

# Evaluation of the sensitivity and specificity of four laboratory tests for detection of occult blood in cockatiel (*Nymphicus hollandicus*) excrement

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**Objective**—To compare sensitivity and specificity of cytologic examination and 3 chromogen tests for detection of occult blood in cockatiel (*Nymphicus hollandicus*) excrement.

**Animals**—20 adult cockatiels.

**Procedures**—Pooled blood from birds was divided into whole blood and lysate aliquots. Excrement was mixed with each aliquot in vitro to yield 6 hemoglobin (Hb) concentrations (range, 0.375 to 12.0 mg of Hb/g of excrement). For the in vivo portion of the study, birds were serially gaged with each aliquot separately at 5 doses of Hb (range, 2.5 to 40 mg/kg). Three chromogen tests and cytologic examination were used to test excrement samples for occult blood. Sensitivity, specificity, and observer agreement were calculated.

**Results**—In vitro specificity ranged from 85% to 100% for the 3 chromogen tests and was 100% for cytologic examination. Sensitivity was 0% to 35% for cytologic examination and 100% for the 3 chromogen tests on samples containing  $\geq 1.5$  mg of Hb/g of excrement. In vivo specificity was 100%, 90%, 65%, and 45% for cytologic examination and the 3 chromogen tests, respectively. Sensitivity was 0% to 5% for cytologic examination and  $\geq 75\%$  for all 3 chromogen tests after birds received doses of Hb  $\geq 20$  mg/kg. Observer agreement was lowest for cytologic examination.

**Conclusions and Clinical Relevance**—Chromogen tests were more useful than cytologic examination for detection of occult blood in cockatiel excrement. The best combination of sensitivity, specificity, and observer agreement was obtained by use of a chromogen test. (*Am J Vet Res* 2006;67:1326–1332)

## ABBREVIATIONS

Hb	Hemoglobin
NSAID	Nonsteroidal anti-inflammatory drug
CI	Confidence interval

species-specific immunochemical assays.<sup>4–6</sup> In addition, colorless chromogens that become colored when oxidized (eg, guaiac and tetramethylbenzidine) have long been used in tests that make use of the peroxidase-like activity of heme to catalyze oxidation.<sup>7–9</sup> Chromogen tests are widely used and cost-effective, but have limited diagnostic sensitivity and specificity.<sup>10,11</sup> In humans, an immunologic assay may be combined with a chromogen test to improve the accuracy of occult blood detection before proceeding to more expensive and invasive diagnostic tests for identifying the source of gastrointestinal tract bleeding.<sup>12–14</sup>

Successful diagnosis of gastrointestinal tract bleeding by use of fecal chromogen tests depends on the site of the lesion, volume of blood loss, composition of the diet, and accuracy of the test. It is commonly accepted that digestive enzymes and bacteria degrade heme in the human gastrointestinal tract,<sup>15,16</sup> so small quantities of blood may not be detected by tests that rely on heme activity, especially after intestinal transit or fecal storage. Also, large amounts of ascorbic acid from citrus fruits and juices may cause false-negative chromogen test results.<sup>17,18</sup> Conversely, peroxidases in certain plants and rare or raw red meat can cause false-positive results, so mammalian patients are generally fed a controlled diet for several days before collection of feces for testing.<sup>13,17,19–23</sup> Many companion birds are omnivorous, and their diets can be easily manipulated and monitored to avoid items that could cause false-positive or false-negative chromogen test results.

Veterinary clinicians generally diagnose blood in avian excrement by means of gross examination,<sup>24</sup> although some authors<sup>25</sup> have advocated more specific

Detection of fecal occult blood has challenged veterinarians and physicians for decades. Detection methods in mammals have included labeling of erythrocytes with chromium 51 and measuring fecal radioactivity,<sup>1</sup> quantifying the fluorescence of Hb-derived porphyrins by use of spectrophotometry,<sup>2,3</sup> and

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methods of testing to verify findings when occult blood is suspected on the basis of visual appearance. This is supported by the observation that some common medications and food items such as salicylates, bismuth, blackberries, raspberries, beets, and many of the dyes used in formulated diets change the color and consistency of avian feces. Also, feces must contain relatively large volumes of blood before a diagnosis of hematochezia or melena can be made on the basis of gross observation.<sup>26</sup> In a pharmacokinetic study<sup>27</sup> of NSAIDs in mallard ducks, the authors used a chromogen test to detect Hb in excrement, and all 48 samples had negative test results. Those findings may have resulted from a true absence of gastrointestinal bleeding, an interfering substance, or inability of the assay to detect mallard duck Hb.

To our knowledge, cytologic examination is the only laboratory method routinely used to detect occult blood in companion bird excrement. The method may be useful when the excrement contains intact RBCs, but excrement could obscure observation of RBCs or interfere with staining. Traditionally, many avian clinicians have assumed that the rapid transit time of fluids through the gastrointestinal tract in psittacine birds (1 to 6 hours, compared with 14 to 45 hours in dogs and 24 to 48 hours in cats)<sup>28–32</sup> enables passage of intact RBCs, regardless of the site of hemorrhage. This may be true for healthy birds but has not been evaluated. In addition, many illnesses, such as proventricular dilation disease, heavy metal toxicosis, gastrointestinal parasitism, ingluvitis, bacterial enteritis, and generalized coelomitis, slow gastrointestinal transit times.<sup>33,34</sup> These and many other diseases can also lead to occult gastrointestinal bleeding, which could be detected and managed earlier if sensitive diagnostic tests were available.

Few veterinary laboratories are equipped to test for the fluorescence of porphyrins in feces, and to our knowledge, no immunochemical test for fecal blood has been developed for use in avian species. Tests for fecal recovery of radioisotope-labeled RBCs are time-consuming, expensive, and considered impractical for routine clinical use in avian patients. Therefore, the most practical and cost-effective avian fecal blood tests presently available are fecal cytologic examination and chromogen tests. Human and veterinary diagnostic laboratories commonly use tetramethylbenzidine-based or guaiac-based chromogen tests. Tetramethylbenzidine-based<sup>a</sup> and guaiac-based<sup>b</sup> tests have the advantage of greater diagnostic sensitivity but also have the disadvantage of being less specific than an enhanced guaiac-based test.<sup>16,23,c</sup> The tetramethylbenzidine-based test is performed by smearing a fecal sample on filter paper, placing a strontium peroxide and tetramethylbenzidine tablet on the filter paper, and moistening the material with water.<sup>35</sup> The guaiac-based and enhanced guaiac-based tests are performed by smearing feces onto guaiac-impregnated paper and developing it with a solution containing hydrogen peroxide.<sup>36,37</sup> The enhanced guaiac-based test includes a proprietary enhancer that improves test sensitivity, readability, and precision over other guaiac-based tests, but may also increase the proportion of false-positive results.<sup>37</sup>

The purpose of the study reported here was to compare the sensitivity and specificity of fecal cytologic examination and 3 commercial chromogen tests in detection of whole blood and blood cell lysate in cockatiel (*Nymphicus hollandicus*) excrement.

## Materials and Methods

**Animals**—Eleven adult female (mean  $\pm$  SD weight,  $98 \pm 6$  g) and 9 adult male ( $88 \pm 5$  g) cockatiels were used in the study. The mean weight of all birds combined was  $94 \pm 7$  g, and age ranged from 1 to 3 years. Birds were obtained from the University of California–Davis Department of Animal Sciences and were maintained in controlled laboratory animal facilities (temperature,  $24 \pm 2^\circ\text{C}$ ; fluorescent lighting, 8 hours light and 16 hours dark). Birds were housed separately in suspended  $40 \times 61 \times 91$ -cm galvanized wire cages and fed a formulated maintenance diet.<sup>4</sup> The study protocol was approved by the University of California–Davis Animal Care and Use Committee (Protocol No. 9456). All birds were assessed as clinically healthy on the basis of results of physical examination, determination of PCV and total plasma protein concentration, direct fecal examination for parasites, and evaluations of a Gram-stained fecal smear and a Wright-stained fecal smear. All 20 birds were used in both in vitro and in vivo experiments.

**In vitro experiments**—Excrement from each bird was collected from generic cellophane sheets placed 10 cm below the cages for 16 hours. To ensure that samples were moist enough to be smeared onto test substrates, an equal amount (wt:vol) of isotonic saline (0.9% NaCl) solution was added to each excrement sample before the tube was sealed with moisture-resistant thermoplastic film<sup>e</sup> and stored at  $4^\circ\text{C}$  for  $< 8$  hours. Birds were manually restrained, and blood (0.5 mL) was collected from the right jugular vein by use of a 26-gauge needle and 1-mL tuberculin syringe and transferred into vials<sup>f</sup> coated with lithium heparin. Samples of whole blood were pooled and divided into 2 aliquots (5 mL each), which were stored for  $< 24$  hours at  $4^\circ\text{C}$ . One aliquot was used as whole blood and the other was used to make a lysate. Hemoglobin concentrations were measured by the standard cyanmethemoglobin method with a hematology analyzer.<sup>5</sup>

### Control specimens

Control excrement specimens ( $n = 20$ ) were prepared by mixing 0.6 mL of isotonic saline solution/g of excrement.

### Whole blood–excrement specimens

Whole blood Hb concentration was adjusted to  $150 \pm 10$  mg/mL by dilution with isotonic saline solution or by concentration via centrifugation and removal of plasma. The adjusted whole blood solution was mixed in glass tubes with aliquots of saline solution–moistened excrement from each cockatiel in varying volumes to yield specimens that contained final concentrations of 0.375, 0.75, 1.5, 3.0, 6.0, and 12.0 mg of Hb/g of excrement ( $n = 20$  specimens/concentration).

### Blood lysate–excrement specimens

Lysates were prepared from RBCs washed 3 times with isotonic saline solution at  $21^\circ\text{C}$  by use of a volume equal to the volume of plasma. Packed cells were lysed by suspension and agitation in a volume of sterile distilled water equal to 50% of the volume of removed plasma, after which they were frozen at  $-70^\circ$  to  $-80^\circ\text{C}$  for up to 8 hours. Frozen lysates were thawed and centrifuged, and Hb concentration was measured. Hemoglobin concentration in the lysate was adjusted to  $150 \pm 10$  mg/mL by dilution with sterile distilled water, when necessary. Lysate was mixed in glass tubes with

aliquots of saline solution–moistened excrement to attain the same range of final Hb concentrations (0.375, 0.75, 1.5, 3.0, 6.0, and 12.0 mg of Hb/g of excrement; 20 specimens/concentration) as for the whole blood–excrement specimens.

#### Testing of specimens

All control (saline-excrement), whole blood–excrement, and lysate-excrement specimens were prepared for testing by smearing onto test papers and glass slides within 1 hour after mixing. All samples were tested by use of the 3 chromogen tests (tests A,<sup>a</sup> B,<sup>b</sup> and C<sup>c</sup>). In addition, the control specimens and whole blood–excrement samples were evaluated by cytologic examination. Two observers conducted each chromogen test and cytologic examination.

**In vivo experiments**—The in vivo experiment began 48 hours after the in vitro experiment was completed and was divided into control and treatment phases. Thus, each bird served as its own control animal.

#### Control phase

Cellophane was placed beneath the cage of each bird, and excrement was collected at 12 hours to obtain pre-gavage samples. Birds were given 2.5 mL of isotonic saline solution via oro-ingluvial intubation (gavage) with a 2.7-mm-diameter rubber feeding tube.<sup>b</sup> Birds were routinely housed, and feed and water were provided before and after the gavage procedure. Clean cellophane was replaced beneath cages immediately after treatment. Excrement samples were collected at intervals typical of those used in clinical settings (8 and 24 hours after gavage), and cellophane sheets were replaced between sample collections. The 3 excrement samples (collected before and 8 and 24 hours after gavage) from each bird were tested for evidence of occult blood by use of the 3 chromogen tests and cytologic examination; 2 observers conducted each test or performed each cytologic examination.

#### Treatment phase

Fresh whole blood and lysate aliquots were prepared as described for the in vitro experiment. Each bird was gaged with sequentially increasing Hb doses calculated to provide 2.5, 5, 10, 20, and 40 mg of Hb/kg of body weight. The series of whole blood doses was administered first and the series of lysate doses was administered second; thus, each bird was treated 5 times with whole blood and 5 times with lysate. Measured aliquots of whole blood or blood lysate were diluted in isotonic saline solution immediately before administration to yield a volume of 2.5 mL/dose. The treatment phase began 48 hours after the control phase, and a minimum 48-hour washout period elapsed between Hb doses. The washout period of 48 hours was at least 8 times the gastrointestinal tract transit time of fluids in healthy psittacine birds and was considered to be sufficient for clearance of Hb from the preceding treatments.<sup>29,30,32</sup> Birds were routinely housed, and food and water were provided before and immediately after each treatment. Excrement was collected by use of the same methods used in the control phase 8 and 24 hours after gavage with each Hb dose for each bird.

Excrement samples collected from birds after administration of whole blood were tested for evidence of occult blood by use of the 3 chromogen tests and cytologic examination; whereas excrement samples collected from birds after administration of blood lysate were tested only by use of the 3 chromogen tests. Each test and cytologic examination was conducted by 2 observers.

#### Testing for occult blood

Three commercial chromogen test kits were evaluated:

test A (lot number 3,600A), test B (lot number 9K07LX), and test C (lot numbers 51,600 and 52,200B). Tests were performed in accordance with manufacturer specifications<sup>35–37</sup> and were rated by 2 observers (PMG and a trained assistant). Tests were coded and randomized so observers were unaware of treatment status. Results were recorded as positive or negative. Duplicate results were compared, and a test result was considered to be positive when at least 1 of the 2 observers observed color development. Birds in the in vivo experiments were considered to have positive results for fecal occult blood when at least 1 of the posttreatment samples (8 or 24 hours after treatment) was rated as having positive results by at least 1 of the observers.

Cytologic specimens were prepared by smearing feces onto glass microscope slides and allowing them to air-dry. One smear was made from each sample. Smears were stained with Wright stain by use of an automated slide stainer.<sup>1</sup> Fecal smears were evaluated microscopically for RBCs by scanning each slide at 400X magnification for 2 minutes. Slides were considered to have positive results for blood when at least 2 intact RBCs were observed during the 2-minute scan. The requirement that at least 2 RBCs be observed was intended to help reduce false-positive results resulting from fecal material or artifacts that appeared similar to RBCs. Smears from all samples were examined microscopically except for in vitro samples mixed with lysate and in vivo samples collected from lysate-treated birds. Smears were examined by 1 of the authors (PMG) and a trained laboratory technician or medical technologist. Slides were coded and randomized so that observers were unaware of treatment group. For samples with observer discrepancy, smears were again reviewed by 1 of the investigators (PMG), and a final determination was made.

**Testing to detect interfering substances**—Uneaten feed was scattered on the cellophane-covered cage floor during normal feeding behavior throughout the experiments. This led to a concern that feed could have mixed with sampled excrement despite careful attempts to avoid this during sample collection. To test whether excrement contamination with feed could contribute to false-positive chromogen test results, feed<sup>d</sup> was mixed with 10 duplicate saline-moistened aliquots of pooled cockatiel excrement at 2 concentrations (0.2 and 0.5 g of feed/g of moistened excrement;  $n = 10$  replicates/concentration). Treated samples were tested by use of all 3 chromogen tests, and results were rated as positive or negative by a single observer (PMG).

**Statistical analysis**—Diagnostic accuracy of the occult blood tests was evaluated by calculating in vitro and in vivo diagnostic sensitivity and specificity. Control samples were used to calculate<sup>e</sup> in vitro and in vivo specificities with 95% CIs for all 4 tests. Diagnostic sensitivity and CI were calculated for each test at each in vitro Hb concentration and at each in vivo Hb dose. Differences in sensitivity or specificity among methods or experimental phases were considered significant when CIs did not overlap ( $\alpha = 0.05$ ). The McNemar test was used to calculate<sup>k</sup> significant differences among test results (ie, in the 6 pairwise comparisons between cytologic examination and test A, cytologic examination and test B, cytologic examination and test C, test A and test B, test A and test C, and test B and test C) with the combined results from all control samples, in vitro Hb concentrations, and in vivo Hb doses and collection times; differences among methods were considered significant at values of  $P < 0.05$ . Interobserver agreement was assessed for the 4 tests by calculating<sup>k</sup> the Cohen  $\kappa$  statistic and CI for each test with the combined results from all control samples, in vitro concentrations, and in vivo doses and collection times; differences among methods were considered significant when CIs did not overlap ( $\alpha = 0.05$ ).

## Results

**Animals**—No clinically important physical examination abnormalities were observed during the initial health evaluations. In addition, plasma total protein concentration and values for PCV were within reference ranges, direct fecal examinations revealed no parasites, Gram-stained fecal smears contained moderate numbers of mostly gram-positive bacilli and cocci, and Wright-stained fecal smears contained no RBCs. Samples of excrement from all 20 birds that were collected during a single randomly selected 24-hour period after treatment weighed  $2.9 \pm 1.0$  g. No signs of illness were noticed in any bird during the study period, and no adverse effects were observed after treatments. However, 2 female birds (each of which was 2 years old during the study) were submitted for necropsy approximately 1 year after completion of the study, and each had severe hepatic lipidosis.

**In vitro experiments**—Diagnostic specificity (95% CI) calculated from control samples was 100% (85% to 100%) for cytologic examination, test A, and test B and 85% (62% to 97%) for test C (Figure 1). False-positive results (3 of 20) were obtained from control samples only with test C.

### Whole blood–excrement specimens

Diagnostic sensitivity (95% CI) of cytologic examination ranged from 0% to 35% (0% to 59%) with no consistent trend relative to whole blood Hb concentration (Figure 2). Diagnostic sensitivity at 0.375 mg of Hb/g of excrement was 80% (56% to 94%) for test A, 60% (36% to 81%) for test B, and 90% (68% to 99%) for test C. At 0.75 mg of Hb/g of excrement, diagnostic sensitivity was 100% (86% to 100%) for tests A and C and 95% (75% to 100%) for test B. All 3 chromogen tests had 100% (86% to 100%) sensitivity at Hb concentrations  $\geq 1.5$  mg/g. The diagnostic sensitivity of cytologic examination was significantly less than that of tests A and C at all Hb concentrations and was significantly less than that of all 3 chromogen tests at Hb concentrations  $\geq 0.75$  mg/g.

### Blood lysate–excrement specimens

Diagnostic sensitivity (95% CI) at 0.375 mg of Hb/g of excrement was 65% (41% to 85%) for test A, 80% (56% to 94%) for test B, and 100% (86% to 100%) for test C (Figure 3). At 0.75 mg of Hb/g of excrement, diagnostic sensitivity was 80% (56% to 94%) for test A and 100% (86% to 100%) for tests B and C. Diagnostic sensitivity for test A was 85% (62% to 97%) at 1.5 mg of Hb/g of excrement, whereas sensitivities for tests B and C were both 100% (86% to 100%) at that concentration. Sensitivity of all 3 chromogen tests was 100% (86% to 100%) at Hb concentrations  $\geq 3.0$  mg/g. Diagnostic sensitivities for the chromogen tests based on Hb concentration did not differ significantly between the whole blood–excrement and lysate-excrement groups.

**In vivo experiments**—Diagnostic specificity (95% CI) calculated from control samples was 100% (86% to 100%) for cytologic examination, 90% (68% to 99%) for test B, 65% (41% to 85%) for test A, and 45% (23% to 68%) for test C (Figure 1). Diagnostic specificity of cytologic examination was not significantly different

than that of test B but was significantly greater than that of tests A and C. Specificities did not differ significantly among chromogen tests, although it was notable that there was very little (1%) overlap in CIs between tests B and C. False-positive results of 2/20, 7/20, and 11/20 were obtained from control samples for tests B, A, and C, respectively.

### Whole blood–treatment phase

Diagnostic sensitivity (95% CI) of cytologic examination ranged from 0% to 5% (0% to 24%). Diagnostic sensitivities of test A were 75% (51% to 91%), 85% (62% to 97%), 90% (68% to 99%), 85% (62% to 97%), and 85% (62% to 97%) at 2.5, 5, 10, 20, and 40 mg of Hb/kg, respectively (Figure 4). Diagnostic sensitivities for test B were 25% (8.7% to 49%), 15% (3.2% to 38%), 40% (19% to 64%), 75% (51% to 91%), and 100% (86% to 100%) at 2.5, 5, 10, 20, and 40 mg of Hb/kg, respectively. Diagnostic sensitivities of test C were 70%

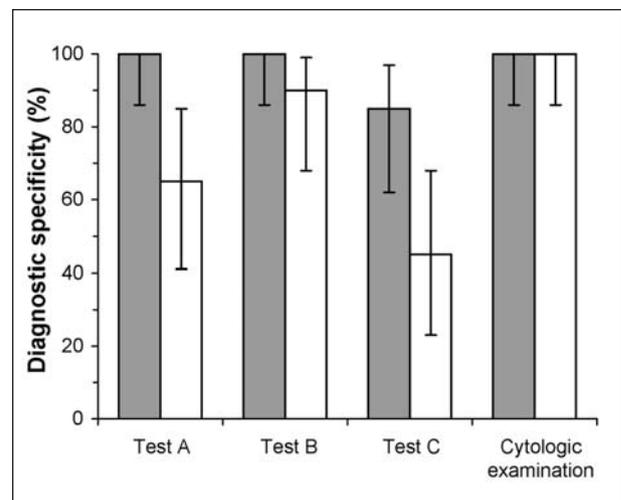


Figure 1—Mean and 95% CI for in vitro (gray bars) and in vivo (white bars) specificities of 3 chromogen tests and cytologic examination used for detection of occult blood in cockatiel excrement. Test A is a tetramethylbenzidine-based test,<sup>a</sup> test B is a guaiac-based test,<sup>b</sup> and test C is an enhanced guaiac-based test.<sup>c</sup>

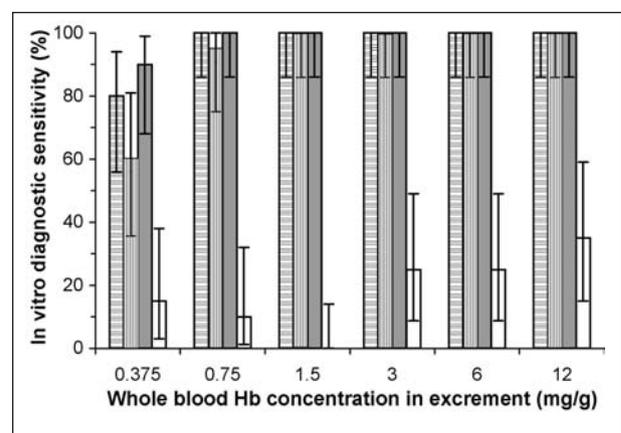


Figure 2—Mean and 95% CI for in vitro sensitivities of 3 chromogen tests (test A [horizontal-striped bars], test B [gray bars], and test C [white bars], respectively) and cytologic examination (vertical-striped bars) used for detection of whole blood mixed with cockatiel excrement to yield specimens containing various concentrations of Hb. See Figure 1 for remainder of key.

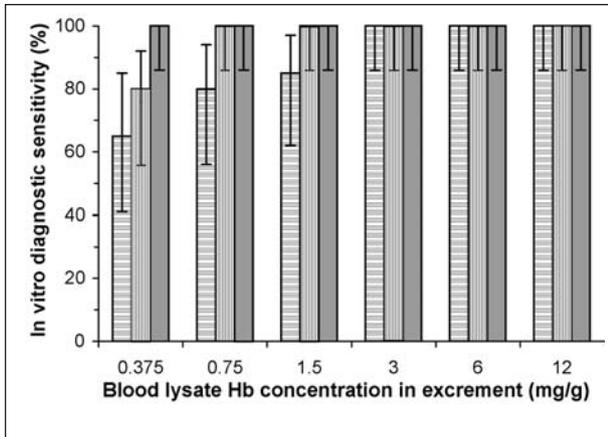


Figure 3—Mean and 95% CI for in vitro sensitivities of 3 chromogen tests used for detection of cockatiel blood lysate mixed with excrement to yield specimens containing various concentrations of Hb. See Figures 1 and 2 for remainder of key.

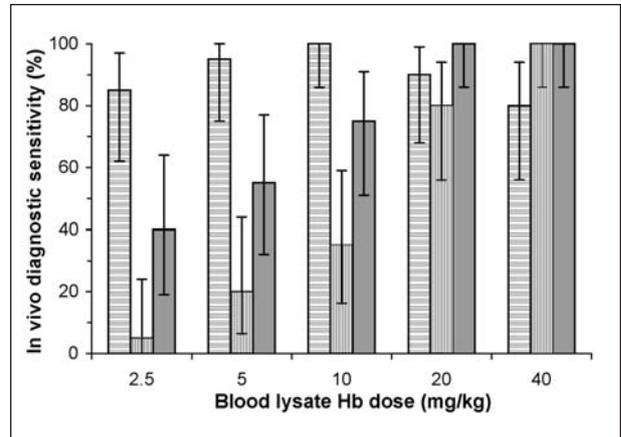


Figure 5—Mean and 95% CI for in vivo sensitivities of 3 chromogen tests used for detection of occult blood in cockatiel excrement that was collected during the 24 hours after gavage administration of blood lysate at various doses. See Figures 1 and 2 for remainder of key.

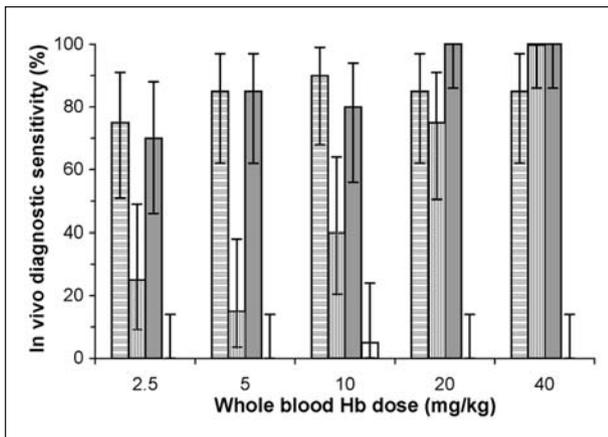


Figure 4—Mean and 95% CI for in vivo sensitivities of 3 chromogen tests used for detection of occult blood in cockatiel excrement that was collected during the 24 hours after gavage administration of whole blood at various doses. See Figures 1 and 2 for remainder of key.

(46% to 88%), 85% (62% to 97%), 80% (56% to 94%), 100% (86% to 100%), and 100% (86% to 100%) at 2.5, 5, 10, 20, and 40 mg of Hb/kg, respectively. The diagnostic sensitivity of cytologic examination was significantly less than that of tests A and C at all Hb doses and significantly less than that of all 3 chromogen tests at doses  $\geq 20$  mg/kg. Diagnostic sensitivities of test B were significantly less than that of test A at 2.5 and 10 mg of Hb/kg and significantly less than that of tests A and C at a dose of 5 mg of Hb/kg. Diagnostic sensitivities of chromogen tests at 20 and 40 mg Hb/kg were not significantly different.

#### Blood lysate-treatment phase

Diagnostic sensitivities (95% CI) of test A were 85% (62% to 97%), 95% (75% to 100%), 100% (86% to 100%), 90% (68% to 99%), and 80% (56% to 94%) at 2.5, 5, 10, 20, and 40 mg of Hb/kg, respectively (Figure 5). Diagnostic sensitivities of test B were 5% (0.12% to 24%), 20% (5.7% to 44%), 35% (15% to 59%), 80% (56% to 94%), and 100% (86% to 100%) at 2.5, 5, 10, 20, and 40 mg of Hb/kg, respectively. Diagnostic sensi-

ties of test C were 40% (19% to 64%), 55% (32% to 77%), 75% (51% to 91%), 100% (86% to 100%), and 100% (86% to 100%) at 2.5, 5, 10, 20, and 40 mg of Hb/kg, respectively. Diagnostic sensitivities of test B were significantly less than those of test A at 2.5, 5, and 10 mg of Hb/kg, but were not significantly different from those of test A at 20 and 40 mg of Hb/kg. Diagnostic sensitivities of tests B and C did not differ significantly. Diagnostic sensitivities of chromogen tests on the basis of Hb dose were not significantly different between the whole blood and lysate treatment phases.

**Testing to detect interfering substances**—Positive test results were obtained in 10 of 10 assays for test A, 0 of 10 assays for test B, and 1 of 10 assays for test C at 0.2 g of feed/g of excrement. In addition, results of 10 of 10 assays for test A but 0 of 10 assays for tests B and C were positive at 0.5 g of feed/g of excrement.

**McNemar test**—The *P* values were calculated by use of pooled results from all in vitro Hb concentrations and all in vivo samples at all Hb doses at all collection times ( $n = 499, 498, 494, 919, 915, \text{ and } 914$  for cytologic examination-test A, cytologic examination-test B, cytologic examination-test C, test A-test B, test A-test C, and test B-test C, respectively). Significant differences in the ability to detect occult blood were observed between all pairwise combinations of tests ( $P < 0.001$  for cytologic examination-test A, cytologic examination-test B, cytologic examination-test C, test A-test B, and test B-test C; and  $P = 0.034$  for test A-test C).

**$\kappa$  statistics**—The Cohen  $\kappa$  statistics were estimated with 95% CIs by use of pooled results from all in vitro Hb concentrations and all in vivo samples at all Hb doses at all collection times ( $n = 499, 1,000, 998, \text{ and } 994$  for cytologic examination and tests A, B, and C, respectively; Table 1). Observer agreement was significantly higher for all 3 chromogen tests than for cytologic examination, but observer agreement was significantly lower for test A than for tests B and C. Observer agreement was not significantly different between tests B and C.

Table 1—Agreement between 2 observers who rated the results of cytologic examination (n = 499 samples), a tetramethylbenzidine-based test (test A<sup>a</sup>; 1,000 samples), a guaiac-based test (test B<sup>b</sup>; 998 samples), and an enhanced guaiac-based test (test C<sup>c</sup>; 994 samples) used for detection of occult blood in cockatiel excrement.

Test	$\kappa$	95% CI
Cytologic examination	0.47*	0.32–0.63
Test A	0.81*	0.77–0.85
Test B	0.97	0.96–0.99
Test C	0.97	0.95–0.98

Denotes significantly ( $\alpha = 0.05$ ) different interobserver agreement.

## Discussion

Analysis of results indicated that commercial chromogen-based tests have acceptable diagnostic specificity and sensitivity for detection of occult blood in cockatiel excrement. In particular, test B had high in vivo diagnostic accuracy of 90% specificity and 100% sensitivity in detecting whole blood or Hb in cockatiel excrement. Furthermore, analysis of results indicated that although highly specific, cytologic examination cannot be used to reliably detect occult blood in cockatiel excrement.

All 3 chromogen tests yielded false-positive results in vivo, and test C also yielded false-positive results in vitro. False-positive results could have resulted from a peroxidase in the diet, traumatic injury during the gavage procedure, hemorrhage from subclinical gastrointestinal tract disease, or normal (trace) amounts of blood in the gastrointestinal tract of the birds. Contamination of excrement with feed could also have contributed to false-positive results. This possibility was investigated, and 20 of 20, 0 of 20, and 1 of 20 results were positive when tested by use of tests A, B, and C, respectively, which confirmed that contamination of collected excrement with feed could have contributed to false-positive results. The label ingredient list for the feed did not include a known peroxidase source, and we did not attempt to determine the source or sources of peroxidase activity in the feed. Avoiding contamination of excrement with feed may decrease the likelihood of false-positive results when chromogen tests are used clinically.

False-positive results could also have resulted from existing gastrointestinal tract hemorrhage caused by subclinical disease. Although we were unable to perform necropsies on all birds used in the study, 2 of the birds were submitted for necropsy approximately 1 year after the experiment concluded; both of those birds had findings consistent with severe hepatic lipidosis. Hepatic lipidosis is often associated with hemorrhage in birds<sup>38–40</sup> and may have accounted for some of the positive test results observed, assuming the birds had that condition at the time of the study. However, there were no indications that the birds were unhealthy during the conduct of the study.

The avian reproductive, urinary, and digestive tracts empty into the cloaca, and products of all these systems pass through the vent. Therefore, expelled excrement can contain materials from any of the 3 systems. Separation of excrement into its constituent parts (ie, feces, urine, urates, and products of the reproductive tract) was not attempted in the study reported

here. Instead, the experimental protocol tested for occult blood in a manner that would be consistent with clinical use. Analysis of results indicated that the chromogen tests, particularly test B, have acceptable diagnostic specificity and sensitivity when there is blood or Hb in unmodified cockatiel excrement as collected from a cellophane-covered cage floor. We did not evaluate whether blood originating from the urinary or reproductive tracts would cause positive chromogen test results, although it is plausible.

Fecal Hb concentration ranges from 0.1 to 2.0 mg/g in healthy humans and dogs, and a concentration of 2.0 mg of Hb/g of feces is generally considered the cutoff value associated with a diagnosis of gastrointestinal disease.<sup>2,4,9,15</sup> It is plausible that there also could be small amounts of blood in the excrement of healthy birds. In the in vivo portion of the study reported here, tests A and C reliably detected fecal occult blood (with diagnostic sensitivities  $\geq 75\%$ ) after an Hb dose of 10 mg/kg was administered via gavage (0.94 mg of Hb/94-g bird). The mean weight of excrement collected from test birds was approximately 2.9 g/24 h, so assuming the administered Hb was evenly distributed throughout the excrement, samples would be expected to have contained approximately 0.32 mg of Hb/g of feces after treatment with 10 mg of Hb/kg of body weight. The relatively low diagnostic sensitivity of test B (ie,  $\leq 40\%$ ) at Hb doses up to 10 mg/kg, but much greater sensitivity (ie,  $\geq 75\%$ ) at doses of 20 mg/kg and higher, may offer a clinical advantage over use of tests A or C in discerning clinically normal from disease-related fecal occult blood.

The 4 tests evaluated differed substantially in their ability to detect occult blood, as determined by results of the McNemar test ( $P < 0.001$ , except for the comparison of tests A and C, for which  $P = 0.034$ ). Therefore, each test differed significantly from the others in ability to detect occult blood in cockatiel excrement. The 4 tests also presented technical challenges that were compared by means of interobserver agreement measured with the Cohen  $\kappa$  statistic. Accurate microscopic identification of blood requires observation of intact RBCs, so cytologic evaluation is not useful when material contained in excrement obscures RBCs, RBCs are not observed because of clumping or nonhomogeneous distribution in the sample, or RBC membranes are disrupted in the gastrointestinal tract or excrement environment. Those challenges likely contributed to the relatively low value for observer agreement ( $\kappa = 0.47$ ) associated with cytologic examination. The chromogen tests require detection of a blue or blue-green color change in the paper adjacent to the fecal specimen. Fecal pigments in many birds are often green, so it can be difficult to determine whether there has been a change in color. It was more difficult to discern chromogen color development for test A than for tests B and C, as indicated by lower observer agreement for test A ( $\kappa = 0.81$ ), compared with agreements for tests B and C ( $\kappa = 0.97$  for both tests).

In the study reported here, we recorded the chromogen test results as positive when rated as such by at least 1 of the 2 observers. This was expected to increase the diagnostic sensitivity and reduce diagnostic specificity of the tests, which could, in clinical patients, lead to unnecessary increases in cost for further diagnostic testing to

identify the source of bleeding. The advantages and disadvantages of tests with increased sensitivity and decreased specificity for detection of fecal occult blood have been studied in humans.<sup>11-15,41</sup> In companion avian medicine, the relatively low per-patient cost of additional diagnostic testing and the expected improvement in disease detection with correspondingly improved success of early treatment should provide sufficient justification for use of a fecal occult blood test with high sensitivity and reduced specificity. We therefore recommend that the results of chromogen-based occult blood tests be recorded as positive when rated as such by at least 1 of 2 observers during clinical use until the advantages and disadvantages of the use of a test with increased diagnostic sensitivity and decreased diagnostic specificity are studied further.

- a. Hematest, Bayer Corp, Elkhart, Ind.
- b. Hemocult, Beckman Coulter Inc, Fullerton, Calif.
- c. Hemocult SENSE, Beckman Coulter Inc, Fullerton, Calif.
- d. Roudybush Inc, Paso Robles, Calif.
- e. Parafilm M, American National Can, Neenah, Wis.
- f. Microtainer, Beckton Dickinson & Co, Franklin Lakes, NJ.
- g. Baker Instruments Corp, Allentown, Pa.
- h. Sovereign feeding tube, Monoject Division of Sherwood Medical, St Louis, Mo.
- i. Aerospray 7100, Wescor Instruments, Logan, Utah.
- j. Stat Xact Turbo, Cytel Software Corp, Cambridge, Mass.
- k. Stat Xact, version 6, Cytel Software Corp, Cambridge, Mass.

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