Chapter 3

Serological Diagnosis of Feline Coronavirus Infection by Immunochromatographic Test

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Abstract

The immunochromatographic assay (ICA) is a simple antibody–antigen detection method, the results of which can be rapidly obtained at a low cost. We designed an ICA to detect anti-feline coronavirus (FCoV) antibodies. A colloidal gold-labeled recombinant FCoV nucleocapsid protein (rNP) is used as a conjugate. The Protein A and affinity-purified cat anti-FCoV IgG are blotted on the test line and the control line, respectively, of the nitrocellulose membrane. The specific detection of anti-FCoV antibodies was possible in all heparin-anticoagulated plasma, serum, whole blood, and ascitic fluid samples from anti-FCoV antibody positive cats, and nonspecific reaction was not noted in samples from anti-FCoV antibody negative cats.

Key words Feline coronavirus, Immunochromatographic assay, Serological diagnosis

1 Introduction

Feline coronavirus (FCoV) is composed of nucleocapsid (N) proteins, membrane (M) proteins, and spike (S) proteins. FCoV has been classified into serotypes I and II according to the amino acid sequence of its S protein [1, 2]. Both serotypes consist of two biotypes: feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV). FECV infection is asymptomatic in cats, whereas FIPV infection causes lethal disease: FIP [3]. FIPV (virulent FCoV) has been proposed to arise from FECV (avirulent FCoV) due to a mutation [4–6]; however, the exact mutation and inducing factors have not yet been clarified.

It is normally comprehensively diagnosed based on the clinical condition, hematological profile, and results of FCoV genomic RNA and anti-FCoV antibody measurements in cats suspected of FIP [7]. An indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA) are used to measure FCoV-antibodies. IFA and ELISA are highly sensitive and specific, but are cumbersome, expensive, and time-consuming.

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A simple and rapid method is necessary to prevent an epidemic of FCoV infection. A low-cost method is also needed to measure anti-FCoV antibodies in cats maintained in multi-cat environments. The most appropriate diagnostic method meeting these conditions may be immunochromatographic assay (ICA). The detection of anti-FCoV antibodies using ICA requires no special device or reagent, and the results can be simply and rapidly obtained. In this chapter, we describe our protocol for the preparation of the ICA to detect anti-FCoV antibodies using recombinant FCoV N protein [8].

2 Materials

2.1 Recombinant FCoV N Protein (rNP)

1. Plasmid DNA: pGEX4T-1 (GE Healthcare) with the N gene of the type I FIPV KU-2 strain (Gene Accession No. AB086881.1) (*see* **Note 1**).
2. Competent *Escherichia coli*, e.g., strain BL-21.
3. LB broth: 1.6 % (w/v) Bacto Tryptone, 1.0 % (w/v) Bacto Yeast Extract, and 0.5 % (w/v) NaCl in ddH_2O, and adjusted to pH 7.0 with 5 N NaOH.
4. 100 mg/ml ampicillin sodium in water.
5. 100 mM isopropyl β-D-L-thiogalactopyranoside (IPTG) in water.
6. 10 mg/ml lysozyme in water.
7. 100 mM phenylmethylsulfonyl fluoride (PMSF) in methanol.
8. 1 mg/ml DNase I in 0.15 M NaCl.
9. 0.1 mg/ml sodium deoxycholate in water.
10. Elution buffer: 0.3 % (w/v) reduced glutathione in 0.1 M Tris–HCl, pH 8.0.
11. Sonicator.

2.2 Capture Agent

1. 0.5 mg/ml purified IgG from serum of FCoV-infected cat (*see* **Notes 2** and **3**).
2. 2.0 mg/ml monoclonal antibody (mAb) YN-2 (anti-FCoV N protein; IgG2a) (*see* **Note 4**).
3. 0.1 mg/ml Protein A.

2.3 ICA Test Strip

1. Sample pad and absorbent pad: C083 Cellulose Fiber Sample Pad Strips (Millipore).
2. Nitrocellulose membrane: Hi-Flow Plus 240 Membrane Cards (Millipore).
3. Automatic cutter, e.g., CM4000 (BioDot) or scissors.
4. Dispensing machine, e.g., XYZ3050 (BioDot) or fine-point brush.

2.4 Colloidal Gold-Labeled rNP

1. Diluting/preserving solution: 20 mM sodium tetraborate, 1 % (w/v) bovine serum albumin (BSA), and 0.1 % (w/v) NaN_3 in water.
2. Colloidal gold solution (40 nm).
3. 10 % (w/v) BSA in water.
4. Borax containing 10 % BSA.

3 Methods

3.1 Preparation of rNP

1. Incubate BL-21 cells containing pGEX4T-1 with the N gene of the type I FIPV KU-2 strain overnight at 37 °C in 10 ml of LB broth containing 100 μg/ml ampicillin (LB/AMP broth).
2. Dilute overnight cultures 1:100 in 100 ml of fresh LB/AMP broth and grow to OD_{600} of 0.4–0.5.
3. Induce expression of GST-tagged rNP by adding 100 μl of 0.1 mM IPTG to the culture.
4. Incubate for 24 h at 25 °C in shaking incubator.
5. Centrifuge at 12,000 × *g* for 15 min and resuspend the cell pellet in 30 ml of PBS.
6. Add 0.8 ml of 10 mg/ml lysozyme and 0.4 ml of 100 mM PMSF to the suspension and mix.
7. Incubate on ice for 20 min.
8. Add 0.3 ml of 0.1 mg/ml sodium deoxycholate.
9. Lyse bacterial cells by three 30 s pulses of sonication on ice using a sonicator.
10. Add 0.2 ml of 1 mg/ml DNase I to the cell lysate and incubate for 30 min at 25 °C.
11. To remove bacterial debris, centrifuge cell lysate at 12,000 × *g* for 15 min at 4 °C.
12. Wash glutathione sepharose beads by adding 40 ml of PBS per 6.7 ml of original slurry of glutathione sepharose (*see* **Note 5**).
13. Add supernatant to the washed glutathione sepharose beads, and rotate overnight at 4 °C.
14. Wash sepharose beads with bound GST-tagged rNP in PBS at for 5 h at 4 °C with rotation.
15. Spin down the beads at 700 × *g* for 5 min at RT, and resuspend in 40 ml of PBS per 6.7 ml of original slurry.
16. Spin down the beads at 700 × *g* for 5 min at RT.

17. Elute GST-tagged rNP from beads using 10 ml of Elution buffer at RT, and 1 ml of fractions (0.2 ml/tube) are collected into test tubes (*see* **Note 6**) and analyzed by SDS-PAGE and Western immunoblotting, using standard procedures.
18. Eluted peak fractions (tube no. 18–22) are pooled and dialyzed against PBS using dialysis tubing for overnight.

3.2 Preparation of the ICA Test Strip

The ICA test strip consists of three main components: a sample pad, nitrocellulose membrane, and absorbent pad (Fig. 1a, b).

1. Stick the sample pad and absorbent pad onto the nitrocellulose membrane using adhesive tape, overlapping by 3 mm.
2. Cut this sheet into 2 cm strips using an automatic cutter or scissors.
3. Dispense 20–30 μl of protein A onto the test line of the nitrocellulose membrane using a dispensing machine or a fine-point brush (*see* **Note 7**).
4. Dispense 20–30 μl of affinity-purified IgG from serum of FCoV-infected cat or mAb YN-2 onto the control line of the nitrocellulose membrane using a dispensing machine or a fine-point brush.
5. Dry the membrane for 30 min at room temperature and then cut into 0.5 cm strips using an automatic cutter or scissors.

3.3 Preparation of Colloidal Gold-Labeled rNP

1. Dilute rNP in PBS to 0.5 mg/ml.
2. Add 30 μl of the diluted rNP to 1 ml of colloidal gold solution.
3. Stir well and incubate for 30 min at room temperature.

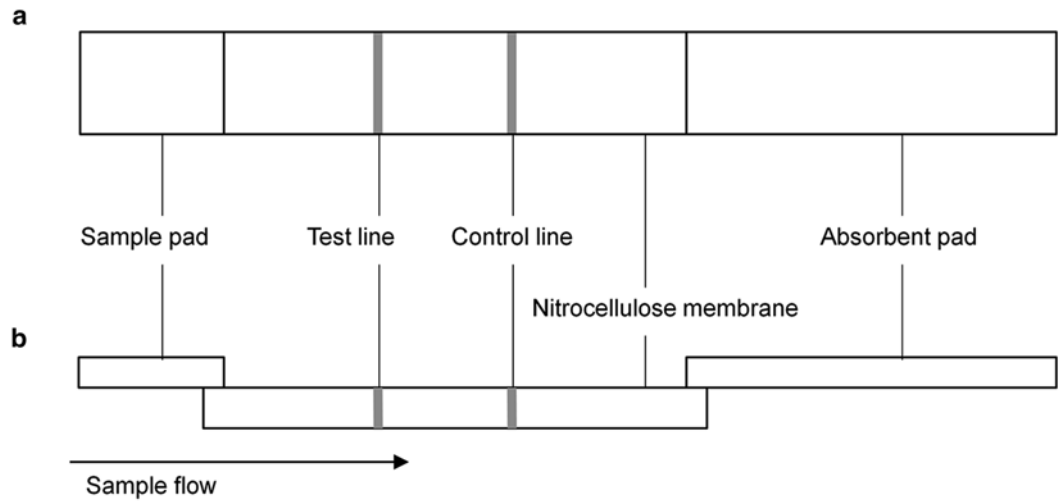

Fig. 1 Schematic diagrams of ICA test strip. (a) Top view and (b) Side view

4. Add 100 µl of 20 mM Borax containing 10 % BSA.
5. Incubate for 30 min at room temperature.
6. Centrifugation at 22,000 × *g* for 10 min and resuspend the pellet in 0.75 ml of 20 mM Borax containing 10 % BSA.

3.4 Procedure for ICA Test

A schematic of the principle of the ICA test is provided in Fig. 2a.

1. Dilute the sample (i.e., plasma, serum, and effusive fluid) 80 times with eluent solution (*see* **Note 8**).
2. Mix 40 µl of this dilution with 20 µl of the colloidal gold-labeled rNP (from Subheading 3.3, **step 6**) in the well of a 96-well plate.
3. Insert the ICA test strip into the well of the 96-well plate and allow mixture to be absorbed (Fig. 2b).
4. The test line or/and control line will appear after 10 min at room temperature (Fig. 2c).

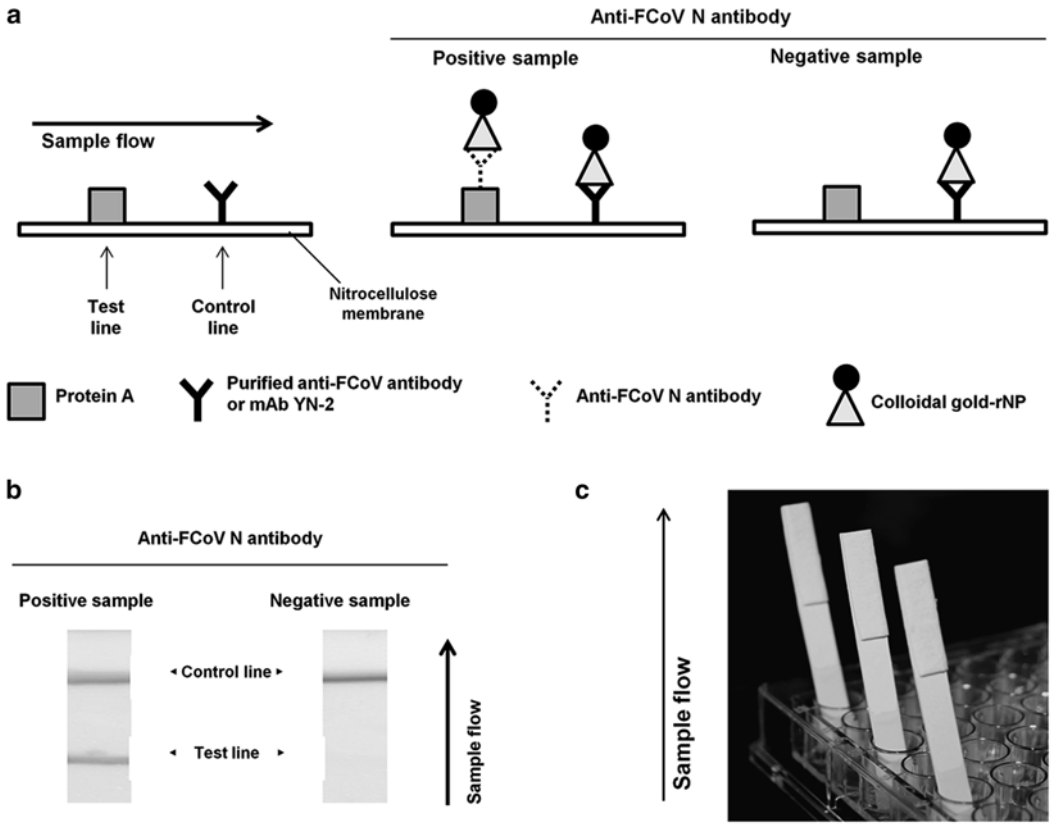

Fig. 2 Principle of the ICA test and example results. (a) Outline of the principle of anti-FCoV antibody detection. (b) The sample pad at the end of the ICA test strip is dipped in the sample mixture. (c) Typical positive and negative results. C: control line (mAb YN-2). T: test line (Protein A)

4 Notes

1. Based on our experience, N protein is more efficiently expressed in *E. coli* than other structural proteins (S protein and M protein) of FCoV.
2. Feline IgG is isolated by affinity chromatography on a Protein G column.
3. Serum and plasma from FCoV-infected cats are good source of cat anti-FCoV IgG. However, large amount of serum and plasma are needed for developing ICA test. It is practically difficult to obtain large amounts of serum and plasma from FCoV-infected cats. Therefore, we recommend using anti-FCoV mAb instead of cat anti-FCoV IgG.
4. The Hybridoma producing mAb YN-2 was prepared following the method reported by Hohdatsu et al. [1]. In our experience, the mAb YN-2 has a higher affinity for the colloidal gold-labeled rNP than other anti-FCoV N mAb (e.g., mAb E22-2).
5. Glutathione sepharose beads are just added to PBS and then they are ready to use.
6. GST-tagged rNP was eluted from the beads by drip-through at a constant flow (2.0–3.0 ml/min).
7. Generally, reagents are dispensed by the dispensing machine on a nitrocellulose membrane. However, this machine is very expensive. If you intend to develop an ICA kit on a trial basis, we recommend using a fine-point brush instead of the machine.
8. The specific detection of anti-FCoV antibodies was possible in all heparin-anticoagulated plasma, serum, whole blood, and ascitic fluid samples from anti-FCoV positive cats. On the other hand, the nonspecific test line formation was noted in EDTA- or sodium citrate-anticoagulated plasma of anti-FCoV negative cats.

Acknowledgments

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