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Evaluation of an in-house dot enzyme-linked immunosorbent assay to detect antibodies against feline panleukopenia virus

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Abstract

Measuring antibody titres to determine a cat's immunity to core diseases instead of just administering annual vaccinations has not been established in Germany so far. An in-house test kit for the detection of antibodies against feline panleukopenia virus (FPV), feline herpesvirus-1 and feline calicivirus – the ImmunoComb Feline VacciCheck – is now available in several European countries. The aim of this study was to assess the quality of the ImmunoComb Feline VacciCheck to determine antibodies by comparing it to a gold standard. The test is aimed for use in practice to assist decision-making when performing an individual health assessment to see whether a cat is potentially unprotected against FPV and requires FPV vaccination. Sera from 347 cats were included in the study. For antibody detection, haemagglutination inhibition (HI) was performed as gold standard. Sensitivity, specificity and positive and negative predictive values of the ImmunoComb Feline VacciCheck were determined for three different HI titre cut-off points (1:20, 1:40, 1:80). In comparison to the HI, the ImmunoComb Feline VacciCheck showed a sensitivity of 79%, 83% and 87%, and a specificity of 89%, 86% and 81%, respectively. Specificity of the ImmunoComb Feline VacciCheck, which was considered the most important parameter, was acceptable in comparison to HI. Especially when considering an antibody titre of 1:20 sufficient for protection (eg in an adult animal), the ImmunoComb Feline VacciCheck can be recommended for use in veterinary practice.

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Introduction

Feline panleukopenia virus (FPV) is a single-stranded DNA virus of the family Parvoviridae and the genus *Parvovirus*. All members of Felidae and cats of all ages can be infected.^{1,2} Owing to high morbidity and high mortality of the infection, the FPV vaccine is considered a core vaccine, and current guidelines on vaccination recommend vaccinating as often as necessary, but not more than necessary. Experts recommend vaccinating kittens every 3–4 weeks up to 16 weeks of age followed by a booster vaccination after 1 year and further vaccinations on a triennial basis.^{3–6} In a population in which the virus is still endemic, many cats are likely to have antibodies and be protected either because of exposure or vaccination. As the presence of antibodies is considered to indicate protection from disease, antibody testing can be used to determine protection or susceptibility of individual cats. Furthermore, it can be used to evaluate the immune response after vaccination and the efficacy of vaccines in

experimental settings.^{7–10} Titre testing to determine whether a cat has specific antibodies against FPV is a useful tool in individualised medicine. However, it has so far not been established in Germany. Its major aim in small animal practice is to determine whether a cat is potentially unprotected against FPV and requires FPV vaccination. Thus, using titre testing instead of just vaccinating a potentially protected cat can prevent over-vaccination in

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the adult cat population. Haemagglutination inhibition (HI) is considered to be the gold standard of measuring antibodies against FPV,^{8,11,12} but the HI titre cut-off point to predict protection is still debated, and different studies consider different HI titre cut-off points as protective.^{8,13,14} While titre determination by HI in a commercial laboratory is time-consuming, an in-house test that provides rapid and reliable results would be useful in everyday practice. Very recently, an in-house test arrived on the German market. The test detects antibodies against FPV, feline herpesvirus-1 (FHV-1) and feline calicivirus (FCV) (ImmunoComb Feline VacciCheck; Biogal). One study investigated the performance of this test in detecting FPV antibodies in young, presumed unvaccinated cats entering a shelter in Florida, USA.¹⁴ Since then, the test has been modified in an aim to increase sensitivity. So far, no study has evaluated this modified antibody in-house test in a diverse population of cats in Europe that included cats of different origin, source area, environment, housing conditions, and health and vaccination status.

Thus, the aims of this study were to evaluate the ImmunoComb Feline VacciCheck in the field by comparing the FPV results to those of the HI (gold standard) using different HI titre cut-off points (1:20, 1:40, 1:80) by measuring sensitivity, specificity, and positive (PPVs) and negative predictive values (NPVs), and furthermore to evaluate the practicability of the test. FHV-1 and FCV results were not evaluated.

Materials and methods

Cats

The study was designed as a prospective cross-sectional study. All cats (n = 347) that were presented from December 2011 to June 2012 to the Clinic of Small Animal Medicine and to the Clinic of Small Animal Surgery and Gynaecology of the Ludwig-Maximilians-University of Munich, Germany, and that needed a blood sample for preventive health assessment or for diagnostic purposes in sick animals, were included in the study. Blood was collected by venepuncture of the vena cephalica antebra-chii, vena saphena, or vena jugularis. For data collection, medical records were studied and missing data were collected via a structured telephone interview. Cats were excluded if there were no historical data available (ie, in stray cats). The study protocol was approved by the ethics committee of the Centre for Veterinary Clinical Medicine, Ludwig-Maximilians-University of Munich, Germany (licence number 3-5-10-2012).

ImmunoComb Feline VacciCheck

After blood sampling, sera were directly separated by centrifugation and stored at -20°C until processed. All samples were analysed with the ImmunoComb Feline VacciCheck according to manufacturer's instructions. Each antibody test kit contained a comb-shaped plastic

card and a multi-compartment developing plate for testing 12 sera in parallel (Figure 1). The manufacturer declares that the positive control of the test would be equivalent to an antibody titre of 1:80 in HI. The test is based on an enzyme-linked immunosorbent assay principle and detects antibodies against FPV, FHV-1 and FCV (FHV-1 and FCV results were not part of this study). After stepwise washing and binding of an enzyme-linked anti-cat immunoglobulin G antibody, a grey colour tone developed in the last step. A colour tone equal to or darker than the positive control was regarded as a positive result; a colour tone paler than the positive control was regarded as a negative result.

HI

All samples were analysed by the gold standard HI. HI is a laboratory test to measure antibodies against FPV. As FPV agglutinates swine erythrocytes, antibodies present in the sample prevent attachment of the virus to these erythrocytes and therefore inhibit haemagglutination. Samples underwent heat inactivation (56°C for 30 mins), were diluted with borate buffered saline (BBS) 1:5 and then pre-adsorbed to $15\ \mu\text{l}$ of a 50% suspension of swine erythrocytes in phosphate buffered saline (PBS) for 1 h at 4°C . After centrifugation for 5 mins, the supernatant was subsequently two-fold diluted serially over 12 steps beginning at 1:10 in 96-well V-bottomed plates (Greiner Bio-One). Eight haemagglutinating units of FPV-b (strain 292, as used and described in previous studies^{15,16}) in BBS were added to each well and incubated for 1 h at room temperature. Then, a 0.5% suspension of swine erythrocytes in PBS was added and incubated at 4°C

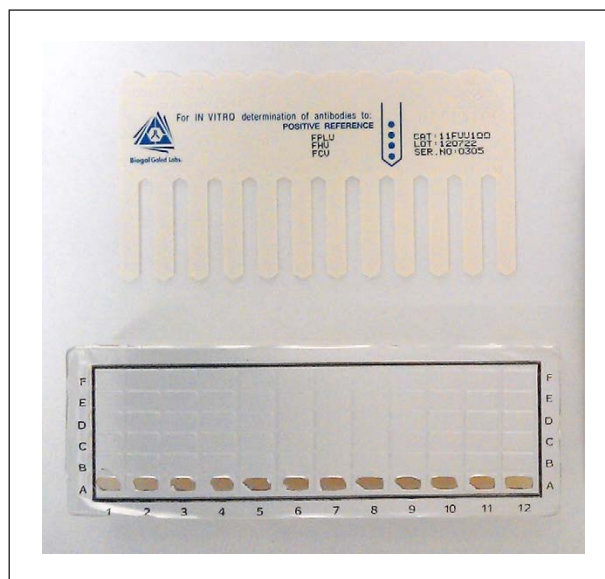


Figure 1 ImmunoComb Feline VacciCheck antibody test kit containing a comb-shaped plastic card and a multi-compartment developing plate

overnight. The reciprocal of the highest dilution of serum that inhibited haemagglutination was defined as the HI titre of the serum. Different antibody titres were used as HI titre cut-off points for a positive result (1:20, 1:40, 1:80).

Statistical analysis

For test evaluation, the following performance parameters were calculated using a 2×2 contingency table: sensitivity, specificity, PPV and NPV. To quantify uncertainty, 95% confidence intervals (CIs) were calculated. The prevalence (and 95% CI) was calculated as the proportion of positive results of the total number of tested sera. Performance parameters and prevalences were calculated for three different HI titre cut-off points (1:20, 1:40, 1:80). Statistical analysis was performed using commercial software (SPSS).

For evaluation of the diagnostic performance specificity was set as the most important parameter. As a predefined criterion, a specificity of $\geq 90\%$ was considered a good performance, a specificity of 80–90% was set as acceptable, and a specificity of $< 80\%$ was considered as unacceptable for recommendation of the test.

Results

Study population

Cats in the study were of a variety of breeds, female ($n = 149$) or male ($n = 198$), neutered ($n = 306$) or sexually intact ($n = 41$). The median age was 9 years and ranged from 6 weeks to 20 years. Cats came from private households ($n = 140$), animal shelters ($n = 41$), breeders ($n = 30$), foreign countries ($n = 59$) or were formerly stray cats ($n = 30$) (origin). Cats lived in either urban ($n = 298$) or rural communities ($n = 49$) (source area) and were kept indoors ($n = 194$) or outdoors ($n = 133$) (environment), as a single cat ($n = 138$) or in multi-cat households ($n = 187$) (housing conditions). At the time of presentation, cats were healthy ($n = 33$), or acutely ($n = 127$) or chronically ill ($n = 187$) (health status). Most cats had a history of prior vaccination ($n = 282$). Twenty-eight cats had never been vaccinated.

Sensitivity, specificity and predictive values

The results of all sera tested with the ImmunoComb Feline VacciCheck compared to HI are shown in Table 1. For the three different HI titre cut-off points (1:20, 1:40, 1:80), the test showed 9, 14 and 23 false-positive results, respectively. Specificities of the ImmunoComb Feline VacciCheck were 89%, 86% and 81%, respectively (Table 2).

Prevalence

Prevalence of antibodies against FPV, when considering a HI titre cut-off point of 1:20, 1:40 or 1:80 as positive, was 77% (267/347), 71% (245/347) and 65% (225/347),

Table 1 Results of all 347 sera in the ImmunoComb Feline VacciCheck compared to haemagglutination inhibition (HI) as gold standard for three different HI titre cut-off points (1:20, 1:40, 1:80)

	ImmunoComb negative	ImmunoComb positive	Total
HI negative ($> 1:20$)	71	9	80
HI positive ($\geq 1:20$)	58	209	267
HI negative ($> 1:40$)	88	14	102
HI positive ($\geq 1:40$)	41	204	245
HI negative ($> 1:80$)	99	23	122
HI positive ($\geq 1:80$)	30	195	225
Total	129	218	347

respectively (Table 2). Antibody prevalence measured by the ImmunoComb Feline VacciCheck was 63% (218/347; 95% CI 58–68).

Practicability of the ImmunoComb Feline VacciCheck

All 347 sera showed valid results in the ImmunoComb Feline VacciCheck and could clearly be classified as positive or negative. Twelve sera could be processed in parallel. The test always delivered results in 21 mins, as described in the manufacturer's instruction manual.

Discussion

FPV is a frequent disease that occurs in young and old cats.² In a retrospective study that investigated prognostic factors for survival of cats with panleukopenia, 244 cats that were presented to the Clinic of Small Animal Medicine, Ludwig-Maximilians-University of Munich, Germany, were diagnosed with FPV between 1990 and 2007.² According to current guidelines, ideally, all cats should be protected against FPV infection at any time, if not through immunity following natural infection, then by vaccination.^{3–6} Although rare in cats, mild-to-severe adverse events after vaccination occur; these include feline injection-site sarcomas (FISS) that often recur after surgery and have a guarded prognosis.^{17–19} FISS occur more commonly after adjuvanted inactivated vaccines,¹⁹ but have also been described after vaccination against FPV, FHV-1 and FCV.²⁰ As immunological protection against FPV is long-lived,²¹ vaccination should ideally only be performed in animals that are unprotected.

In adult vaccinated cats, regardless of vaccine type or vaccination interval, or cats that overcame infection, detection of FPV-specific antibodies is predictive of protection. Thus, measurement of antibodies against FPV can be used to assess the immune status in these cats.^{8,9,21} A fast and reliable in-house test would be an excellent tool for veterinarians to perform modern individualised medicine and avoid over-vaccination.

Table 2 Performance parameters of the ImmunoComb Feline VacciCheck based on results given in Table 1: sensitivity, specificity, and positive (PPV) and negative predictive value (NPV) (and 95% confidence interval [CI]), calculated using haemagglutination inhibition (HI) at three different HI titre cut-off points (1:20, 1:40, 1:80) as gold standard

HI titre cut-off point	Antibody prevalence in % (95% CI)	Sensitivity in % (95% CI)	Specificity in % (95% CI)	PPV in % (95% CI)	NPV in % (95% CI)
1:20	77 (73–83)	78 (73–83)	89 (82–96)	96 (93–99)	55 (46–64)
1:40	71 (66–75)	83 (79–88)	86 (80–93)	94 (90–97)	68 (60–76)
1:80	65 (60–70)	87 (82–91)	81 (74–88)	89 (85–94)	77 (69–84)

The intended use of the test evaluated in this study is to assess the specific immune status of cats by detecting antibodies, either before or after regular vaccinations in veterinary practice. When evaluating a test, samples should be representative for the intended use of the test.²² Thus, a diverse population of cats that mimics the actual population in a small animal practice regarding signalment, origin, source area, environment, housing conditions, and health and vaccination status was chosen in the present study. However, this clinic population cannot be assumed to be representative for the national cat population.

In this study, 347 sera were analysed. Prevalence of antibodies against FPV was 77%, 71% and 65%, depending on the chosen HI titre cut-off point. Similar FPV antibody prevalences were described in 267 client-owned cats in the USA (67%).⁸ A remarkably lower antibody prevalence was found in cats entering a Florida (USA) animal shelter (40%). In that study, 67% of the cats entering the shelter were stray cats. In these cats, a low vaccination rate is the most reasonable explanation for the low antibody prevalence at the time of blood sampling. In addition, differences in environmental exposure to FPV can be a reason for the different prevalences.

For the detection of antibodies against FPV, HI served as the gold standard in this study.^{8,11,12} The method is very specific for the virus, and the technique is simple and well established. In addition, equipment and reagents are quite inexpensive. However, this test cannot be performed in practice. Another limitation of the method is that reading the plate is subjective, which could lead to false-positive or false-negative results. However, to minimise subjective evaluation, HI plates were read by two independent people: one was the first author (KM) and one was an experienced laboratory technician. Divergent results were checked by a second laboratory technician.

Specificity was set as the most important parameter in this study. For use in the context of an individual health assessment and as a tool for deciding whether a cat needs vaccination, it is essential to obtain a low number

of false-positive test results, so that potentially unprotected cats can be identified.

The manufacturer of the ImmunoComb Feline VacciCheck declares a HI titre cut-off point of $\geq 1:80$ as a positive result. However, when considering a HI titre cut-off point of 1:80 as a positive result, the specificity of the ImmunoComb Feline VacciCheck in this study would only be 81%. There was a relatively high number (23) of false-positive test results. The PPV was still relatively high (89%), which, however, is influenced by the high antibody prevalence in this study. According to the predefined criterion, the specificity of the test was not good when basing the result on a HI titre cut-off point of 1:80. This specificity would be remarkably lower than those described previously for the ImmunoComb Feline VacciCheck, for example, in the shelter study in Florida, USA (99%),¹⁴ and in the manufacturer's product information (98%). On the contrary, the sensitivity of the test (87%) was higher than in the shelter study in Florida (49%),¹⁴ but not as good as declared by the manufacturer (90%). In this study, 30 sera tested false-negative. False-negative results, however, are not as problematic as false-positive results. Cats with false-negative test results will receive a booster vaccination even if they are protected against infection at the time of blood sampling. The difference between test results in the shelter study and the present study, as well as the declaration by the manufacturer concerning sensitivity and specificity, could be due to recent modifications to the test by the manufacturer.

It is still debated which antibody titre is equivalent to protection in adult cats. In former studies, even lower ($< 1:80$) antibody titres were considered to be predictive for protection against FPV infection in cats that were formerly vaccinated or who had overcome infection.^{13,23} In one study, cats were considered to be protected against infection with FPV if they had a titre of $\geq 1:40$ in HI before vaccination.¹³ Considering a HI titre cut-off point of 1:40 as protective, the specificity of the ImmunoComb Feline VacciCheck in this study would be 86%.

At present, however, the presence of antibodies even at a low concentration (such as titres of 1:20) is considered protective in cats that have been vaccinated after maternally derived antibodies have dropped, or in cats that have overcome infection, as these indicate a response of the immune system to an antigen.^{8,24,25} In challenge experiments, the presence of antibody titres of 1:20 in previously vaccinated cats was predictive for protection against disease.⁸ When basing the results of the present study on a HI titre cut-off point of 1:20, specificity of the ImmunoComb Feline VacciCheck was 89% and thus almost reached the predefined criterion for a good test performance. Based on this HI titre cut-off point, the test showed a low number (nine) of false-positive results, which is important in helping to decide whether a cat requires FPV vaccination.

The test has some benefits concerning the practicability compared with titre testing at a laboratory. As the test can be performed in about 21 mins, results are available during consultation and can be used immediately for decision-making. Furthermore, up to 12 samples can be processed in parallel. Another advantage is the low amount of blood required. The test just requires 5 µl of serum or plasma, or 10 µl of whole blood, whereas for HI only serum can be used and at least 100 µl is needed.

As FHV-1 and FCV results were not evaluated in this study, no recommendation can be given about the test's usefulness in determining FHV-1 and FCV antibodies in the field of small animal practice.

One limitation of the study is that the amount of antibodies indicating protection is still unclear. A true protection can only be determined by challenge experiments, which, of course, was not possible in this study with privately owned cats.

Conclusion

The ImmunoComb Feline VacciCheck showed a high specificity to detect antibodies against FPV. When considering an antibody titre of 1:20 in HI to be protective, the test almost reached the predefined criterion of 90% (89%) for a good performance and at least delivered acceptable results. Thus, the test can be recommended for use in veterinary practice to help in deciding whether a cat requires FPV vaccination. However, further modification by the manufacturer aiming for an even higher specificity of the test would be desirable to reduce the risk of missing and not vaccinating unprotected cats.

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Conflict of interest The authors do not have any potential conflicts of interest to declare.

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