serum groups (H5N1, H7N7 and H5N2 groups), respectively, while the specificities of kits B, C, D and E were higher than those of kit A (67–100%) for every serum group for which they were tested.

The non-specific reactivities of ELISA kits A, B and C were also determined using 204 serum samples obtained from 350–450-day-old AI-free, conventional, adult layer chickens. Kits D and E were not evaluated, because, in this study, these kits generally showed the lowest sensitivity of the 5 kits for both inactivated vaccinations (H5N1, H7N7). The non-specific reaction rates of ELISA kits A, B and C were 1.5%, 0.5% and 0%, respectively. Also, hemolyzed sera (63 samples) and muddy sera (56 samples) were not observed to cause nonspecific reactions in kits A, B and C. All of the reference antisera were positive by the 5 ELISA kits (data not shown).

Generally, ELISA kits can be used as screening tools, because of their rapidity, sensitivity and handling simplicity. Several AI antibody detection ELISA kits have been reported to show high relative sensitivities compared with both AGP and HI tests when using sera from chickens at 19 or 21 dpi with AI virus and when using reference sera obtained by administration of inactivated antigen [4]. However, this study showed that kits B and C showed lower performance than the AGP and HI tests when the sera used were serially collected from infected chickens (Fig. 1c). Kits D and E may also be lower performing than the AGP and HI tests, because these kits had sensitivities similar to kits B and C (Fig. 1a and 1b).

Discrepancies in the reported performance of AI antibody detection ELISA kits between this study and previous ones may be related to the manner in which the AGP and HI tests were executed. The AGP test used in this study is more sensitive than a conventional AGP test, because positive reference antiserum was placed next to the sample well. For the HI test in this study, the same virus strains were used for both immunization of the chickens and preparation of the antigens for the HI test. In a previous study, homologous and heterologous HA subtypes of the virus strains were used [4]. In this study, 3 sets of antisera were tested; 2 were serially collected from chickens inoculated with inactivated H5N1 or H7N7 antigens (Fig. 1a and 1b), and 1 was serially collected from chickens experimentally infected with live H5N2 virus (Fig. 1c). The relative sensitivities of ELISA kits B and C compared with the AGP test were higher at most test points when the sera from birds immunized with the inactivated vaccines were tested (Fig. 1a and 1b). However, the sensitivities were lower when the sera from chickens infected with live virus were tested (Fig. 1c). This study indicates that these ELISA

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- a: The number of samples positive in both the ELISA kit and AGP test / the number of samples positive in AGP test × 100.
- b: The number of samples negative in both the ELISA kit and AGP test / the number of samples negative in AGP test × 100.
- c: The number of samples positive in both the ELISA kit and HI test / the number of samples positive in HI test × 100.
- d: The number of samples negative in both the ELISA kit and HI test / the number of samples negative in HI test × 100.

NT: Not tested.
kits, except for kit A, cannot always detect AI antibodies. Therefore, unexpectedly, these kits (B, C, D and E) may not be suitable for monitoring for AI infection, when compared with the AGP test. On the other hand, ELISA kits have some advantages over the AGP test. For example, ELISA kits can usually detect AI virus antibodies for a longer period after infection than the AGP test [11]. This study also showed that the ability of the AGP method to detect antibodies declined after 14 dpi, whereas those of the ELISA tests did not (Fig. 1c), indicating that ELISA kit A could be expected to detect AI antibody over a longer period than the AGP test.

In this study, each of the sensitivities for the ELISA kits, AGP test and HI test was different between H5N1 vaccination, H7N7 vaccination and H5N2 virus inoculation. These differences may arise from the structural diversity of the NP protein between these virus strains; however, the truth is unknown. We used only 1 H5N2 subtype virus to evaluate the performance of ELISA kits for serum obtained from AI-infectious chickens, and so those results should be considered as a case. Therefore, we have to determine whether or not those results are specific for this strain or can be applied to all of the AI viruses by gathering more data about various subtypes of AI virus. Considering the different capabilities of each ELISA kit to detect the AI antibodies, it is necessary to choose an ELISA kit that is suited for the desired purpose.

REFERENCES