Evaluation of an in-house indirect enzyme-linked immunosorbent assay of feline panleukopenia VP2 subunit antigen in comparison to hemagglutination inhibition assay to monitor tiger antibody levels by Bayesian approach

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Abstract

**Background:** Feline panleukopenia virus (FPV) is an etiologic pathogen of feline panleukopenia that infects all members of Felidae including tigers (*Panthera tigris*). Vaccination against FPV among wild felid species has long been practiced in zoos worldwide. However, few studies have assessed tiger immune response post-vaccination due to the absence of a serological diagnostic tool. To address these limitations, this study aimed to develop an in-house indirect enzyme-linked immunosorbent assay (ELISA) for the monitoring of tiger antibody levels against the feline panleukopenia vaccine by employing the synthesized subunit capsid protein VP2. An in-house horseradish peroxidase (HRP) conjugated rabbit anti-tiger immunoglobulin G (IgG) polyclonal antibody (HRP-anti-tiger IgG) was produced in this study and employed in the assay. It was then compared to a commercial HRP-conjugated goat anti-cat IgG (HRP-anti-cat IgG). Sensitivity and specificity were evaluated using the Bayesian model with conditional dependence being assumed between both HRP-conjugated antibody-based ELISAs and hemagglutination-inhibition (HI) tests.

**Results:** The posterior estimates for sensitivity and specificity of two indirect ELISA HRP-conjugated antibodies were higher than those of the HI test. The sensitivity and specificity of indirect ELISA for HRP-anti-tiger IgG and HRP-anti-cat IgG were 86.5%, 57.2% and 86.7%, 64.6%, respectively, while the results of the HI test were 79.1 and 54.1%. In applications, 89.6% (198/221) of tiger serum samples were determined to be seropositive by indirect ELISA testing.

**Conclusion:** The results support evidence that an in-house indirect ELISA developed in this study could be used as a serological tool for the effective detection of tiger antibodies.

**Background**

Feline panleukopenia, also referred to as feline distemper or feline infectious enteritis, is a contagious disease that is common among domestic cats (*Felis catus*) and other Felidae with high rates of morbidity and mortality [1-4]. Feline panleukopenia virus (FPV) is an etiologic agent of the disease. The mortality rate can range from 25 to 100% depending on the severity of the clinical signs. However, the current prevalence of subclinical infections is unknown [5]. Cats of all ages can be infected with FPV, but young animals are the most susceptible. Previous investigations have reported...
incidences of fatal FPV infections among captive felids. Duarte et al. (2009) [6] reported on the death of a white tiger (*Panthera tigris*) and an African lion (*Panthera leo*) from FPV infection at the Lisbon zoo in Portugal. Dissanayake et al. (2017) [7] reported on the death of an unvaccinated Bengal tiger cub (*Panthera tigris tigris*) and severe illness in an unvaccinated leopard cub (*Panthera pardus*) at a zoological garden in Sri Lanka, both of which were caused by FPV. These incidences indicate that tigers, which are the largest cat species, appear to be particularly susceptible to FPV infection. Moreover, feline panleukopenia in tigers should be of significant concern because tigers are an endangered species according to the **International Union for Conservation of Nature’s (IUCN) Red List of Threatened Species** [8].

An effective vaccination policy is the most important strategy for the prevention of FPV infection [5]. Vaccinations against FPV have been a routine element of feline preventative medicine for the past 40 or more years [9]. Despite the frequent use of effective vaccines in domestic cats, there are no vaccines and vaccination programs that have been approved for use in non-domestic felids. Importantly, vaccination policies have long been practiced in zoos worldwide. Therefore, the application of vaccines and vaccination programs for cats are now being suggested for use among wild felids. In practice, modified live vaccines (MLV) are commonly used on domestic cats. However, inactivated vaccines are recommended for use among tigers and other wild felids that are held in captivity [10-11]. This is due to the potential risk of inducing a range of diseases, or the fact that they may lead to mutations when MLVs are used among species upon which the vaccines have never been tested. Many zoological parks, including some in Thailand, have also used inactivated vaccines to prevent FPV infection in tigers. However, the effectiveness of using a cat’s inactivated FPV vaccine on captive tigers is unknown. Additionally, suitable vaccine protocols have not yet been clarified due to the lack of an established specific serological method for the evaluation of tiger antibodies post vaccination.

Generally, the detection of antibodies against FPV is commonly achieved by the hemagglutination-inhibition (HI) test or enzyme-linked immunosorbent assay (ELISA). The recombinant capsid protein or viral protein 2 (VP2) protein of FPV has been used as a coating antigen for ELISA in order to detect the
level of FPV-specific immunoglobulin G (IgG) in cats [12]. The capsid protein VP2, a major structural protein of FPV, is a well characterized gene that has been widely used for phylogenetic analysis because it encodes the major protein that determines the host’s range, along with the relevant viral pathogenicity and immune response [4, 6]. Therefore, to address the limitations of the present serological diagnostic tools for tigers, this study aimed to develop an in-house indirect ELISA test kit for the monitoring of tiger antibody levels against the feline panleukopenia vaccine by employing the VP2 protein as a coating antigen. An in-house horseradish peroxidase (HRP) conjugated rabbit anti-tiger IgG polyclonal antibody was also employed in this study as a secondary antibody. This in-house indirect ELISA test kit will be particularly helpful in laboratory investigations involving disease surveillance of FPV among tigers.

Results

**Generation and binding capability of HRP-conjugated rabbit anti-tiger IgG antibody**

The purified rabbit anti-tiger IgG was conjugated with HRP. The binding capability of HRP-conjugated rabbit anti-tiger IgG antibody was evaluated by western blotting analysis. The result showed the reactive protein bands at approximately 170 kDa under non-reducing conditions as the expected size of the tiger IgG, as well as, HRP-conjugated goat anti-cat IgG antibody (Fig. 1). The results demonstrated the capturing ability and specificity of HRP-conjugated antibody to purified tiger IgG antibody.

**Optimal concentration of in-house indirect ELISA reagents and cut-off value**

The signal to noise (S/N) ratio between the optimal density (OD) value of the vaccinated and non-vaccinated sera at 450 nm is shown in Table 1. The optimal conditions for the indirect ELISA were 1 ng of capsid protein VP2 in 100 µl of the coating buffer as a coating antigen. A dilution of 1:200 for tiger serum with 1:1000 HRP-conjugated goat anti-cat IgG antibody, and a dilution of 1:100 for tiger serum with 1:2000 HRP-conjugated rabbit anti-tiger IgG antibody, were selected for use as the primary and secondary antibodies in this study.

Average OD values at 450 nm and the standard deviation of HRP-conjugated goat anti-cat antibody were 0.265 and 0.043, respectively. Thus, the cut-off point of the indirect ELISA using HRP-conjugated
goat anti-cat antibody was 0.400. Additionally, the average OD value at 450 nm of HRP-conjugated rabbit anti-tiger antibody was 0.069, and the standard deviation value was 0.006. Therefore, the cut-off point of the indirect ELISA using HRP-conjugated rabbit anti-tiger antibody was 0.100.

**Seroprevalence of antibody titers against feline panleukopenia vaccine in tigers**

The results of an indirect ELISA using two different HRP-conjugated antibodies where compared to the HI test and are shown in Table 2. Considering the HI titer at 1:20, the seropositive of tiger antibodies against FPV vaccine was 80% (178/221). In contrast, the seropositive measured by in-house indirect ELISA tests with either anti-tiger or anti-cat antibody was 89.6% (198/221).

**Sensitivity and specificity of an in-house indirect ELISA**

The posterior estimates for sensitivity and specificity of each test and the prevalence of tiger antibodies against the FPV vaccine are shown in Table 3. Posterior sensitivity estimates for anti-cat ELISA, anti-tiger ELISA and the HI test were found to be higher than the previously recorded figures with median values of 86.7% [95% posterior probability interval (PPI) = 82.0-90.8%], 86.5% (95% PPI = 81.8-90.8%) and 79.1% (95% PPI =73.4-84.5%), respectively. Posterior specificity estimates for anti-cat ELISA and HI test were close to the prior values with median values of 64.6% (95% PPI = 47.5-80.0%) and 54.1% (95% PPI = 30.0-76.2%), respectively. Posterior specificity estimates for anti-tiger ELISA were similar to the prior values with a median value of 57.2% (95% PPI = 41.7-71.8%). Lastly, posterior estimates for the prevalence of tiger antibodies against FPV vaccine were higher than the prior estimates with a median value of 94.6% (95% PPI= 87.6-98.4%). The conditional dependence among two ELISA tests and the HI test was low among both seropositive and seronegative tigers, with probability intervals of the conditional covariance being 0. The conditional independent model, which did not include a covariance term among all three tests, had a higher the deviance information criterion (DIC) value than the conditional dependent model (120 vs. 58, respectively). Therefore, the conditional dependent model was preferred as a final model.

The model converged properly and autocorrelation was eliminated after omitting the first 10,000 iterations (Additional file 1: Fig. S1). In the sensitivity analysis, no major changes (changes in median or 95% probability percentiles >25%) were observed in the posterior sensitivity estimates for all three
tests and specificity estimations of both ELISA tests when non-informative distributions were used as the prior values for any parameter. This result was interpreted as evidence of model robustness. In contrast, a change in the posterior estimates of specificity for the HI test was observed with a higher estimated posterior specificity (from 54.1 to 72.9%) when a non-informative prior value was used. Therefore, it is suggested that prior values of this parameter had a stronger influence on the results of the model.

Discussion

Tigers are the largest cat species and an important member of the ecosystem. Previous studies have identified the FPV infection in several populations of tigers [6-7]. This evidence indicates that tigers appear to be susceptible to FPV infection. Vaccination against FPV among wild felid species has long been practiced in zoos worldwide, but few studies have assessed tiger immune response post-vaccination. Considering serum antibody titer, it has been shown to be useful for the determination of immune responses against viruses or vaccination. Moreover, it is very important to predict the required frequency for the administration of the vaccine [13-15]. Therefore, an in-house indirect ELISA was developed in this study.

FPV is a non-enveloped single-strand deoxyribonucleic acid (ssDNA) virus that is classified in the family Paroviridae, genus Parovirus. The paroviruses are small viral particles containing two major open reading frames (ORF), which encode non-structural (NS) proteins and capsid proteins including VP1 (10%) and VP2 (90%) [9]. VP2 protein is a major structural protein of the parovirus and has been highly conserved which involves in receptor recognition and nuclear translocation [16]. Amino acid at positions of 80, 564, and 568 are important for efficient viral replication in cats and are conserved among all canine parovirus (CPV) and FPV viruses [17-19]. Previous studies have identified the capsid protein VP2 of FPV as a coating antigen for ELISA for the detection of antibodies against feline panleukopenia in cats [12]. In addition, VP2 subunit protein exhibited good antigenicity in ELISA development detecting antibodies against parovirus in minks causing Aleutian Mink disease with high sensitivity and specificity [20]. On that basis, the amino acids 545-585 of VP2, which covered the epitope region and were specific to the FPV, were selected and employed as an antigen in an indirect
ELISA test in this study. The results showed that an in-house ELISA test using the VP2 peptide had better sensitivity and specificity when compared with the HI test. Therefore, it could be preferable over the HI test for the serological screening of tiger antibodies against FPV vaccine or infection.

ELISA and HI tests have been developed to assess antibody titers against feline parvovirus infections in cats [21-22]. However, the specificity of the serum neutralizing (SN) test is the highest among viral serologic testing [23]. Since the SN test is considered time-consuming and costly, the HI test is being widely used to determine antibody titer against FPV [14, 23-25]. The ELISA test has been recognized as a sensitive and reliable method to determine humoral antibody response against parvovirus, possibly allowing for the calculation of the time that vaccinations can be performed [14, 26]. Bayesian latent class analysis is a statistical procedure for estimating the sensitivity and specificity of a diagnostic test in the absence of a gold standard assay. A Bayesian model has been used to estimate the sensitivity and specificity of various diagnostic techniques [27-31]. In this study, a Bayesian model was performed to estimate the sensitivity and specificity of an in-house indirect ELISA with two HRP-conjugated antibodies, and these values were compared to the HI test. The estimated sensitivity of anti-cat ELISA (86.7%) and anti-tiger ELISA (86.5%) tests were higher than that of HI test (79.1%). In addition, the estimated specificity of ELISA on anti-cat and anti-tiger antibodies (64.6% and 57.2%, respectively) were higher than that of HI test (54.1%) as well. Therefore, the developed in-house ELISA appears to be more efficient for the screening and monitoring of antibody levels against FPV vaccination or infection in tigers.

The posterior estimates for the prevalence of tiger antibodies against FPV were significantly higher than the prior estimates. This finding can be explained by the fact that most of the blood samples in this study were collected from vaccinated tigers. Thus, posterior estimates of the prevalence of tiger antibodies were higher than initially expected. For the sensitivity analysis, an important change was observed only in terms of the specificity of the HI test when non-informative prior distributions were applied in the model. The model was found to be sensitive to the prior selections for some parameters. There were no previous reports on the performance of the HI test for FPV detection in tigers. Consequently, prior values of the HI test were estimated based on expert opinions due to a
lack of available information. This suggests that the HI test may be associated with higher specificity values than initially expected.

Methods

**Production of rabbit anti-tiger IgG polyclonal antibody**

Purified Bengal tiger IgG was obtained from a previous study [32]. New Zealand white rabbits were weekly subcutaneously immunized with purified tiger IgG formulated with Montanide™ ISA 206 VG (Seppic, Paris, France; 1:1 v/v, 100 µg/ml) for 4 weeks. Rabbit antisera were then purified using a Melon™ Gel IgG Purification Kit following the manufacturer’s instructions. Concentrations of purified rabbit anti-tiger IgG polyclonal antibody was measured using the bicinchoninic acid (BCA) protein assay (BCA protein assay kit; Pierce, Rockford, IL, USA) according to the manufacturer’s instructions and then stored at −20°C for further analysis.

**Preparation of HRP-conjugated rabbit anti-tiger IgG antibody**

The two-step glutaraldehyde method was used to couple HRP with rabbit anti-tiger IgG as has been previously described by Chansiw et al. (2008) [33]. Briefly, 2 mg of HRP (Sigma-Aldrich, St. Louis, Missouri, USA) were dissolved in 200 µl of 0.1 M potassium phosphate buffer (pH 6.8) in 1.25% glutaraldehyde 200 µl and incubated at 4°C for 18 hrs with an end-over-end rotator. Excess free glutaraldehyde was removed by 10K Vivaspin® 500 (Sartorius Stedim Biotech Gmbh, Goettingen, Germany) and the specimens were centrifuged at 10,000 rpm for 30 min. They were then resuspended with 200 µl of 0.1 M sodium carbonate-bicarbonate buffer (pH 9.5) with 30% sucrose and collected in a microcentrifuge tube. Micro Bio-Spin™ Chromatography Columns (Bio-Rad Laboratories, Hercules, CA, USA) were used for buffer exchange to the appropriate buffer for the antibody according to the manufacturer’s instructions. Subsequently, 500 µl of 0.15 M NaCl were added to the column, and the column was then centrifuged at 1,000 × g for 1 min and washed three times. Rabbit anti-tiger IgG 1 mg/100 µl was carefully added directly to the center of the column, and the column was then centrifuged at 1,000 × g for 4 min. Rabbit anti-tiger IgG (1 mg/100 µl) was mixed with HRP (2 mg/200 µl) and incubated overnight at 4°C using an end-over-end rotator. Additionally, 15 µl of 2 M
glycine were added and the specimens were centrifuged by 50K Amicon® Ultra-15 (Merck Millipore Ltd, Cork, Ireland) at 3,400 rpm for 30 min to eliminate the excess HRP and other buffers. Subsequently, 100 μl of the phosphate-buffered saline (PBS, pH 7.2) was resuspended. Next, 500 μl of PBS buffer (pH 7.2) were added to the Micro Bio-spin® Chromatography Column for buffer exchange. The column was then centrifuged at 1,000 × g for 1 min and washed three times. HRP-IgG solution was then carefully added to the column and it was centrifuged at 1,000 × g for 4 min in order to allow the solution to collect in a microcentrifuge tube. HRP-conjugated rabbit anti-tiger IgG was obtained and then stored at -20°C for further analysis.

**Determination of avidity and specificity of tiger IgG and rabbit anti-tiger IgG polyclonal antibody by western blot**

Immunochemical analysis of purified protein was done by western blot technique according to the method of Towbin et al. (1979) [34] with some modifications. Briefly, the purified tiger IgG was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) [35]. Samples were prepared in sample buffer and boiled at 95°C for 5 min and then analyzed on 10% SDS-PAGE gel slabs in a mini-slab apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The protein on SDS-PAGE gel was electrically transferred onto a nitrocellulose membrane (Merck Millipore™, Merck KGaA©, Darmstadt, DEU). The blotting time was 60 min at a constant voltage of 10 V. The membrane was incubated with blocking buffer (5% skim milk in PBS) for 1 hr at room temperature with gentle shaking. After being washed three times with washing buffer (PBS containing 0.05% Tween® 20; PBST), the membrane was probed with HRP-conjugated rabbit anti-tiger IgG (1:500 dilution) obtained from previous step and HRP-conjugated goat anti-cat IgG (1:2000 dilution; KPL, Gaithersburg, MD, USA) antibodies, separately. The membrane was then incubated with gentle shaking at room temperature for 1 hr and washed three times. Finally, the reactions were visualized using a solution containing 3,3´-diaminobenzidine (DAB; Invitrogen, Carlsbad, CA, USA) with hydrogen peroxide (H₂O₂; Merck, Germany).

**Animals**
All of tigers included in the study belonged to the Tiger Kingdom, Mae Rim, Chiang Mai, Thailand. During the study, the tigers were fed, housed and managed according to Tiger Care Manual [11]. All sampling procedures were monitored and controlled under veterinarian. After the study, all of tigers, a protected wildlife animals in Thailand, were kept and looked after at Tiger Kingdom, Mae Rim and Tiger Kingdom learning centre, Mae Tang, Chiang Mai.

**Vaccination and blood sampling**

Forty-three tigers were included in this study. All tigers were vaccinated with an inactivated vaccine (Fel-O-Vax® 4 Vaccine; Boehringer Ingelheim Vetmedica, Inc. St. Joseph, MO, USA) according to the vaccination protocol described in the Tiger Care Manual [11]. Blood samples were collected from the saphenous vein after administering anesthesia. A blood sample was taken from each tiger prior to the vaccinations and every 3 months for 1 year. Sera were prepared by centrifugation and stored at -20°C for further analysis.

**Hemagglutination Inhibition (HI) test**

Tiger sera were tested using the HI test following the method that had been previously described [36]. HI titer that was higher than 1:20 was considered as seropositive.

**Capsid protein or viral protein 2 (VP2) subunit antigen prediction and synthesis**

The VP2 subunit antigen was predicted based on the amino acid sequence accession number ABN70938.1 using the linear and conformational epitope prediction software as was described in the previous report [37]. The selected amino acid sequence (WNPIQQMSINVDNQFNYPNNIGAMKIVYEKSLAPRKLY) was then further synthesized (Genscript, Piscataway, New Jersey, USA).

**In-house indirect ELISA**

The reaction was performed in 96-well plates (Nunc-Immuno™ MaxiSorp™; Sigma-Aldrich). Each well was coated with an optimal concentration of synthetic VP2 capsid protein with coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6). Each plate was incubated overnight at 4°C and then washed three times with 200 µl of washing buffer (PBST, pH 7.2). Non-specific binding sites were blocked with
100 µl per well of blocking buffer (1% skim milk in PBS) for 1 hr at 25°C. After being washed three times with PBST, 100 µl of tiger serum that was diluted in blocking buffer was added to each well and the plate was incubated for 1 hr at 25°C. After washing three times with PBST, 100 µl of either HRP-conjugated goat anti-cat IgG (KPL) or HRP-conjugated rabbit anti-tiger antibody diluted in blocking buffer were added to each well. The reaction was performed for 1 hr at 25°C. After completion, any excess antibody was washed out with PBST over the course of three washings. One hundred µl of tetramethylbenzidine (TMB; KPL) was added to each well as a substrate. The reaction was performed in the dark for 15-30 min at room temperature before being stopped by adding 50 µl of 2 N H₂SO₄.

The absorbance was measured at a wavelength of 450 nm using an automatic ELISA plate reader (AccuReader; Metertech, Taipei, Taiwan). The results were expressed as an OD value.

**Optimization of an in-house indirect ELISA**

Checkerboard titration was performed according to Crowther (2009) [38] to determine the optimal dilutions of the sera and conjugated antibody. Pooled vaccinated sera and pooled non-vaccinated sera were analyzed according to the ELISA method as has been previously described and ranged from 1:100 to 1:500 dilutions. The HRP-conjugated antibody was varied from 1:1000 to 1:4000 dilutions. The optimal dilutions of serum and HRP-conjugated antibody were defined as those at which the ratio was the greatest between the vaccinated and non-vaccinated sample OD values.

**Calculation of cut-off value**

The cut-off value was obtained by OD value at a wavelength of 450 nm and calculated from the mean plus 3 standard deviations (SD) of the non-vaccinated group [38]. For further interpretation, any tiger sera with an OD value of more than or equal to the cut-off value were classified as seropositive. Tiger sera with an OD value that was less than the cut-off value were classified as seronegative.

**Determination of sensitivity and specificity**

A latent class analysis was performed using a Bayesian model to determine the sensitivity and specificity of two indirect ELISA tests and the HI test. Since all three tests are based on the detection of antibody response, their results were considered conditionally dependent upon each other [39]. Therefore, a Bayesian model for three diagnostic tests was implemented in one population in order to...
determine the sensitivity and specificity of each test.

Prior information on test performance and the prevalence of antibodies was introduced into the analysis using probability distributions (prior distribution). As a result of a lack of prior information on three tests, the sensitivity and specificity of prior values for indirect ELISA and HI tests were modeled as beta distributions based on three expert opinions. Prior value for the prevalence of antibodies against FPV vaccine in tigers in Chiang Mai was also selected based on opinions from three zoo and wildlife experts due to a lack of published information. Prior values used for analysis (sensitivity, specificity, and prevalence) are listed in Table 4. All analyses were implemented in JAGS 3.4.0 [40] via the rjags and R2jags packages [41-42] obtained from R 3.2.2 software [43]. Posterior distributions were computed after 100,000 iterations of models with the first 10,000 being discarded as the burn-in phase.

The model convergence was assessed by visual inspection of the Gelman-Rubin diagnostic plots [44-45] using three sample chains with different initial values. The goodness of fit of the models was evaluated using DIC [46], and the number of effectively estimated parameters (pD) [47] served as the calibrating parameters. The model sensitivity analysis was performed to assess the influence of prior information and the assumption of conditional dependence between the two ELISA tests and the HI test on the posterior estimates [27, 48]. These analyses were performed by replacing each prior value with a non-informative uniform 0-1 distribution and by comparing the DIC between models with and without the covariance term [27].

Abbreviations

BCA: Bicinchoninic acid; CPV: Canine parvovirus; DIC: Deviance information criterion; DAB: 3,3´-diaminobenzidine; ELISA: Enzyme-linked immunosorbent assay; FPV: Feline panleukopenia virus; HI: Hemagglutination-inhibition; HRP: Horseradish peroxidase; H$_2$O$_2$: Hydrogen peroxide; IUCN: International Union for Conservation of Nature; IgG: Immunoglobulin G; MLV: Modified live vaccines; NS: Non-structural; pD: Number of effectively estimated parameters; ORF: Open reading frames; OD: Optical density; PBS: Phosphate-buffered saline; PBST: Phosphate-buffered saline containing 0.05% Tween® 20; PPI: Posterior probability interval; SN: Serum neutralizing; S/N: Signal to noise; ssDNA:
single-strand deoxyribonucleic acid; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SD: Standard deviations; H₂SO₄: Sulfuric acid; TMB: Tetramethylbenzidine; VP2: Viral protein 2

Declarations

Ethics approval and consent to participate

All procedures used in this study were approved of by the Animal Care and Use Committee (FVM-ACUC), Faculty of Veterinary Medicine, Chiang Mai University (Ref. No. R6/2560). The written informed consent for the use of animals was obtained from the owner of the Tiger Kingdom.

Consent for publication

Not applicable.

Availability of data and materials

All relevant data in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interest.

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Authors’ contributions

CA, TS, CS, WP, PV and NS designed the study. CA collected the blood sample, performed the experiments, carried out the statistical analysis, and drafted the manuscript. PV and NS contributed to the experiments. TS carried out the statistical analysis and contributed to writing manuscript in statistical part. CA, AR, BN and IF contributed to writing, reviewing and editing the manuscript. All authors have read and approved the final manuscript for submission and publication.
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Tables
Table 1    Prior values for sensitivity and specificity of ELISA (anti-cat and anti-tiger antibodies) and HI tests, and prevalence of antibodies against FPV
## Diagnostic tests

| Parameters   | Mode (%) | 95% CI |
|--------------|----------|--------|
| Prevalence   | 50       | < 80   |
| ELISA (anti-cat) |          |        |
| Sensitivity  | 75       | > 63   |
| Specificity  | 66       | > 50   |
| ELISA (anti-tiger) |        |        |
| Sensitivity  | 68       | > 53   |
| Specificity  | 57       | > 43   |
| HI           |          |        |
| Sensitivity  | 56       | > 40   |
| Specificity  | 50       | > 30   |

*95% CI = 95% confidence interval

Table 2  
Optimal HRP-conjugated antibody dilution and serum dilution in 1 ng/100 µl of coating buffer

| HRP-Conjugated antibody | Serum dilution / HRP dilution |
|-------------------------|------------------------------|
|                         | 1:100 | 1:200 |
| Anti-cat                |       |       |
| 1:1000                  | 2.460 | 3.095*|
| 1:2000                  | 2.310 | 2.724 |
| 1:3000                  | 2.350 | 2.641 |
| 1:4000                  | 2.300 | 2.730 |
| Anti-tiger              |       |       |
| 1:1000                  | 3.812 |       |
| 1:2000                  | 3.160*| 2.600 |
| 1:3000                  | 2.577 | 2.132 |
| 1:4000                  | 2.651 | 2.283 |

* Represents suitable S/N ratio obtained from this study

Table 3  
Results of all 221 tiger sera in indirect ELISA tests compared to HI test
Table 4  Posterior estimates for sensitivity and specificity of the ELISA (anti-cat and anti-tiger antibodies) and HI tests, and prevalence of antibodies against FPV

| Diagnostic tests | Parameters | Posterior estimates |
|------------------|------------|---------------------|
|                  |            | Median (%)          | 95% PPI               |
|                  | Prevalence | 94.6                | 87.6 - 98.4           |
| ELISA (anti-cat) | Sensitivity| 86.7                | 82.0 - 90.8           |
|                  | Specificity| 64.6                | 47.5 - 80.0           |
| ELISA (anti-tiger) | Sensitivity | 86.5                | 81.8 - 90.8           |
|                  | Specificity| 57.2                | 41.7 - 71.8           |
| HI               | Sensitivity| 79.1                | 73.4 - 84.5           |
|                  | Specificity| 54.1                | 30.0 - 76.2           |

\(^{\text{a}}\) 95% PPI: 95% posterior probability interval

**Figures**
Figure 1

Western blot analysis of binding capability of HRP-conjugated IgG antibody against Bengal tiger IgG. Lane M: Protein marker; Lane 1: HRP-conjugated goat anti-cat antibody, Lane 2: HRP-conjugated rabbit anti-tiger IgG antibody

Supplementary Files
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