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Evaluation of Rapid Immunochromatographic Tests for Serological Diagnosis of Feline Immunodeficiency Virus and Feline Leukemia Virus Infection in Cats

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Abstract: Two one-step immunochromatographic (IC) tests were developed for the detection of Feline Immunodeficiency Virus (FIV) antibodies and Feline Leukemia Virus (FeLV) antigen respectively, on serum (for FIV) and on serum and whole blood (for FeLV) feline samples. Two panels of sera were evaluated by a comparative test with 3 commercially available IC tests and 4 commercially available enzyme-linked immunosorbent assays (ELISA). The results of the evaluation showed an agreement between the FIV-IC and the other IC tests and the ELISA between 95.4 and 97.3%, whereas the agreement between the FeLV-IC and the other IC tests and ELISA varied between 93.7 and 100%. Further analysis of serum samples comparing the FIV-IC test with an ELISA reference test yielded a sensitivity of 94.7% and a specificity of 96.1%, whereas comparing the FeLV-IC test with an ELISA reference test, the same samples yielded both a sensitivity and a specificity of 100%. The advantages offered by the IC test (rapidity, easiness to perform and to interpret, as wells as low cost) make the test itself a useful tool for diagnosing feline retroviral infections.

Key words: Feline, retroviral infections, FIV, FeLV, cats

Introduction

Feline Immunodeficiency Virus (FIV) is an enveloped single-stranded RNA virus belonging to the lentivirus subgroup of retroviruses, responsible for a severe acquired chronic immunodeficiency in cats, leading to death of infected animals (Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988 and Pedersen *et al.*, 1989). Although the definitive test for FIV is represented by isolation of infective virus from peripheral blood lymphocytes (Pedersen *et al.*, 1987), this method is time-consuming and too expensive for routine laboratory practice. Fortunately, almost all infected cats develop antibodies directed against several viral proteins; for diagnostic purposes, the major p24 protein and, at a lesser extent, the p17 protein are considered to be valuable antigens for immunodetection tests (Pedersen *et al.*, 1989 and Reid *et al.*, 1991).

Several FIV antibody tests are now available, based on various techniques: enzyme-linked immunosorbent assay (ELISA), one-step immunochromatographic test (IC), immunofluorescence assay (IFA) using FIV-infected lymphocytes, Western blot, radio-immunoprecipitation assay and polymerase chain reaction (Pedersen *et al.*, 1987; Gruffydd-Jones *et al.*, 1988; Lutz *et al.*, 1988a; Lutz *et al.*, 1988b; Yamamoto *et al.*, 1988; O'Connor *et al.*, 1989; Reid *et al.*, 1991; Furuya *et al.*, 1992; Hohdatsu *et al.*, 1992; Mermer *et al.*, 1992; Calzolari *et al.*, 1995; Sibille *et al.*, 1995 and Robinson *et al.*, 1998).

As FIV, Feline Leukemia Virus (FeLV) belongs to the family Retroviridae, but is included in the subfamily Oncornaviridae, due to its well defined oncogenic properties (Jarret *et al.*, 1964 and Hardy, 1981). Infected cats show several different syndromes, including both proliferative and degenerative conditions; persistently viremic animals have a half life of 1 year. Due to the profound immunosuppressive properties of the virus, FeLV-infected cats do not usually produce high levels of antibodies, making serological tests for diagnostic purposes not very widely used. The most used sample for FeLV testing is represented by blood, as cats with persistent infection are viremic; both free viral antigen and cells loaded with FeLV antigen are present in blood.

Virus isolation, detection of plasma antigen by ELISA , detection by IFA of FeLV in neutrophils on blood smears, as well as IC for detection of blood antigen may be employed for diagnostic purposes (Hardy *et al.*, 1973; Kahn *et al.*, 1980; Hirsch *et al.*, 1982; Jarret *et al.*, 1982; Waits *et al.*, 1982; Lopez *et al.*, 1989; Hardy and Zuckerman, 1991; Hawks *et al.*, 1991; Swango, 1991 and Robinson *et al.*, 1998).

Of these tests, the most widely used in the veterinary practice for both FIV and FeLV are represented by ELISA and IC. Since a few years, the latter test has become particularly popular in the veterinary laboratory for "in practice" testing, due to the easiness of handling, low cost, sensitivity, reproducibility and rapidity (giving a result in a few minutes of time). Several commercially available kits, based on the IC technique for detection of FIV antibodies and FeLV antigens, have already been investigated for their

performances, showing different degrees of sensitivity and specificity (Swango, 1991 and Robinson *et al.*, 1998).

The aim of this study is to compare the performance of two one-step immunochromatographic tests for the detection of FIV antibodies and FeLV antigens respectively in serum or whole blood of cats, in comparison with other commercially available established tests based on ELISA and IC techniques.

Materials and Methods

Samples: A first panel of sera (panel 1) from naturally infected and uninfected cats presented for veterinary consultation was used for the study. The sera samples were coded, stored frozen at -70°C and tested concurrently with FIV-IC, FeLV-IC, 2 commercially available IC tests (Witness FeLV-FIV, Synbiotics, San Diego, USA; Speed Duo FeLV-FIV, Bio Veto Test, La Seyne-sur-Mer, France;) and 5 commercially available ELISA (Snap Combo Plus, Idexx, Westbrook, USA; Ingezim FIV-Vet, Ingenasa, Madrid, Spain; Ingezim FeLV-Vet, Ingenasa, Madrid, Spain; ViraCHECK/FIV, Synbiotics, San Diego, USA; ViraCHECK/FeLV, Synbiotics, San Diego, USA).

A second panel of sera or whole blood (panel 2) from naturally infected and uninfected cats presented for veterinary consultation was used. The samples were coded and tested concurrently soon after submission with FIV-IC, FeLV-IC and a commercially available ELISA (Speed Duo FeLV-FIV, Bio Veto Test, La Seyne-sur-Mer, France).

All the tests were performed according to the manufacturer's instructions.

In case of discrepant results, samples from panels 1 and 2 were further tested by Western blotting for antibodies to FIV protein p24 (Laemmli, 1970 and Towbin *et al.*, 1979) (courtesy of Prof. H. Lutz, University of Zurich, Switzerland).

FIV-IC: The basic component of the test (FIV-IC Agrolabo, Romano Canavese, Italy) is represented by a chromatographic 7x53 mm strip with a FIV peptide cocktail immobilized to the test zone (T) (Fig.1). Polyclonal antibodies raised in goat directed against mouse IgG (Sigma, St. Louis, USA) are permanently attached to the chromatographic strip in a control zone (C). A mouse monoclonal antibody directed against cat IgG has been conjugated with 20nm colloidal gold particles (Biocell, Cardiff, U.K.) and bound onto a conjugate pad (CP). An absorbent pad (AP) is positioned on one extremity of the chromatographic strip. The assembly is sealed within a small plastic device provided of three windows (Fig. 2). After adding 10 μl of serum sample diluted 1:50 on the chromatographic strip in windows 3, a few drops of diluent (PBS, 1% Tween 20, 1% BSA) added in window 1 solubilize the colloidal gold conjugated antibody and the complex migrates up the chromatographic strip. If anti-FIV antibodies are present in the sample, they will be captured by the antigen in the test zone (window 2), to form a pink line. The remaining conjugate will be captured by the control zone reagent to form a second pink line in window 3. If no serum antibodies are present, only the control line will be visible. The test results are visually interpreted 10 minutes after adding the sample.

FeLV-IC: The device (FeLV-IC Agrolabo, Romano Canavese, Italy) is based on the same principle of the FIV IC test, having instead two anti-FeLV monoclonal antibodies directed against different viral epitopes of p27 protein in the test zone and in the conjugate pad respectively, the latter conjugated with 40 nm gold particles. After adding the serum or the whole blood and the diluent on the conjugate pad in window 1, the sample solubilizes the colloidal gold antibody. If FeLV antigen is present in the serum, a pink line will appear in association with the test line.

In order to perform a preliminary evaluation of the sensitivity of the test itself, an assay was run using a panel of two different antigens, represented by a FeLV p27 recombinant protein (at a twofold dilution range from 1:25, to 1:200) and a gradient-purified fraction of FeLV (at a twofold dilution range from 2 μg ml^{-1} to 128 μg ml^{-1}), on both the FeLV-IC and the Ingezim FeLV-Vet. The higher dilution of the FeLV p27 recombinant protein detected by the FeLV-IC test was 1:200.

Determination of Agreement, Sensitivity and Specificity: The agreement between the FIV-IC and FeLV-IC tests and the other commercially available ELISA and IC tests was separately determined for the two panels of sera under investigation.

Separate sets of sensitivity and specificity values were also calculated for the sera samples from panel 1 in comparison with the Ingezim FIV-Vet and Ingezim FeLV-Vet.

Sensitivity and specificity calculations were determined utilising the following formulas (Hardy, 1991):

$$\text{sensitivity} = \frac{nA}{nA + nB}$$

$$\text{specificity} = \frac{nC}{nC + nD}$$

where: nA = number of samples resulted positive with the reference test.

nB = number of samples resulted positive with the

observed. The cell monolayer was washed with deionized water until excess violet was removed. After drying, the plate was observed under inverted microscope and data was calculated to determine $TCID_{50}$. The final $TCID_{50}$ was the mean of four counts.

Serum neutralization Test (SNT): The cell suspension was prepared as the same way of $TCID_{50}$ assay. A 96 well plate was recognized as rows 1–6 for homologous SNT and rows 7–12 for non-homologous SNT. Then 180 µl of pre-warmed HBBS was added to all wells of column A and 100 µl of pre-warmed HBBS was added to all the remainder wells of plate. Twenty µl reovirus-specific serum (anti-reovirus antibody previously collected form Professor M. Yoshima, (Consultant, Japan International Co-operation Agency {JICA}, BLRI, Savar, Dhaka) was added to all wells of column A except A6 and A12 because they were serum control. Wells of row 6 and 12 and column H did not contain any serum because they were control. Then 100 µl serum suspension from column A was transferred to column B. Two fold dilution of serum through column A to G was performed by transferring 100 µl of suspension from column B to C, C to D and so on. Hundred µl virus suspension (10^2TCID_{50}) was added to all wells of the plate and 100 µl suspension was poured off from all wells. The plate was incubated at 37° C for 45 min. Then 200 µl suspension of vero cell ($5X10^5$ cells ml^{-1}) was added to all wells of the plate. The plate was incubated at 37° C for 3 days and observed twice per day for CPEs. Finally, the plate was stained as the same way of $TCID_{50}$ assay and observed under microscope to obtained data. In this test, wells of column 1-5 were for SNT against reovirus, column 7–11 were against Newcastle disease virus (NDV) and wells of row 6 and 12 were control.

Results

Cytopathic Effects (CPEs): Cytopathic effects involved rounding, granulation, vacuolization, clumping, syncytia formation of vero cell monolayer due to infection by reovirus. Following 24 h of infection no CPEs was found, the cells were looked as confluent monolayer (Fig. 1). After 24 h of infection, the cells were gradually started to changes in shape to produce CPEs. CPE was characterized by granularity in cytoplasm, rounding of infected cells, development of microplaque, clustering of infected cells, intracytoplasmic bridge connecting those clusters, vacuolization in the cell system and formation of syncytia. After formation of syncytia, the nuclei of the few Vero cells were aggregated in several places on the monolayer. These multinucleated aggregated cells were defined as giant cells (Fig. 2). During the terminal stage of CPE, the whole monolayer showed maximum degeneration of cells and large gaps throughout the monolayer, which are called plaque (Fig. 3). Plaques were formed during 36 to 42 h following infection. The plaques of Reovirus on vero cells have special characteristics that are clear, red as well as intermediate turbid form and several size classes ranging from 0.5 to 4.0 mm in diameter.

Day-by-day observed CPES were

* About 0 to 24 h following infection: Vero cell monolayer
* About 24 to 28 h following infection: Rounding, granulation and vacuolization of cells
* About 30 h following infection: Syncytia formation
* About 36 h following infection: Giant cells formation
* About 36 to 72 h following infection: Plaque formation

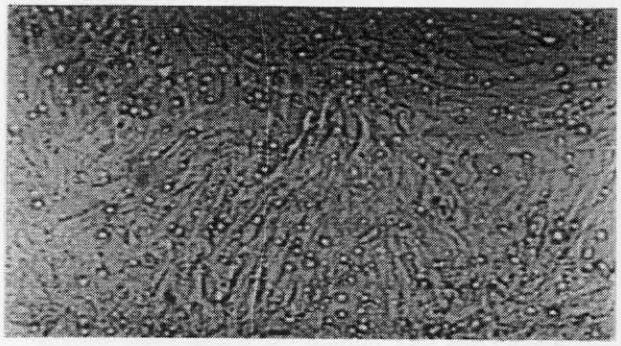

Fig. 1: Vero cells infected by reovirus following 24 h of infection

Outcome of $TCID_{50}$: About 36 h after infection reovirus produced suitable CPEs on vero cells. Stained plate was observed under microscope for percentage of CPE or cell damage obtained at each well (Table 1). Column H and row 6 did not produce any cell damages, because they were virus control. Wells of column A, B, C and D showed 100% CPE and column G did not produce any CPE. Wells of column E and F produced fractionated CPE, that is, column E showed 90% CPE,

Table 1: Data obtained from $TCID_{50}$ assay

Column	Log_{10} titer of virus dilution	Observed CPE percentage
A	0	100
B	-1	100
C	-2	100
D	-3	100
E	-4	90
F	-5	30
G	-6	0
H	No virus	0

Table 2: Data obtained from SNT

Type of SNT	No. of row	No. of column	Dilution of serum	Observed CPE percentage
Homologous SNT		A	1:10	0
		B	1:20	0
		C	1:40	0
		D	1:80	0
	1-5	E	1:160	20
		F	1:320	40
		G	1:640	80
		H	No serum	100
	6	A, B, C, D, E, F, G, H	No serum	100
Non-homologous SNT		A	1:10	90
		B	1:20	100
		C	1:40	100
		D	1:80	100
	7-11	E	1:160	100
		F	1:320	100
		G	1:640	100
		H	No serum	100
	12	A, B, C, D, E, F, G, H	No serum	100

F showed 30%. These observed data were sufficient to calculate $TCID_{50}$ titer of P3 adapted reovirus sample by using Karber method (Karber, 1931) and it was found that the infectivity titer of P3 adapted reovirus sample was $10^{-5.5}TCID_{50}$.

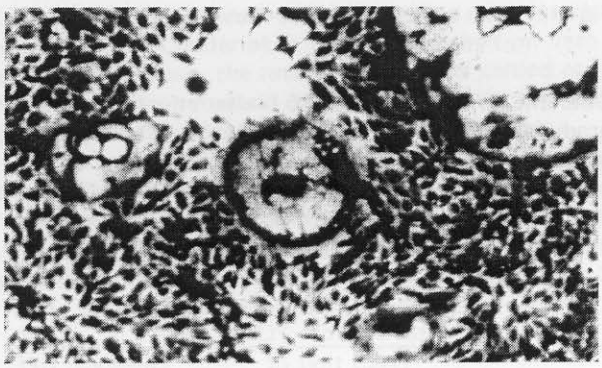

Fig. 2: Giant cell formation in vero cell monolayer following 30-36 hr of infection by reovirus

Outcome of SNT: It is found that all control wells (wells of row 6, 12 and column H) showed 100% CPE, because they did not contain any serum and all the viruses were active. In case of homologous SNT (row 1-5), column A, B, C and D did not show any CPE, because high dilution of serum present at that dilution which inactivated the virus. Column E, F and G showed 20%, 40% and 80% CPE respectively. In case of Non-homologous SNT (row 7-11), about 100% CPE was found all over the wells, because reovirus-specific serum can not neutralize any other virus. From the data obtained from SNT (Table 2), it is found in case of homologous SNT, CPE was obtained at 1:80 dilution of serum and the titer of virus used was 10^2TCID_{50}. The

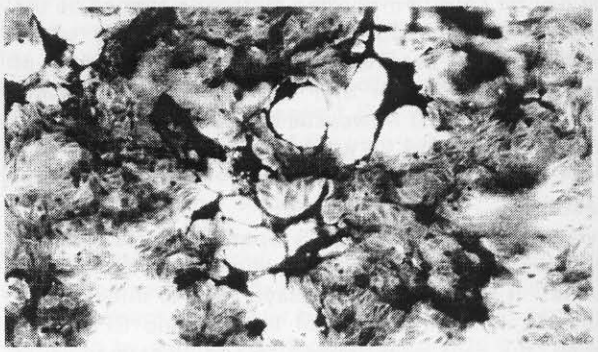

Fig. 3: Plaque formation in vero cell monolayer following 72 hr of infection by reovirus

homologous SNT against reovirus was $0.8x10^4$ units per ml. In case of non-homologous SNT, CPE was observed only in 1:10 dilution and the titer of virus used was 10^2TCID_{50}. The non-homologous SNT against NDV was $0.1 x10^4$ units per ml and it was negative.

Discussion

To adapt reovirus on vero cell line, the collected reovirus was given three serial passages on vero cells.

The resultant CPEs on each passage was observed carefully. Virus from each passage were harvested and clarified by centrifugation. The infectivity titer of reovirus present in each passage suspension was measured by $TCID_{50}$ assay. Homologous SNT as well as non-homologous SNT against reovirus recognized the purity of reovirus on passaged suspension, which was prevalent at that laboratory. During the first passage of reovirus on vero cells, P1 virus did not produce clear evidence of CPEs. The virus started to adapt on vero cells and their infectivity to vero cells were low. During the second passage, some changes in vero cell monolayer began to develop after 30 h of incubation following infection. Monolayer showed rounding, granulation, clumping of infected cells and vacuolization in the cell cytoplasm. During the third passage, CPE was rapid and consistent. In addition to above changes in cell monolayer, a large number of clear syncytia were observed after about 30-36 h of infection which were followed by formation of multinucleated giant cells. A large number of clear, red as well as intermediate turbid form plaques were also observed after 72 h of infection. The size classes of plaque ranged from 0.8 to 4.4 mm in diameter. These P3 viruses were well-adapted on vero cell lines.

Sil *et al.* (1996) studied the adaptation of Pesti des Petits Ruminants (PPR) virus on vero cell line after 5 serial passages. Virus produced typical CPEs in cells, such as formation of dendritic shaped cells, syncytium and gaint cells. Highest titre of virus was found on 96 hours post-infection, which decreased with increasd time of incubation. According to the findings of Peilin *et al.* (1997), complete CPEs of IBDV on vero cell line was stably produced in 65 to 72 h of inoculation during 4th passage. The present experiment showed that complete CPEs of reovirus were observed on vero cell line following 72 h of incubation during 3rd passage. This observation was found consistent with the findings of Sil *et al.* (1996) and Peilin *et al.* (1997).

Following three serial passage of reovirus on vero cell line, P3 viruses became progressively more cytopathogenic or infective but less virulent to vero cells. The infectivity titer of P3 viruses was $10^{-5.5}$ $TCID_{50}$. It indicates that $10^{-5.5}$ times diluted vero cell adapted P3 reoviruses were able to produce CPE in the 50% of the cell cultures inoculated. The purity of these P3 viruses was tested by SNT by using specific serum against reovirus. In case of homologous SNT test against reovirus, the result found was $0.8x10^4$ units per ml, which was very positive. In case of non-homologous SNT against reovirus, the result was $0.1x10^4$ units per ml almost negative. Compared to heterologous serum (anti-reovirus antibody), which clearly indicated that the resultant virus following adaptation in ver cell was reovirus.

Further study may help to determine the attenuation of virus for the production of live vaccine and mass production of test antigen.

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