The reverse zoonotic transmission of the pandemic H1N1 2009 influenza virus to swine necessitates enhanced surveillance of swine for influenza virus infection. Using a well-characterized panel of naturally infected swine sera, we evaluated and optimized the performances of three commercially available competitive enzyme-linked immunosorbent assays (ELISAs), namely, the IDEXX Influenza A Ab test, IDEXX AI MultiS-Screen Ab test, and IDVet ID Screen influenza A antibody competition ELISA, for detecting influenza A virus-reactive antibodies in swine. Receiver operating characteristic (ROC) analysis suggests that adjustment of the manufacturer-recommended cutoff values optimizes the sensitivity and specificity of these assays, making them applicable for seroepidemiology studies of swine influenza. Using such optimized cutoff levels, the sensitivity and specificity of the IDEXX Influenza A Ab test were 86% and 89%, respectively; those for the IDEXX AI MultiS-Screen Ab test were 91% and 87%, respectively; and those for the IDVet ID Screen influenza A Ab test were 95% and 79%, respectively.

The emergence of the pandemic H1N1 2009 (H1N1pdm) influenza virus of swine origin (3) and its transmission back to swine (6–8) heightened the need for global surveillance of swine influenza (http://www.oiflu.net/fileadmin/home/en/meeting-reports/pdf/SIV-Summary.pdf). Seroepidemiology provides useful information on swine influenza virus activity. However, hemagglutination inhibition (HI) or microneutralization assays are subtype and partially virus lineage specific, and studies carried out with a limited or poorly chosen panel of virus antigens may underestimate the true seroprevalence. This is particularly relevant where there is a paucity of virological data, e.g., many parts of Asia, Africa, and South America.

There are limited data on the performances of influenza A virus-specific enzyme-linked immunosorbent assays (ELISAs) for seroepidemiology analysis of swine. ELISAs specific for H1N1 and H3N2 swine influenza virus antibodies had poor sensitivities for use on pigs experimentally infected or vaccinated with Eurasian avian-like swine influenza viruses (1). Competitive ELISAs that detect cross-reactive antibodies to type A influenza viruses are used for surveillance of avian influenza in poultry and would potentially be useful for seroepidemiology of swine. The IDEXX AI MultiS-Screen Ab test gave a good sensitivity and specificity with sera from pigs with a known infection history for North American swine influenza viruses, provided that adjustments were made to the specimen/negative control ratio (S/N) cutoff by reference to the receiver operating characteristic (ROC) curve (2).

The aim of the present study was to evaluate the test performance characteristics of three commercially available competitive ELISAs which detect antibodies cross-reactive with influenza A virus for detection of influenza A virus antibody in swine sera.

MATERIALS AND METHODS

ELISAs. The tests evaluated were the IDVet ID Screen influenza A antibody competition ELISA (IDVet-Innovative Diagnostics, France) and two IDEXX tests, the IDEXX AI MultiS-Screen Ab test (USDA licensed for serology of five domestic poultry species but not of swine) and the IDEXX Influenza A Ab test (IDEXX, ME) (marketed outside North America with claimed applicability to avian, canine, feline, and swine species; uses a different cutoff for swine sera). These tests were used according to the manufacturers’ instructions.

Panel of sera used for test evaluation. We used a panel of well-characterized sera from our 13-year systematic abattoir-based virological and serological surveillance for swine influenza in southern China, where all three major virus H1 lineages, viz, classical swine (CS), Eurasian avian-like swine (EA), and North American triple reassortant (TRIG) viruses, as well as EA H3N2 and human-like H3N2 viruses, were found to cocirculate (9). The sera were collected at the largest abattoir in Hong Kong, where approximately 4,000 pigs are slaughtered daily, with 95% of the pigs being sourced from 12 provinces across China. The pigs were apparently healthy on arrival at the abattoir, and their past infection status was determined by detection of HI antibodies to a range of well-chosen viral antigens (see below). We selected a panel of 116 sera that had been tested in H1 assays using a panel of 9 swine influenza viruses chosen to represent the different virus subtypes and antigenically variant virus lineages known to be active in our study area (9). Virus antigens from six H1 influenza A viruses were used: CS lineage, A/swine/HK/4167/1999 (H1N1) and A/swine/HK/1304/2003 (H1N2); TRIG lineage (H1N2), A/swine/HK/1110/2006; EA lineage (H1N1), A/swine/HK/NS29/2009 and A/swine/HK/1559/2008; and an H1N1pdm virus, A/CA/04/2009. Three H3N2 viruses, A/swine/HK/5212/1999 (Eurasian avian-like), A/swine/HK/1128/2003 (human-like), and A/swine/HK/2422/1998 (human-like), were also used. Thirty-eight sera were seronegative with all 9 antigens, and the other 78 sera were selected to represent sera that were seropositive for one or more of the viruses tested.
Table 1 presents test characteristics of three competitive ELISAs for detection of influenza A virus antibody.

The sensitivity of the IDVet, IDEXX AI Multi-Screen Ab, and IDEXX Influenza A Ab tests were 69% (95% confidence interval [CI], 58 to 79%), 82% (95% CI, 73 to 91%), and 86% (95% CI, 76 to 93%), respectively (Table 1; Fig. 1). The specificities of the three assays were 89%, 87%, and 79%, respectively. The ROC curve (5) identified test kit cutoff values for each test kit that may provide optimal test sensitivity and specificity (Table 1; Fig. 1), with the optimal S/N cutoff ratio for the IDVet test being 0.71 (the kit-recommended cutoff is 0.5) and that for both IDEXX assays being 0.56. Table 1 presents test characteristics using (i) the manufacturer’s recommended cutoff S/N ratio, (ii) the optimal S/N ratio derived from the ROC curve and the Youden index, and (iii) the S/N ratios giving specificities of 95% and sensitivities of ≥96% are included.

**RESULTS**

At the manufacturer’s recommended cutoff for each test (see the table legend for details), the sensitivities of the IDVet, IDEXX AI MultiS-Screen, and IDEXX Influenza A Ab tests were 69% (95% confidence interval [CI], 58 to 79%), 82% (95% CI, 73 to 91%), and 86% (95% CI, 76 to 93%), respectively (Table 1; Fig. 1). The statistical analysis was carried out using the software program MedCalc. The sensitivity, specificity, positive and negative predictive values, ROC curve, and Youden index were computed as described elsewhere (4).

**analysis.** To allow comparison between the IDVet and IDEXX tests, we expressed the results of the IDVet assay as S/N ratios rather than % competition. The kit-recommended S/N cutoff for a positive result was then <0.5 for both the IDVet and IDEXX AI MultiS-Screen Ab assays (for avian sera) and <0.6 for the IDEXX Influenza A Ab test (for swine sera). The optimal cutoff for each assay determined by the Youden index analysis of our data is indicated in bold. In addition, the lowest S/N ratios giving specificities of ≥97% and sensitivities of ≥96% are included.

**TABLE 1 Test performance characteristics of three competitive ELISAs for detection of influenza A virus antibody**

| Parameter | IDVet ID Screen ELISA | IDEXX AI Multi-Screen Ab ELISA | IDEXX Influenza A Ab ELISA |
|-----------|------------------------|-------------------------------|-----------------------------|
| S/N ratio | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
| 0.23 | 45 (34–57) | 97 (86–100) | 94 | 56 | 45 (31–54) |
| 0.5 | 69 (58–79) | 89 (75–97) | 93 | 59 | 67 (46–88) |
| 0.71 | 95 (87–99) | 79 (63–90) | 97 | 86 | 70 (50–90) |
| 0.74 | 96 (89–99) | 76 (60–89) | 97 | 93 | 80 (60–100) |

Abbreviations: CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; J, Youden index.

To allow comparison between the IDVet and IDEXX tests, we expressed the results of the IDVet assay as S/N ratios rather than % competition. The kit-recommended S/N cutoff for a positive result was then <0.5 for both the IDVet and IDEXX AI Multi-Screen Ab assays (for avian sera) and <0.6 for the IDEXX Influenza A Ab test (for swine sera). The optimal cutoff for each assay determined by the Youden index analysis of our data is indicated in bold. In addition, the lowest S/N ratios giving specificities of ≥97% and sensitivities of ≥96% are included.

**FIG 1 ROC curve for panel of sera tested with three competitive ELISAs which detect influenza type A virus cross-reactive antibodies. (a) IDVet ID Screen influenza A antibody competition ELISA kit (IDVet, France); (b) IDEXX Influenza A Ab test (IDEXX, ME); (c) IDEXX AI Multi-Screen Ab test (USDA licensed for serology of five domestic poultry species but not of swine). The readout for all three tests is the specimen/negative control (S/N) ratio. The sensitivity and specificity at different S/N ratios are shown. The % true-positive results (sensitivity) and % false-positive results (100% – % specificity) at different S/N ratios are shown. The recommended S/N cutoff for a positive result in the IDVet and IDEXX AI Multi-Screen tests was 0.5, and that for the IDEXX Influenza A Ab test was 0.6 for swine sera. The boxed line indicates the sensitivity and specificity calculated for each serum sample, the dotted lines are the 95% confidence intervals, and the diagonal line represents what would be expected if the test results were generated at random. The area bounded by the boxed line and the diagonal is the area under the curve (AUC) for the ROC curve. A larger AUC implies that the test results are not likely to be random. The arrows denote the S/N ratios corresponding to those shown in Table 1. The AUC for the IDVet ID Screen, IDEXX AI Multi-Screen Ab, and IDEXX Influenza A Ab tests were 0.922, 0.915, and 0.901, respectively. The standard errors for the results were 0.0314, 0.0327, and 0.0351, respectively (see reference 4).
manufacture of these two assays are the same, though the test kits are packaged in two separate locations and are targeted at two different markets.

DISCUSSION

The findings of this study indicate that the competitive ELISAs we evaluated can be used for seroepidemiological studies of swine influenza. The sensitivity and specificity of the tests for use with swine sera can be improved by selecting the S/N ratio (test cutoff) based on the ROC curve we generated rather than using the S/N ratio recommended by the manufacturer. The reason for the suboptimal test cutoff S/N ratios for some tests is probably a consequence of the tests being designed and validated mainly with poultry sera rather than swine sera, and indeed, two of the tests are not marketed for use on swine sera. For seroepidemiological studies, however, positive results by these ELISAs would provide evidence of the circulation of influenza viruses in swine, even when local swine influenza virus isolates are unavailable. Sera that are positive in the ELISAs can then be tested further in HI tests with representative virus antigens to identify the virus lineages that are likely to be prevalent in a given geographic region.

Three of the 38 sera that were seronegative in HI tests with the antigen panel were positive in all three ELISAs (Fig. 2), which may be evidence of seropositivity for an influenza virus (of swine or even avian origin) that is not represented in the HI test reference antigen panel used for this study. This may provide an impetus for further serological testing against other influenza viruses. For the analysis of test performance characteristics (Table 1; Fig. 1), we assumed that sera that were seronegative by HI tests with all of the test antigens were truly negative. However, because the ELISAs tested here target conserved anti-influenza A virus nucleoprotein antibodies, it is possible that some sera which are HI negative but ELISA positive (i.e., currently regarded as false-positive sera) may be truly positive against a swine influenza virus antigen not represented in our panel. The specificity and negative predictive values in our analysis must be interpreted with this caveat in mind.

The false-negative results were randomly distributed across the different virus lineages. Many (but not all) of them had ELISA optical density values that were close to the cutoff level, but there was no correlation with the HI titers of respective sera (data not shown).

In summary, our results indicate the feasibility of using competitive ELISAs as screening tests for seroepidemiology of swine influenza, provided that their limitations are recognized. The detection of swine influenza virus antibody by the application of such competitive ELISAs with broad influenza A virus reactivity would provide an impetus for virological surveillance to detect the viruses circulating in a given geographic region. In time, such an effort would allow the assembly of panels of relevant viral HI antigens that cover swine influenza virus activity in a regional (if not global) context. As with any serological test, alternative explanations for seropositivity, such as vaccination, need to be consid-
ered, but vaccination of swine for influenza is uncommon in many Asian and African countries.

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