Binding of radiolabeled porcine motilin and erythromycin lactobionate to smooth muscle membranes in various segments of the equine gastrointestinal tract

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Objective—To identify and characterize motilin receptors in equine duodenum, jejunum, cecum, and large colon and to determine whether erythromycin lactobionate competes with porcine motilin for binding to these receptors.

Sample Population—Specimens of various segments of the intestinal tracts of 4 adult horses euthanatized for reasons unrelated to gastrointestinal tract disease.

Procedure—Cellular membranes were prepared from smooth muscle tissues of the duodenum, jejunum, pelvic flexure, and cecum. Affinity and distribution of motilin binding on membrane preparations were determined by use of ^{125}I-labeled synthetic porcine motilin. Displacement studies were used to investigate competition between ^{125}I-labeled synthetic porcine motilin and erythromycin lactobionate for binding to motilin receptors in various segments of bowel.

Results—Affinity of ^{125}I-labeled synthetic porcine motilin for the equine motilin receptor was estimated to be 6.1nM. A significantly higher number of motilin receptors was found in the duodenum than in the pelvic flexure and cecum. The jejunum had a significantly higher number of motilin receptors than the cecum. Erythromycin lactobionate displacement of ^{125}I-labeled porcine motilin from the equine motilin receptor did not differ significantly among various segments of bowel.

Conclusions and Clinical Relevance—Motilin receptors were found in the duodenum, jejunum, pelvic flexure, and cecum of horses. The highest number of motilin receptors was in the duodenum, and it decreased in more distal segments of bowel. Erythromycin lactobionate competed with motilin binding in the equine gastrointestinal tract. This suggests that 1 of the prokinetic actions of erythromycin in horses is likely to be secondary to binding on motilin receptors. (*Am J Vet Res* 2002;63:1545–1550)

Gastrointestinal ileus has been defined as the functional inhibition of propulsive bowel activity, irrespective of its pathophysiologic basis.[1] Terms used to describe the various clinical manifestations of motility disorders in horses are confusing, with ileus, postoperative ileus, endotoxemic ileus, idiopathic ileus, and adynamic ileus used inconsistently.[2] When duration of clinical signs is used to classify ileus, it can be characterized as adynamic (resulting from short-term alterations of motility) or paralytic (resulting as a consequence of loss of gastrointestinal motility for > 72 hours).[3] Ileus can result from diseases of the digestive system or can develop secondary to diseases of other systems.[4] Reflex postoperative ileus has been documented in humans and horses.[5] It has been postulated that this occurs as the result of an imbalance between sympathetic and parasympathetic nervous input to the bowel. Sympathetic (adrenergic) hyperactivity results in splanchnic vasoconstriction, reduction of propulsive motility, and an increase in tone of the gastrointestinal sphincter. Parasympathetic (cholinergic) hypoactivity results in a decrease in gastrointestinal motility and in a decrease in secretion.[4,6] However, it is likely that abnormalities involving the enteric nervous system, which uses several neuropeptides and nitric oxide as neurotransmitters,[7,8] and damage to the muscle cells that normally generate slow waves also contribute to the development of ileus.[9] Shock,[10] intestinal ischemia,[11] endotoxemia,[12] prolonged distention, and inflammation of the intestinal tract[13] have all been implicated as factors that contribute to the pathogenesis of ileus in horses.

Various pharmacologic agents have been used in horses to promote motility of the gastrointestinal tract.[2,4] Erythromycin lactobionate, a macrolide antibiotic, has been characterized as a prokinetic agent in humans,[14,15] dogs,[14] cats,[16] rabbits,[17,18] and horses[19-21] in vivo and in vitro. In clinically normal horses, erythromycin lactobionate accelerates gastric emptying,[21] whereas in healthy ponies, erythromycin lactobionate can increase cecal emptying as well as ileocecocolic myoelectric activity.[19] Interestingly, during the first 24 hours after abdominal surgery in horses, erythromycin increases myoelectric activity of the ileum and pelvic flexure, but not the cecum. However, administration of erythromycin initiates myoelectric activity in the cecum and is initiated within 8 days after surgery.[20] Unfortunately, the investigators of that study did not report results of myoelectrical activity recordings between 24 hours and 8 days after surgery; thus, it is unknown whether erythromycin has an effect on the cecum during that time.

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In other species, the action of erythromycin seems to be secondary to binding to smooth muscle motilin receptors[14-18] as well as neural motilin receptors.[22,23] In horses, IV administration of motilin increases regular spiking activity in the jejunum and ileum,[a] and it has been hypothesized that the prokinetic action of erythromycin is a result of binding to motilin receptors,[19] similar to other species. Interestingly, in 1 in vitro study,[24] porcine motilin did not have an effect on muscle strips from the equine pyloric antrum. Horses possess intestinal endocrine cells that are immunoreactive for motilin, but to our knowledge, the density and distribution of motilin receptors throughout the gastrointestinal tract has not been studied.[25] It is unknown whether erythromycin promotes motility in horses by binding to these motilin receptors. The purpose of the study reported here was to use an in vitro model to identify and characterize motilin receptors in the equine duodenum, jejunum, cecum, and large colon and to determine whether erythromycin lactobionate competes with porcine motilin for binding to these receptors.

Materials and Methods

Collection of tissue samples—Tissue samples were collected from 4 horses. Horses were 2 to 8 years old and weighed between 420 and 512 kg. None of the horses had gastrointestinal tract disorders or evidence of systemic disease and had not been treated with erythromycin or similar compounds. Horses were euthanatized for reasons unrelated to the gastrointestinal tract. Horses were euthanatized by IV administration of an overdose of pentobarbital sodium.

The technique for preparation of crude membranes has been described for other species.[18] Immediately after horses were euthanatized, various segments of the gastrointestinal tract were exteriorized through a ventral midline incision. Full-thickness sections (5 × 5 cm) of duodenum (10 cm orad to the duodenocolic ligament), jejunum (approx 6 m aborad to the duodenum), cecum (between the cecocolic and the ileocolic fold), and large colon (area of the pelvic flexure) were harvested. Segments were gently lavaged with physiologic saline (0.9% NaCl) solution to remove ingesta. Mucosa and submucosa were dissected free from the smooth muscle tissue with scissors or a scalpel blade. Each smooth muscle sample was rinsed again with physiologic saline solution, wrapped in aluminum foil, snap-frozen in liquid nitrogen, and stored at –70°C until binding studies were conducted.

Binding studies—The technique for binding studies by use of ^{125}I-labeled synthetic porcine motilin has been described for other species.[18] Each section of smooth muscle tissue was weighed (range, 2.2 to 3.5 g; mean, 2.65 g), finely minced, and homogenized in 10 volumes of buffer (250mM sucrose, 50mM tris-HCl buffer [pH, 7.4]), using a homogenizer[b] at 1,500 rounds/min for 2 minutes. Homogenates were centrifuged at 1,000 × *g* for 15 minutes and washed 4 times in 150nM sodium chloride. Homogenates were resuspended in 5 volumes of 50mM tris-HCl-buffer (1.5% bovine serum albumin, 10mM $MgCl_2$ [pH, 8.0]) and divided into 1-mL aliquots for use in binding and displacement studies.

Preliminary experiment—A preliminary experiment was used to evaluate whether there was binding of porcine motilin in various bowel segments, to ensure that the various segments of bowel did not have significant differences for the **equilibrium dissociation constant (K_D)**, and to ensure that free ligand was not depleted during the experiments. Total binding was determined at 20°C by incubating 1.25, 2.5, 5, 10, 20, and 40nM ^{125}I-labeled synthetic porcine motilin[c] with crude membrane preparations of the duodenum, jejunum, cecum, and large colon in 50mM tris-HCl buffer. Identical preparations were incubated with a 100-fold excess of unlabeled motilin to assess nonspecific binding. Three repetitions were performed for each concentration for each segment of bowel from each of the 4 horses. After incubation for 60 minutes, bound and unbound motilin were separated by centrifugation (1,000 × *g* for 15 minutes). The supernatant (unbound motilin) was aspirated, and radioactivity of the pellet (bound motilin) and supernatant was measured in a gamma counter.[d] Measurement of the radioactivity of the supernatant was performed to ensure that depletion of the free ligand (unbound motilin) was maintained at < 5%.[26] Mean total protein content of each pellet was determined by use of a protein assay.[e] Binding values were expressed per milligram of protein. Specific binding was determined by subtracting nonspecific from total binding. The K_D for each segment of bowel (duodenum, jejunum, cecum, and large colon) was obtained for a quadratic model[27] by use of a commercially available statistical program.[f]

Affinity for the motilin receptor—An experiment was designed to reduce the SEM and obtain a more accurate estimate of the affinity of ^{125}I-labeled synthetic porcine motilin for the equine motilin receptor. Total binding was determined at 20°C by incubating 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6nM ^{125}I-labeled synthetic porcine motilin with crude membrane preparations of duodenum from 1 horse (9 repetitions) in 50mM tris-HCl buffer. In addition, membrane preparations of duodenum from the other 3 horses were incubated with 3.2 and 6.4nM ^{125}I-labeled synthetic porcine motilin (5 repetitions). Incubation of identical samples with a 100-fold excess of unlabeled motilin was used to assess nonspecific binding. After incubation for 60 minutes, bound and unbound motilin was separated by centrifugation at 1,000 × *g* for 15 minutes. The supernatant (unbound motilin) was discarded, and radioactivity of the pellet (bound motilin) was measured in the gamma counter. Mean total protein content of each pellet was determined by use of a protein assay.[e] Binding values were expressed per milligram of protein. Specific binding was determined by subtracting nonspecific from total binding. The K_D and **number of binding sites (B_{max})** of the membrane preparations of duodenum were determined by use of nonlinear least-squares analysis.[26,f] The K_D obtained from this analysis was used for the subsequent experiment.

Distribution of motilin receptors—Total binding, nonspecific binding, and specific binding for crude membrane preparations of duodenum, jejunum, cecum, and large colon were determined by incubating 3.2 and 6.4nM ^{125}I-labeled synthetic porcine motilin,[c] using the same technique described previously. We conducted 5 repetitions at each concentration for each segment of bowel from each of the 4 horses. The B_{max} values of the duodenum, jejunum, cecum, and large colon were calculated by use of the following formula[26]: binding = (B_{max} • concentration)/(K_D • concentration). The K_D from the previous experiment was used to solve this equation.

Displacement experiments—Competition for binding sites was evaluated. Displacement curves were obtained by incubating crude membrane preparations of duodenum with 3.2nM ^{125}I-labeled synthetic porcine motilin and increasing concentrations (10^2, 10^3, 10^4, and 10^5nM) of erythromycin lactobionate[g] in 50mM tris buffer. We conducted 15 repetitions for each concentration with tissues from 1 of the horses, but we only conducted 5 repetitions at each concentration for tissues from the other 3 horses. Nonspecific binding was assessed by incubation of identical preparations with 50nM

unlabeled motilin. After incubation for 60 minutes at 20°C, bound and unbound motilin was separated by centrifugation at 1,000 × *g* for 15 minutes. Radioactivity of the pellet (bound motilin) was determined in the gamma counter. Specific binding was determined as described previously. The **negative logarithm of the concentration of erythromycin lactobionate that displaced 50% of the labeled ligand** (**pIC_{50}**) was obtained from the displacement curve.[26,28] Competition of erythromycin lactobionate and ^{125}I-labeled synthetic porcine motilin for motilin receptors of the jejunum, cecum, and large colon was assessed by incubating each of the various crude membrane preparations (3 repetitions for each tissue from each of the 4 horses) with 3.2nM ^{125}I-labeled synthetic porcine motilin and 10^2 and 10^3nM of erythromycin lactobionate by use of the same technique outlined previously. The pIC_{50} values were obtained from the resulting binding curves.

Statistical analysis—Statistical analysis was performed by use of a randomized complete block design to determine whether there was a significant difference between various segments of bowel for values of K_D, B_{max}, and pIC_{50}. Tukey multiple means comparison tests were used when a significant difference was detected.[f] A value of $P < 0.05$ was considered significant.

Results

Preliminary experiment—Analysis of results of the preliminary experiment revealed that motilin receptors were found in the duodenum, jejunum, cecum, and large colon. Mean ± SEM K_D of ^{125}I-labeled synthetic porcine motilin for the equine motilin receptor did not differ significantly among the duodenum (4.85 ± 0.32nM), jejunum (5.12 ± 0.47nM), cecum (6.31 ± 2.13nM), and large colon (7.61 ± 2.16nM). Thus, the affinity of ^{125}I-labeled synthetic porcine motilin for the equine motilin receptor did not change among the various segments of the gastrointestinal tract that were evaluated.

Affinity of the motilin receptor—Affinity of ^{125}I-labeled synthetic porcine motilin (ie, K_D) for the equine motilin receptor was determined by use of nonlinear least-squares analysis. The value determined was 6.1nM **(Fig 1)**.

Distribution of motilin receptors—Mean ± SEM B_{max} for the duodenum, jejunum, cecum, and large colon was 325.1 ± 28.9, 310.1 ± 33, 203.4 ± 15.7, and 239.3 ± 21.1 fmol/mg of protein, respectively. Mean B_{max} differed significantly among various segments of bowel, as indicated on the basis of results of the *F* test. The duodenum had a significantly higher B_{max}, compared with values for the cecum and large colon. The B_{max} for the jejunum was significantly higher, compared with the value for the cecum **(Fig 2)**.

Displacement experiments—Erythromycin lactobionate displaced ^{125}I-labeled synthetic porcine motilin from the equine motilin receptor. Mean pIC_{50} in the duodenum was 2.97 (95% **confidence interval [CI]**, 2.82 to 2.94), which was similar to the value in the jejunum (mean pIC_{50}, 2.91; 95% CI, 2.85 to 2.97), cecum (mean pIC_{50}, 2.89; 95% CI, 2.82 to 2.94), and large colon (mean pIC_{50}, 2.87; 95% CI, 2.8 to 2.94), respectively **(Fig 3)**. The concentration of erythromycin lactobionate required to displace porcine motilin from the equine motilin

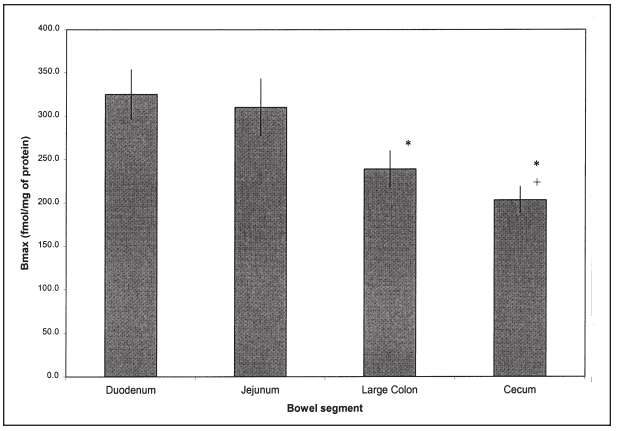

Figure 2—Number of equine motilin receptors (B_{max}) in various segments of the gastrointestinal tract of 4 horses. Values reported are mean ± SEM for 10 measurements of each crude membrane preparation for each segment of bowel obtained from each of the 4 horses. *Segment of bowel has a significantly ($P < 0.05$) lower number of motilin receptors than the duodenum. +Segment of bowel has a significantly ($P < 0.05$) lower number of motilin receptors than the jejunum.

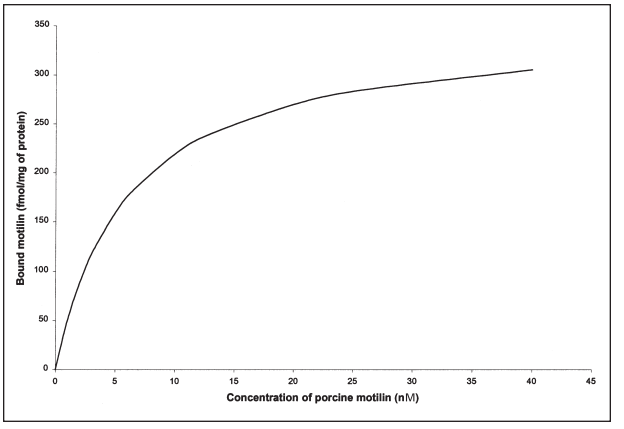

Figure 1—Binding of various concentrations of ^{125}I-labeled synthetic porcine motilin to crude membrane preparations of duodenum obtained from 4 horses. Mean binding values were generated by use of a nonlinear least squares analysis. Mean equilibrium dissociation constant (K_D) of ^{125}I-labeled synthetic porcine motilin for the equine motilin receptor was estimated to be 6.1nM.

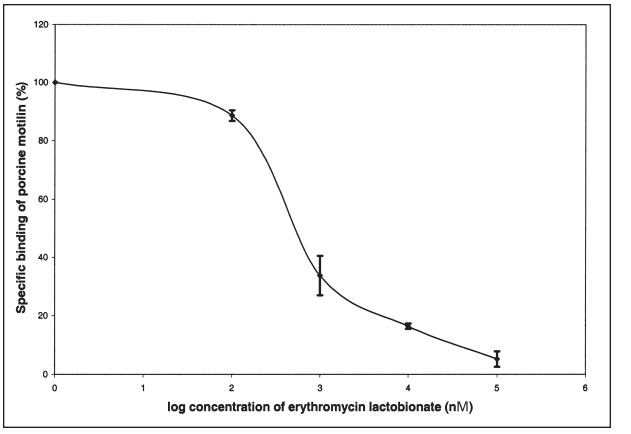

Figure 3—Displacement of ^{125}I-labeled synthetic porcine motilin for various concentrations of erythromycin lactobionate in crude membrane preparations of duodenum from 4 horses. Values reported are mean ± SEM for 15 measurements for each concentration of erythromycin for 1 horse, and 5 measurements for each concentration of erythromycin for each of the other 3 horses.

receptor did not differ significantly among the various segments of bowel.

Discussion

Radioligand binding to receptors was first described in 1965 by Paton and Rang,[29] who investigated binding of ^{3}H-atropine to muscarinic receptors in smooth muscle. Since then, this technique has become widely accepted for use in receptor studies.[26] The existence of motilin receptors in crude membrane preparations of rabbit intestine was first described in 1986 in a study[18] that used a radiolabeled ligand-binding model. We used this technique to obtain biochemical evidence for motilin receptors in horses in the study reported here. Various techniques have been reported for the use of ^{125}I-labeled motilin. Determination of affinity and maximum number of receptors was performed in saturation experiments that used increasing concentrations of radiolabeled motilin[17] or in competition experiments that used a fixed concentration of radiolabeled ligand and increasing concentrations of unlabeled motilin.[30] Large errors in estimated binding values have been reported for saturation experiments.[31] However, comparison of the accuracy of saturation and competition experiments for use in estimating binding values did not reveal significant differences between the techniques.[18] Accordingly, we used a saturation technique to characterize motilin receptors in the equine gastrointestinal tract.

Crude membrane homogenates contain muscle, nerve, and supporting cell membranes. Hence, the precise cellular location of the motilin receptor cannot be determined.[28] Peeters et al[30] had difficulty detecting binding with purified membrane fractions. Furthermore, when tissue is homogenized, cells may be disrupted and expose receptors that were not originally on the cell surface. This would lead to an overestimate of the density of receptors on the cell surface.[32] The goal of the study reported here was to detect motilin receptors in the gastrointestinal tract of horses and their distribution in equine duodenum, jejunum, cecum, and large colon. Our smooth muscle homogenates were all prepared in the same manner, and the results reported were not representative of the absolute number of receptors; however, they provided a good estimate of the relative amount in each tissue.

Nonlinear least-squares analysis was used to estimate that the affinity of ^{125}I-labeled synthetic porcine motilin (K_D) for the equine motilin receptor was 6.1nM. Use of porcine motilin in an equine system may explain the slightly lower affinity observed here, compared with affinity for some other systems.[18,22] For example, chicken motilin had a much higher affinity for the motilin receptor in chicken ileum than did canine or porcine motilin.[33] To our knowledge, equine motilin has not yet been synthesized, although the sequence of DNA encoding the equine motilin precursor has been identified.[34] Comparison of amino acid and nucleotide sequences of the signal peptide and motilin from various mammalian species reveals considerable homology.[34,35] Porcine and equine motilin are 86% homologous with regard to amino acid sequence. The homology is even higher when the N-terminal regions of porcine and equine motilin are compared.[34] The N-terminal region is the bioactive portion of motilin that binds to the motilin receptor.[35]

Mean ± SEM affinity of porcine motilin for the motilin receptor in rabbit stomach and small intestine is 1.1 ± 0.3nM.[18] In another study[17] that used porcine motilin, investigators found an affinity of 1.1 ± 0.22nM for the rabbit motilin receptor in the small intestine, colon, and rectum. In both of those studies, affinity for the motilin receptor did not differ significantly among various segments of bowel. This is in agreement with findings of the study reported here in that we did not detect a significant difference in the K_D between the various segments of the equine gastrointestinal tract.

The highest number of motilin receptors in our study was detected in the duodenum. A significant difference in B_{max} was detected for the duodenum, compared with the cecum and large colon. There also was a significant difference in B_{max} between the jejunum and cecum, but not the large colon. Use of more horses may have increased statistical power and enabled us to detect smaller differences in B_{max} between the jejunum and large colon. Examination of the data revealed that B_{max} was higher, but not significantly different, in the small intestine, compared with the large intestine. The fact that the number of motilin receptors decreased in more aborad segments of the bowel in horses is consistent with similar findings in most other species.[16,36] Rabbits are the only species in which a higher number of motilin receptors has been reported in the colon than the large intestine.[17,18,22]

Erythromycin lactobionate competed with porcine motilin for binding to the equine receptor. These results support the hypothesis of other investigators of horses, namely, that 1 of the prokinetic actions of erythromycin is mediated through binding on the motilin receptor.[19,21,24] However, erythromycin can displace motilin bound to smooth muscle homogenates of cats,[16] rabbits,[17] and humans.[37] The pIC_{50} in the horses reported here was slightly higher than the value reported for cats,[16] rabbits,[17] or humans.[37] This difference may be attributed to slight differences in experimental protocols.

We did not find any differences among the duodenum, jejunum, cecum, and large colon with regard to the ability of erythromycin to displace radiolabeled motilin. Depoortere et al[17] stated that the ability of erythromycin to displace motilin did not vary among various segments of bowel obtained from rabbits. The magnitude of smooth muscle contraction after administration of erythromycin depends on the number of motilin receptors in each segment of bowel.[14] A decrease in the density of motilin receptors in the small intestine of rabbits correlates well with a decrease in contractile response to the erythromycin derivative, EM-523.[37] Although the colon of rabbits had a higher number of motilin receptors then the duodenum, there was not a significant difference in extent of contractile response. On the basis of that fact, it was concluded that receptor density and biological response are related but not directly proportional.[17]

In the study reported here, the highest number of motilin receptors was in the duodenum, and ery-

thromycin displaced motilin from the duodenal receptor. On the basis of these findings, it could be expected that erythromycin would cause a contractile response in smooth muscle strips obtained from the duodenum. It has been reported[24] that erythromycin does not cause contraction of smooth muscle strips obtained from the duodenum; rather, it causes contraction only of smooth muscle strips from the midjejunum and the longitudinal layer of the pyloric antrum. In that study, duodenal muscle strips were harvested at a location 2 to 5 cm aborad to the pylorus. In the study reported here, we collected samples 10 cm orad to the duodenocolic ligament, and it is possible that the smooth muscle of the proximal portion of the duodenum does not have motilin receptors.

We detected motilin receptors in the equine cecum and documented that erythromycin displaced motilin from these receptors. Interestingly, in an in vivo study[38] in healthy horses, motilin injected IV induced strong phase-3 contractions in the jejunum but had no effect on the cecum. On the other hand, erythromycin given IV to healthy ponies promotes cecal emptying and cecocolic myoelectric activity.[19] In another study,[20] there was a prokinetic effect of erythromycin on the cecum 8 days after surgery but not in the early postoperative period. The prokinetic effect of erythromycin in various species is mediated by direct action on motilin receptors[30] or through a mechanism that involves cholinergic and serotonergic nerves.[39-41] Even though we documented that erythromycin binds to motilin receptors in the cecum, the action of erythromycin in the cecum in vivo may be mediated largely through another pathway.

Analysis of results of the study reported here indicated that horses possess motilin receptors. The highest concentration of receptors is in the duodenum, with decreasing numbers in the jejunum, large colon (ie, pelvic flexure), and cecum, respectively. Erythromycin displaces porcine motilin from the equine receptor, indicating that 1 of the prokinetic actions of erythromycin in horses is likely to be secondary to binding of erythromycin to the motilin receptor.

[a]Coatney R, Adams S. The effect of motilin on equine small intestinal motility, in *Proceedings*. 3rd Equine Colic Res Symp 1988;29.
[b]Polytron, Kinematica AG, Litau, Switzerland.
[c]M4505, Sigma-Aldrich Canada Ltd, Mississauga, ON, Canada.
[d]1272 Clinigamma, Perkins-Elmer Wallac, St Laurent, QC, Canada.
[e]BIO RAD protein assay, BioRad laboratories, Toronto, ON, Canada.
[f]SAS, version 6, SAS Institute Inc, Cary, NC.
[g]Erythromycin Lactobionate, Novopharm Ltd, Scarborough, ON, Canada.

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