Original Article

A rapid fecal immunochemical test for gastrointestinal bleeding detection in cynomolgus macaques (*Macaca fascicularis*)

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Abstract

We used the Fecal Immunochemical Occult Blood Test (FIT) kit, a lateral flow immunochromatographic assay specific for human hemoglobin, on cynomolgus monkeys (*Macaca fascicularis*). We aimed to employ this test to detect gastrointestinal injury in captive animals at the National Primate Research Center of Thailand-Chulalongkorn University (NPRCT-CU). The cross-reactivity and sensitivity of the test for monkey blood were determined and compared with the human blood. The anti-human hemoglobin antibody of the FIT kit reacted with the monkey blood in a similar way as it did with humans and the intensity (T/C ratio) values between the two data sets closely correlated ($R^2 = 0.9324$, $p>0.05$). Although the specificity for monkey blood was 4.2 times lower than for human blood, monkey blood with a dilution as low as 1:256,000 could be detected in monkey fecal samples. Thus, we used the FIT kit to determine the gastrointestinal adverse effects of the NPRCT excipient which was orally administered daily to four female cynomolgus monkeys for 28 days. A daily visual inspection of freshly defecated fecal samples did not detect any blood but with the high sensitivity of the FIT kit the presence of hemoglobin in the feces was shown once in three of the four monkeys (3 of the 112 specimens determined). In conclusion, the human-FIT can be an applicable tool for early detection of gastrointestinal injury in captive as well as wild cynomolgus monkeys. The advantage of this kit is that it requires non-invasive fecal sample collection, requires no additional equipment and gives results that can be read by the naked eye immediately.

Keywords: gastrointestinal bleeding, diarrhea, FIT, hemoglobin, cynomolgus macaque

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Introduction

Gastrointestinal (GI) disease is one of the most frequently encountered problems seen in non-human primates (Brady *et al.*, 1998). Clinical signs of gastroenteritis may be induced by lesions within the primary GI tract or may result from other accessory organs (or the secondary GI tract) or from infectious pathogens such as bacteria. The common clinical sign of gastroenteritis is diarrhea and, to a lesser extent, vomiting (Hird *et al*., 1984; Paul-Murphy, 1993). Up to 15% of captive non-human primates exhibit diarrhea and this has raised serious concern in many primate facilities and zoos (Holmberg *et al.*, 1982; Hird *et al.*, 1984; Brady *et al.*, 1998). Some GI diseases such as gastric ulceration, inflammation of the small and large intestines from bacterial infection and GI neoplasia (e. g., adenocarcinoma) cause bleeding, melena or hematemesis together with diarrhea (Courtney, 2013). Shigellosis and campylobacteriosis are common enteric bacterial pathogens isolated from nonhuman primates (Fortman *et al*, 2018). Clinically, animals with shigellosis have voluminous diarrhea containing blood, mucous and/ or sloughed mucosal tissue (Mulder, 1971). Thus, the early detection of the blood contaminated with feces is highly critical for diagnostic, prognostic and treatment purposes and sensitive and accurate methods of detection are of the utmost necessity (Cooper *et al*., 2019).

Fecal occult blood tests (FOBTs) have been widely used in human medicine for colorectal cancer screening. Two types of FOBTs are commonly used; guaiac FOBT (gFOBT) and the fecal immunochemical test (FIT). The gFOBT may give a false-positive reading from dietary substances containing peroxidase activity such as plant peroxidase and red meat. Additionally, antioxidants such as ingested ascorbic acid can suppress peroxidase activity and cause false-negative results. Conceptually, FIT uses antibodies specific to human hemoglobin (globin chain content) in feces and, therefore, is less likely to be susceptible to non-human hemoglobin peroxidase activity (Sokoro *et al.*, 2020). Although some researchers have used gFOBT in diagnosing GI disease in rhesus (*Macaca mulatta*) and cynomolgus macaques (*M. fascicularis*) (Suzuki *et al.*, 1983; Dubois *et al.*, 1996; Valverde *et al.*, 2000; Lang *et al.*, 2013; Cooper *et al.*, 2019), no FIT has been used in non-human primates.

Cynomolgus macaques are the most encountered species among the 17 species of non-human primates in Thailand. Their distribution ranges from the lower north (Pichit province) to the south (Yala province) of Thailand (Malaivijitnond et al., 2011). The Cynomolgus monkey is one of the most suitable species used for non-clinical pharmaceutical safety studies because of its similarity in the pharmacology of therapeutic targets to the humans (Bluemel *et al.*, 2015). In some oral pharmaceutical product tests, stomach and intestinal mucosal injury can occur and fecal occult blood detection is needed (Endo *et al*., 2015). Thus, this study aimed to test if the fecal immunochemical occult blood test (FIT) kit, which is specific to human blood and declares no cross-reaction with hemoglobin from various species of animals, can be used in cynomolgus monkeys. The hypothesis was initiated by the fact that amino acid sequences of α and β hemoglobin chains are highly similar between monkeys and humans (Barnabas *et al*., 1971; Hardison 2012). Thus, the cross-reactivity and sensitivity of the FIT kit for monkey blood in comparison with human blood and the efficiency of the detection for fecal occult blood in cynomolgus monkey was investigated.

Materials and Methods

Blood and fecal sample collection for the fecal immunochemical occult blood test: Monkey blood and monkey fecal samples were collected from one healthy juvenile female cynomolgus monkey, aged 3.5 years old and 2.8 kg body weight. The animal was housed individually in a stainless-steel cage at the National Primate Research Center of Thailand (NPRCT), Chulalongkorn University, Saraburi, Thailand. The animal holding area was environmentally controlled with 15 air changes per hour; the temperature setting was 25 ± 1 °C; and the relative humidity was set at 50 ± 10%. Artificial lighting was controlled to give a cycle of 12 h dark and 12 h light (light on at 0600 h). The monkey was fed twice daily with a commercial diet for primates (Perfect Companion Group Co., Ltd, Thailand) in the morning (0900 – 1000 h) and fresh fruit in the afternoon (1400 – 1500 h). Water was provided ad libitum. The facility had been AAALAC International Accredited (1752). All animal procedures were approved by the NPRCT, Chulalongkorn University Animal Care and Use Committee (Protocol review no. 2075008).

The monkey was anesthetized by intramuscular injection of tiletamine/zolazepam (Zoletil 100®: Virbac animal health; Inc.) 3 mg/kg mixed with dexmedetomidine hydrochloride (Dexdomitor®: Zoetis; Inc) 0.03 mg/kg. A blood sample was collected by saphenous venipuncture with 23G x 1″ (Nipro Corporation, Japan), transferred into a lithium heparin tube (Zhejiang Kangshi Medical Devices, Co., Ltd, China) and mixed well before use. After the blood collection had been completed, the monkey received an antidote by intramuscular injection of atipamezole hydrochloride (Antisedan®: Zoetis; Inc) in the same volume as the dexmedetomidine hydrochloride, then the veterinarian carefully observed the monkey until she had fully recovered.

Freshly defecated fecal samples were collected from a tray under the cage. Fecal samples were collected following the manufacturer's instruction (One Step Fecal Occult Blood Test Device; ABON®-Abon Biopharm (Hangzhou) Co., Ltd., China). Briefly, the samples were randomly stabbed with a collection stick connected to the cap of the collecting tube provided in the kit, at at least 3 different sites, the cap screwed on and tightened on to the tubes; the tube was shaken vigorously to mix the fecal specimen and extraction buffer. The monkey fecal solution was then ready for the FIT.

Human blood (500 μL) was collected from a healthy adult male human (TK; aged 30 years; weight: 72 kg) from a median cubital vein with 23G x 1″ (Nipro Corporation, Japan), transferred into a lithium heparin tube (Zhejiang Kangshi Medical Devices, Co., Ltd, China) and mixed well before use.

Fecal immunochemical occult blood test: The One Step Fecal Occult Blood Test Device (ABON®- Abon Biopharm (Hangzhou) Co., Ltd., China) was used in this study. The assay system was a lateral rapid chromatographic immunoassay where the membrane was pre-coated with the anti-hemoglobin antibody on the test line region (T). The control line region (C) located upwardly from the T-line was the internal control for the test. The test was interpreted as positive, negative or invalid, if two distinct colored lines, only the C-line or no line appeared, respectively. The sensitivity of the assay in detecting human-fecal occult blood was 50 ng/mL or 6 µg/g feces. The company declares that the coated anti-hemoglobin antibody in the test had no cross-reactivity with bovine hemoglobin, chicken hemoglobin, pork hemoglobin, goat hemoglobin, horse hemoglobin, rabbit hemoglobin and turkey hemoglobin.

Specimen preparation for FIT: The monkey and human blood samples were diluted with normal saline solution (0.9% NaCl) at dilutions of 1:2, 1:4, 1:8, 1:16, and 1:128. For the blood test, we followed the protocol provided by the manufacturer with slight modifications. Briefly, 100 µL of each serial diluted blood sample was pipetted into extraction buffer (2 mL) in a specimen collection tube. The negative control was prepared by pipetting 100 µL of normal saline into the specimen collection tube. The specimen collection tube was held upright and the upper cap was unscrewed and opened. The specimen collection tube was inverted, squeezed to transfer 2 full drops of the solution into the loading well of the test device and the result was read within 5 – 10 minutes.

Since the C-line and the T-line were clearly seen at a dilution of 1:128 for the monkey blood test, 100 µL of monkey blood sample at a dilution of 1:128 was pipetted into the specimen collection tubes containing monkey fecal solution prepared as above (see **Blood and fecal sample collection**). This was to mimic the condition of the fecal occult blood. The test was done as had been done for the blood specimens.

After the tests, we photographed and used the Image Studio Lite (version 5.2.5; Biomedia Thailand Co., Ltd, Thailand) to analyze the C- and T-line signals (intensity) of all human and monkey blood samples. The T/C ratios were calculated.

Specificity and sensitivity of the FIT: After we had found that the antibody raised for human hemoglobin in the human-FIT kit could cross-react with the monkey hemoglobin, the specificity of the antibody was tested. Two-fold serial dilutions of the human blood (1:100 – 1:40,9600; in total 13 dilutions) and the monkey blood (1:100 – 1:25,600; in total 9 dilutions) samples were prepared. 100 µL each of the serial diluted blood samples was pipetted into the specimen collection tubes. Note that the monkey blood was pipetted into two sets (mixed and not mixed with monkey feces) of the specimen collection tubes (9 tubes for each set). After 5-10 mins of the reaction, the C-line and the T-line were read, photographed, the intensity measured and the T/C ratios were calculated. The parallelism of the dose-response curves (serial dilutions versus T/C ratios) between human and monkey blood was assessed and %cross reactivity was calculated from the following equation.

$$\%\text{cross reactivity} = \frac{\text{dilution of human blood giving 50\% T/C ratio}}{\text{dilution of monkey blood giving 50\% T/C ratio}} \times 100$$

The correlation between the T/C ratios of the human and monkey blood was tested using SPSS Statistics version 24.0. A P value less than 0.05 was accepted as a significant difference.

Using FIT to determine the gastrointestinal bleeding in cynomolgus monkeys during 28-day oral administration of the excipient: After the specificity and sensitivity of the FIT had been tested and accepted, we used the test to determine the gastrointestinal bleeding of cynomolgus monkeys after being orally administered with the enteric coated capsules comprising GRAS listed excipients (hereafter termed NPRCT excipient). A total of four sub-adult/adult female cynomolgus monkeys, aged 4 to 6 years, with body weight between 3.8 to 4.0 kg were selected from the breeding facility of the NPRCT, housed in individual cages at the research building and daily orally administered with the NPRCT excipient at a fixed dose of 56 mg for 28 days. Daily visual inspection of feces for blood and the presence of hemoglobin in the feces tested using FIT was done after the monkeys had freshly defecated on the trays under the individual cages.

Results

Cross- reactivity of the anti- human hemoglobin antibody of the human-FIT kit with monkey hemoglobin: The T-lines were visible at all dilution levels (1:2 – 1:128) of the human and monkey blood samples, except for the negative control (Fig 1). This indicates that the anti-human hemoglobin antibody of the human-FIT kit can cross-react with monkey hemoglobin. Note that at a dilution of 1:4 or lower the T line was not clearly seen. Considering the dilution factor at a range of 1:2 – 1:128, the T/C ratios tended to be increased when the dilution factors were higher and the values for both human and monkey blood samples were highest at a dilution of 1:128 (Table 1). After the monkey blood had been tested positive with the FIT, the test was done further with the monkey fecal samples containing 100 µL of monkey blood at a dilution of 1:128. At a dilution of 1:128, the T/C ratio of the monkey fecal sample (0.33) was lower than that of the monkey and human blood samples (0.45 and 0.55, respectively; Table 1).

Specificity and sensitivity of the FIT: Human and monkey blood samples showed a parallelism of the dose-response curves (serial dilutions versus T/C ratios) but not for the monkey fecal occult blood (Fig. 2). The 50% T/C ratio of human blood was at a dilution of 7,179 and at a dilution of 1,716 for the monkey blood. Thus, the % cross-reactivity for human blood was 4.18 times higher than in monkey blood. T/C ratio values of the human blood highly correlated with the monkey blood (R2 = 0.9324; Fig. 3). Since the dose-response curve of the monkey fecal occult blood did not show

any parallelism with the other two sets of data, the %cross-reactivity was not calculated (Fig. 2). The sensitivity of the detection of the monkey fecal occult blood was as low as 1:256,000 dilution level (100 µL of 1:12,800 diluted blood sample mixed with 2 mL of buffer solution).

Using FIT to determine the gastrointestinal bleeding in cynomolgus monkeys during 28-day oral administration of the NPRCT excipient: During 28-day daily oral administrations of the NPRCT excipient to four female cynomolgus monkeys, no blood in the feces was observed. However, from 112 feces determined using the FIT kit, the presence of hemoglobin was detected once in three monkeys (3 out of 112 samples), on Day-3, Day-8 and Day-16, respectively.

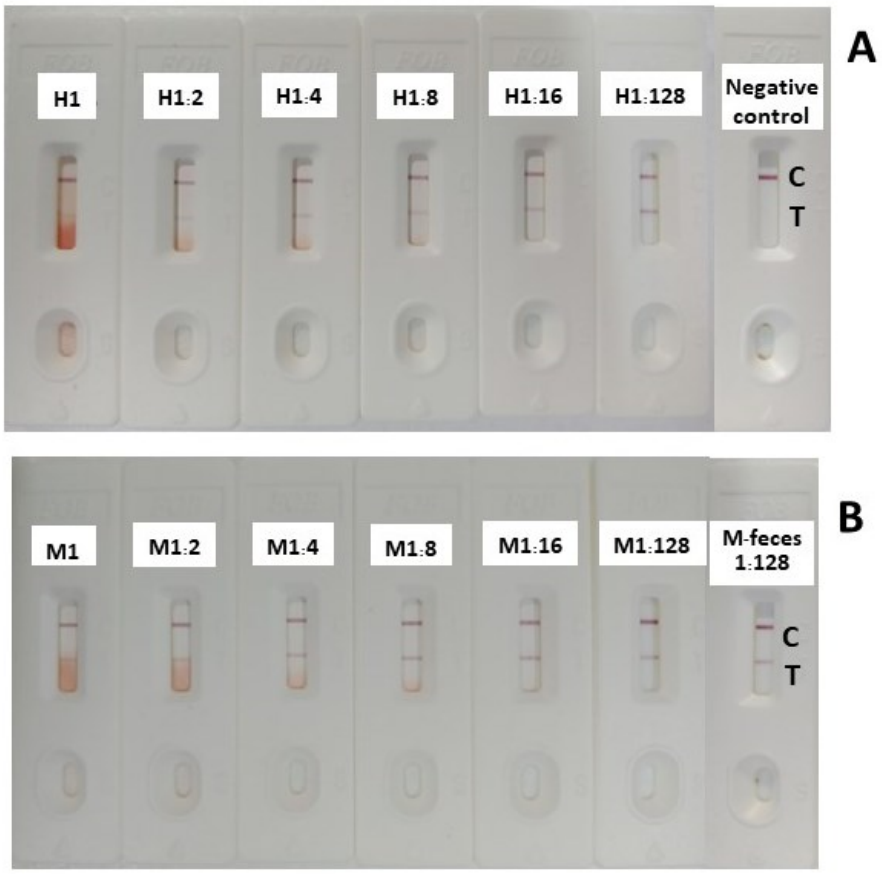

Figure 1 Fecal immunochemical occult blood test. A) Human blood samples (H), B) Cynomolgus macaque blood samples (M) and monkey fecal solution mixed with monkey blood (M-feces) at a serial two-fold dilution from 1:2 to 1:128. C and T indicate control and test lines.

Table 1 Intensities of C- and T-line and the calculated T/C ratios of human and monkey blood samples and monkey fecal occult blood samples.

Samples	Dilution factor	C-line intensity	T-line intensity	T/C ratio
Human blood	1:1	73325	21475	0.29
	1:2	93425	33075	0.35
	1:4	116750	44775	0.38
	1:8	91950	34150	0.37
	1:16	96875	42075	0.43
	1:128	77525	42875	0.55
Monkey blood	1:1	73175	18500	0.25
	1:2	79775	18400	0.23
	1:4	92775	38200	0.41
	1:8	92875	37650	0.41
	1:16	96600	43575	0.45
	1:128	81275	36400	0.45
Monkey fecal occult blood	1:128	140750	46425	0.33
	Normal saline	200750	0	0.00

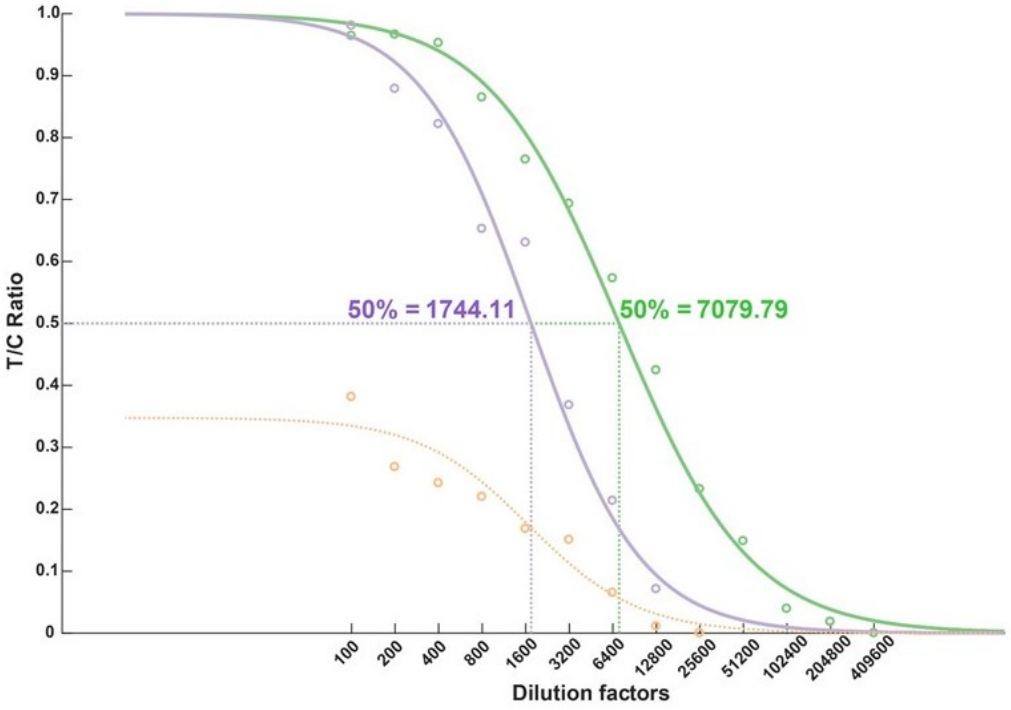

Figure 2 The parallelism of the dose-response curves (serial dilutions versus T/C ratios) between human (green line) and monkey (purple line) blood, and monkey fecal occult blood (orange line).

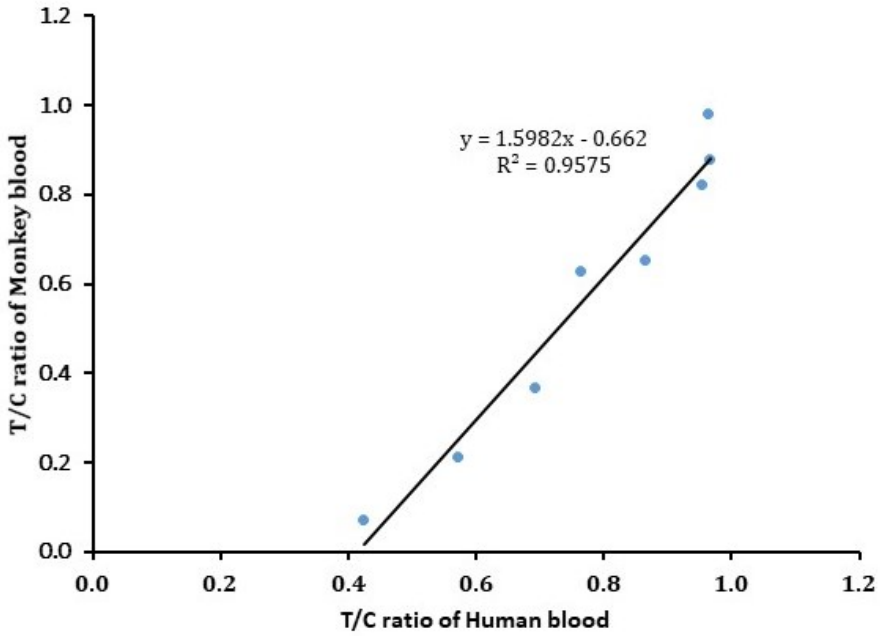

Figure 3 The correlation of the T/C ratios between human and monkey blood samples.

Discussion

This study demonstrated that the fecal immunochemical occult blood test (FIT) that has been widely used in human medicine for colorectal cancer screening can be used in cynomolgus monkeys. Its anti-human hemoglobin antibody which has been marked as no-cross-reactivity with many species of domestic animals and rabbits reacted very well with monkey hemoglobin, but the specificity was 4.2 times lower than in human blood. Although the sensitivity of the kit was reduced if the monkey feces were mixed into a buffer solution, the blood sample at a dilution of 1:256,000 could be detected. It should be kept in mind that the blood samples at a dilution of 1:4 or lower showed low values of T/C ratios indicating that accuracy of the measurement could become lower when the blood concentration is too high. In this situation when confirmation is needed, dilution of the specimen is recommended for accuracy. One simple explanation is that large amounts of blood sample also contain high inhibiting factors. Thus, the kit is not suggested for use with animals exhibiting the condition of menstruation or hemorrhoids.

Hemoglobin is a molecule transporting oxygen in the red blood cells of all vertebrates, except for the fish in family Channichthyidae. The hemoglobin molecule is made up of four polypeptide chains: two α-chains of 141 amino acid residues each and two β-chains of 146 amino acid residues each. Previous studies have reported that the amino acid sequences of the α-chain of the horse, bovines, rabbits and chickens (Schroeder *et al*., 1967; Heindell *et al*., 1978; Dodgson *et al*., 1983; Clegg 1987) had amino acid differences from humans (Robert *et al*., 1963) for 17, 17, 25 and 42 amino acids. Thus, there should theoretically be no cross reaction between anti-human hemoglobin antibody with hemoglobin of those animals. However, when we compared the amino acid sequences of α- and β-hemoglobin chains of the cynomolgus monkeys with that of humans (Takenaka *et al*. 1988; α-chains: accession nos. P69905 and D1MGQ2 for humans and A0A2K5WIF5, G7Q018 and G7Q017 for cynomolgus macaques; β-chains: accession nos. P69892 and E7CYP2 for humans and A0A2K5WIZ9 and G7PQY2 for cynomolgus macaques), only 4 amino acid differences for each chain were detected. Those 4 amino acid differences had not fallen into the 87th residue of the α-chain and the 92nd residue of the β-hemoglobin chains, thus it had no effect on the oxygen binding capability of the hemoglobin (Shaanan 1982). Besides, the hemoglobin gene clusters reflecting hemoglobin function in non-human primates (including cynomolgus macaques) are evolutionarily closely related to the humans (Hardison 2012). The above-mentioned reasons should help to explain why we detected the hemoglobin of cynomolgus monkey using a FIT kit that was specific for the human hemoglobin. After we tested this FIT in four female cynomolgus monkeys orally administered with the NPRCT excipient for 28 days, it was indicated that the FIT was very useful for the detection of gastrointestinal bleeding in cynomolgus monkeys and the sensitivity was very high and blood in the feces was not visually detected. The NPRCT excipient tested in this study will be used as a permeation enhancer for drugs that aim to be administered to humans via the oral route. Although the constituent of the NPRCT excipient is generally regarded as safe (GRAS) by US-FDA, it is occasionally induces gastrointestinal adverse effects such as diarrhea and vomiting if high doses are used (Hardee *et al*., 2006; Hardee *et al*., 2008; Granhall *et al*. 2019), thus a safety test is needed.

In conclusion, the human-FIT kit can be an applicable tool for the early detection of disease in the gastrointestinal tract such as intestinal adenocarcinoma (Valverde *et al*., 2000) and gastrointestinal bleeding in cynomolgus monkeys for laboratory research. The advantages of this kit are that it is simple, fast and easy to use (no need for high skill of user nor other assisting equipment because the results can be read by the naked eye). In particular, this non-invasive screening method can be very useful in the field where free-ranging non-human primates freshly defecate and drop samples on to the ground. It needs to be borne in mind that a false-positive might be encountered if the fecal specimen has been collected from unidentified monkeys which exhibit the condition of menstruation or hemorrhoids. It is of note that in the animals that the anti-hemoglobin of the FIT kit reported, there was no cross-reaction with livestock that we generally ingest, however, if patients unintentionally consume cynomolgus monkeys as seen in many locations in South and South-east Asian countries, false-positive results might also be detected.

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