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Short communication

Development of an enzyme-linked immunosorbent assay for equine neutrophil elastase measurement in blood: Preliminary application to colic cases

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ABSTRACT

Equine neutrophil elastase (NE) is a protease released in inflammatory diseases and participating in tissue destruction. To measure NE in horse plasma to assess its role in pathological conditions, we purified elastase from equine neutrophils by a double step chromatography and obtained a pure protein of 27 kDa, 4 kDa smaller than the NE 2A previously purified (Scudamore et al., 1993; Dagleish et al., 1999), which was likely to be NE 2B. We developed an ELISA by using two specific polyclonal antibodies obtained from rabbit and guinea pig. The sandwich complex was detected using a secondary antibody conjugated to alkaline phosphatase. The ELISA showed good precision and accuracy, with intra- and inter-assay coefficients of variation below 10% for equine NE concentrations ranging from 1.875 to 60 ng/ml. A stable plasma NE value, unaffected by the delay of centrifugation (over 4 h), was obtained with plasma from EDTA anticoagulated blood. The mean value (±SEM) measured in 37 healthy horses was 32.53 ± 4.6 ng/ml. NE level in plasma of horses with colic at the time of admission was significantly higher than in healthy horses. Our results indicate that the ELISA technique we developed to measure plasmatic NE is a powerful tool for studying the role of elastase in equine inflammatory disease. In future, the application will be extended to other equine biological fluids.

1. Introduction

Neutrophil elastase (NE) is a serine protease present in high concentration in the azurophilic granules of the polymorphonuclear neutrophils (Shapiro, 2002), which has been isolated in several animal species. In horse 2 types of neutrophil elastase were identified (Dubin et al., 1976). Because of its abundance in polymorphonuclear neutrophils, NE is thought to serve important biologic functions such as tissue remodelling and turnover (Chua and Laurent, 2006), and pathogen killer; NE is a main

Abbreviations: α_1PI, alpha-1 antitrypsin; α2M, alpha-2 macroglobulin; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate solution; SLPI, secretory leukocyte protease inhibitor; MPO, myeloperoxidase; NBT, nitro blue tetrazolium; NE, neutrophil elastase; RAO, recurrent airway obstruction; ROC, receiver operating characteristic.

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component of the oxygen independent microbicidal pathway (Shapiro, 2002). NE was also shown to enhance intercellular adherence molecule-1 expression by endothelial cells (Ishihara et al., 2006) but its role in the neutrophil migration to extravascular compartments remains debated (Henson and Johnston, 1987; Huber and Weiss, 1989; Delacourt et al., 2002; Scholz et al., 2003). Its main natural inhibitors are alpha-1 antitrypsin (alpha-1 proteinase inhibitor, α_1PI) and alpha-2 macroglobulin (α2M) but NE is also inhibited by secretory leukocyte protease inhibitor (SLPI) found in the mucus secretions and elafin isolated from the lung and the skin (Bieth, 2001).

NE released by activated neutrophils is recognized as an inflammatory and prognostic marker in various conditions (Gross et al., 1993; Zorn et al., 2003; Braga et al., 2006; Langhorst et al., 2008) and is implicated in inflammatory tissue damage (Jochum et al., 1994; Ginsburg, 1999). This enzyme plays a role in the ischaemia-reperfusion injuries (Bzeizi et al., 1996; Okajima et al., 2004; Aoki et al., 2005), and is especially implicated in the human respiratory inflammatory disorders such as acute respiratory distress syndrome, chronic obstructive pulmonary disease and cystic fibrosis (Ohbayashi, 2002). According to the protease–antiprotease imbalance theory, tissue destruction by NE would occur only when the proteolytic activity exceeds the protease inhibitor capacity of the connective tissue, usually when antiproteases are ineffective due to oxidation or genetic defect (Clark et al., 1981; Stockley, 1999).

In horses, NE might intervene in recurrent airway obstruction (RAO) and play a role greater than expected in human asthma, a disease presenting many similarities with RAO (Dagleish et al., 1999; Sampson, 2000; Brazil et al., 2005). An in vitro study by Dagleish et al. (2003) indicates that the NE monitoring could be a potential diagnostic tool for endotoxaemia, a frequent complication in horse colic (Moore and Morris, 1992). However, few data are currently available about the role of NE in equine inflammatory diseases.

Studies performed using NE activity assays probably underestimate the role of elastase because of its interaction with inhibitors. Therefore, the development of an immunological method for the specific measurement of NE in horse plasma will be a strategic advance. We describe here the development and evaluation of an enzyme-linked immunosorbent assay (ELISA) for routine measurement of plasmatic equine NE.

2. Materials and methods

2.1. Reagents

NaCl, KCl, Na_2HPO_4, KH_2PO_4, Na-acetate, acetic acid, cetyltrimethylammonium bromide, ethanol, Coomasie Brillant Blue and Tween 20 were from Merck (Darmstadt, Germany). NaOH was from VWRI (Leuven, Belgium). BSA (Fraction V) was from Roche (Mannheim, Germany). Paranitrophenyl phosphate was from D-tek (Mons, Belgium). Ficoll-paque, tris(hydroxymethyl)aminomethane, porcine pancreatic elastase (type IIA), equine serum albumin, N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, immobilon™-P (PVDF), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate solution (BCIP) were from Sigma–Aldrich (Bornem, Belgium). Ion exchange Hiload SP Sepharose column, Hiload Superdex 200 gel filtration column, Protein A Sepharose column, molecular weight markers were from GE Healthcare (Diegem, Belgium).

2.2. Purification of equine neutrophil elastase

Neutrophil elastase (NE) was purified as previously described (de la Rebière de Pouyade et al., 2009) with minor modifications. Briefly, NE was extracted from equine polymorphonuclear neutrophils isolated from whole blood by sedimentation on Ficoll-Paque density gradient. The pellets of neutrophils were homogenized in sodium acetate buffer (25 mM Na-acetate; 1 M NaCl; pH 4.7) containing 1% cetyltrimethylammonium bromide. The supernatant was collected by centrifugation and dialyzed against the starting buffer (25 mM Na-acetate, 0.2 M NaCl, pH 4.7) to precipitate many proteins. NE was then purified by a double step chromatography using successively an ion exchange Hiload SP Sepharose column (1.6 cm $\times$ 15 cm) and a Hiload Superdex 200 gel filtration column (1.6 cm $\times$ 60 cm). The presence of NE was monitored by the enzymatic assay using the specific substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide. The purity of NE was assessed by enzymatic assays and by 12% SDS-PAGE. The pool of purified NE was used for immunization.

2.3. Immunization and purification of polyclonal antibodies

Animal housing, immunization, and rabbit and guinea pig anti-equine NE antiserum collections were performed at the Laboratory of Hormonology (Centre d'Economie Rurale, Marloie, Belgium). Antisera were raised in 1 rabbit and 1 guinea pig by intradermal injections (1 ml total volume) with a total amount of 100 μg pure equine NE dissolved in 500 μl of 25 mM Na-acetate buffer, 0.2 M NaCl, at pH 5.5, and emulsified with 1 equiv. of Freund complete adjuvant. Booster injections were given at days 14, 28, 42, and 56 with 50 μg of emulsified pure NE. Blood samples were collected at the marginal vein of the ear, 10 days after each booster injection. Twenty-four days after the last booster injection, the animals were exsanguinated by heart puncture under general anaesthesia. The immunoglobulins (IgG) were extracted from the rabbit or guinea pig antiserum by affinity chromatography on Protein A Sepharose column. Anti-NE IgG were conjugated to alkaline phosphatase (AP) by Zentech (Liège, Belgium) and specificity was tested by Western blot on 0.45 μm PVDF membrane against the purified equine NE and an extract of 150×10^6 equine neutrophils, obtained by 2 cycles of freezing/thawing. Revelation was performed with an NBT/BCIP solution.

2.4. Sandwich ELISA for neutrophil elastase

The microplate wells (Nunc, MaxiSorp, Denmark) were coated (overnight, at 4 °C) with 150 μl of the rabbit anti-NE IgG solution (primary antibody). Several

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concentrations (0.5, 1, 2, 3, 4 and 5 μg/ml) of the primary antibody dissolved in the coating buffer (10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) were tested to select the optimal (saturating) concentration for further use in the ELISA. After the primary antibody coating, the plates were washed 4 times with 300 μl of the first washing buffer (154 mM NaCl solution with 0.1% Tween 20). Two hundred microlitres of the blocking buffer (coating buffer with 5 g/l BSA) were then added and the plates were incubated for 150 min at room temperature (20 °C). After 4 washes with the first washing buffer, 100 μl of equine NE standards or tested samples were added to the wells and incubated overnight at 4 °C. Control (blank) and dilutions of the samples were made with the dilution buffer (blocking buffer added with 0.1% Tween 20). After 4 washes with 300 μl of the second washing buffer (150 mM NaCl, 50 mM Tris–HCl, 0.1% Tween 20, pH 7.5), the plates were incubated (2 h, 37 °C) with 100 μl (3 μg/ml) of the secondary antibody conjugated to AP and diluted with the second washing buffer. After washing (second washing buffer), phosphatase activity was detected by incubation (30 min, 37 °C, in the dark) with the substrate paranitrophenyl phosphate. The reaction was stopped with 2.5 M NaOH, and the absorbance was read at 405 nm with the Multiscan Ascent plate reader (Thermo Labsystems, Helsinki, Finland). A standard curve was generated to allow determination of NE concentrations in samples.

2.5. Effect of the anticoagulant and the delay between blood sampling and centrifugation

Blood samples were drawn from the jugular vein of 5 healthy horses into plain tubes or tubes containing EDTA, heparin, or citrate as anticoagulant (Vacuette, Greiner bio-one, Belgium), and centrifuged at 0.5, 1, 2 and 4 h after sampling. The plasma and serum were kept frozen in small aliquots at −20 °C until use.

2.6. Validation of the ELISA

The cross-reactivities of the anti-elastase antibodies were tested with human NE, porcine pancreatic elastase, equine albumin, equine myeloperoxidase (MPO) and extracts from horse erythrocytes and lymphocytes, as previously described (Deby-Dupont et al., 1998). The detection limit was defined as the lowest point of the NE standard curve with an absorbance value that was statistically different from the absorbance value obtained with dilution buffer alone. The standard curve of the ELISA was calculated and plotted point to point using the Ascent software for the Multiscan Ascent plate reader. The precision and reproducibility were estimated by the coefficients of variation (CV) within or between assays calculated from several measurements of the same samples. The accuracy was assessed by measuring NE in successive dilutions (10, 20 and 40 times) of 4 samples. We also evaluated the accuracy by estimating the recovery of known amounts of NE (2.5, 10 and 20 ng/ml) spiked into 4 equine plasma samples ($n = 12$).

2.7. Mean normal and pathological values of equine NE in blood

Blood samples from 37 healthy horses were drawn into EDTA tubes (mixed breeds, mean age ± SD: 6.2 ± 2.3 years). Horses were considered as healthy if no clinical signs of acute disease were observed at the time of sampling.

To assess the capacity of the new ELISA to measure blood NE in pathological conditions, EDTA blood samples were also obtained from 49 horses admitted for colic at the Equine Clinic of the Faculty of Veterinary Medicine of Liège (Belgium) between 2005 and 2008 (mixed breeds, mean age ± SD: 8.9 ± 5.2 years). Horses suffered either from strangulated small ($n = 20$) or large ($n = 16$) intestine diseases, or from medical inflammatory diseases (peritonitis, diarrhea, proximal enteritis) ($n = 13$). The blood was immediately centrifuged and the plasma frozen until assay.

2.8. Statistical analyses

Statistical treatments were performed using Instat 3.05 or Medcalc 7.6 software. One-way ANOVA with Tukey's post-test was used to evaluate the differences in NE measurements over the time for the same anticoagulated samples. The differences in NE measurements according to the type of anticoagulant at the same time were assessed by using unpaired *t*-test with Welch correction when necessary.

Differences in plasma NE levels between healthy and pathological horses were evaluated by using Mann–Whitney test. The significance was set at $p < 0.05$.

The sensitivity level, the specificity and the ideal cutoff value to distinguish healthy from pathological horses were performed by using receiver operating characteristic (ROC) curve analysis.

3. Results and discussion

As assessed by enzymatic assay and 12% SDS-PAGE, that showed a unique band, we purified a protein with elastase activity and a molecular weight around 27 kDa (Fig. 1). We previously reported equine elastase (from the same batch) with a molecular weight around 29 kDa, as determined on 8–18% polyacrylamide gradient gel (de la Rebière de Pouyade et al., 2009). This variation was attributed to the difference in the electrophoresis technique. Dubin et al. (1976) reported that 2 elastase-like enzymes are present in horse neutrophils, elastase 2A and 2B with MW of 24.5 and 20.5 kDa, respectively, that is a difference of 4 kDa in the MW of the 2 types of NE. Additionally, other articles, with similar resolving gel of electrophoresis that we used, reported the purification of elastase 2A with a MW of 31 kDa (Scudamore et al., 1993; Dagleish et al., 1999). Thus, we observed between our purified NE (27 kDa) and the latter NE 2A (31 kDa) the same MW difference than that reported by Dubin et al. (1976). Consequently, despite of discrepancies in the literature regarding the MW of elastases, we were likely to have purified the NE 2B.

We developed polyclonal antibodies against purified NE in 2 animal species to assure the specificity of the test. The Western blot confirmed the specificity of the antibodies for

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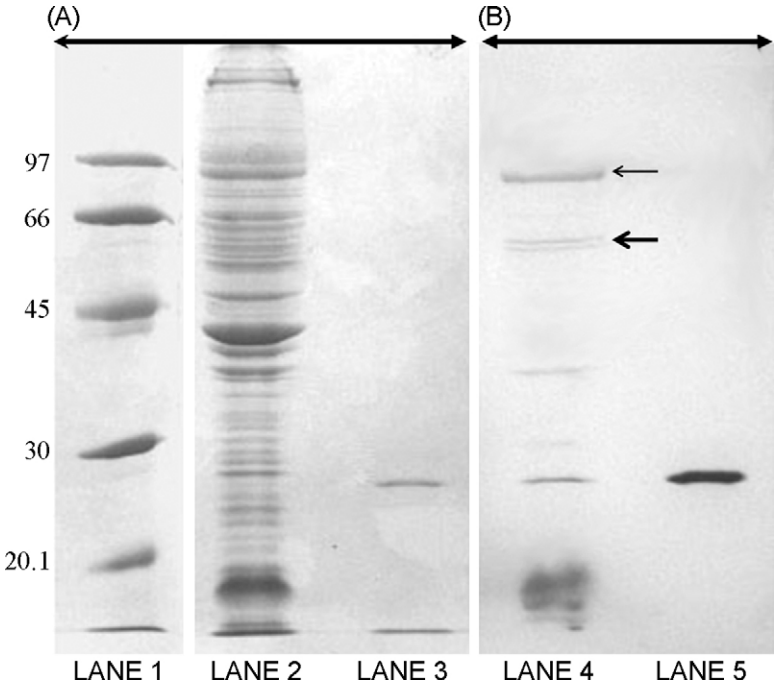

Fig. 1. Characterization of NE and NE antibodies. (A) 12% SDS-PAGE of a mixture of molecular weight markers (MW in kDa), a neutrophil extract (10 μl/lane), and purified equine neutrophil elastase (5 μg/lane) in lanes 1–3, respectively. (B) Western blot performed with guinea pig anti-NE antibodies: neutrophil extract (lane 4) and purified equine neutrophil elastase (lane 5). Thin arrow indicates the probable NE–α_1PI complex and the thick arrow the NE–HLEI complex.

NE, and identified on neutrophil extract a major band corresponding to free elastase, but also accessory bands with MW ranging from 61.5 to 81 kDa, which are consistent with NE–inhibitor complexes (α_1PI and HLEI, Horse Leukocyte Elastase Inhibitor) as previously described (Dagleish et al., 1999). The diffuse reaction observed in the area of the low molecular weight proteins was likely to represent degradation products of NE or NE–inhibitor complexes (Fig. 1). Immuno-specificity was further demonstrated by the absence of cross-reactivities of the antibodies with human NE, porcine pancreatic elastase, equine albumin, MPO and components from equine erythrocytes and lymphocytes, as shown by the lack of response to the ELISA (data not shown).

The presence of the α_1PI in the serum and neutrophil cytoplasm (Scudamore et al., 1993; Dagleish et al., 1999) could justify the use of polyclonal antibodies able to capture the complex NE–α_1PI. The reaction of anti-NE antibodies with the complex NE–α_1PI was previously reported, and no significant difference was found between samples containing either NE or NE with an excess of α_1PI (Dagleish et al., 1999). This observation is supported by the accuracy of our test, in which the recovery of pure NE added to plasma containing α_1PI was excellent ($n = 12$; $100.98 \pm 8.01\%$ [SD]; range: 91.55–119.14) and by the Western blot which showed bands consistent with NE–inhibitor complexes (Fig. 1).

The optimal concentration of the primary antibody (rabbit anti-NE IgG) was 2 μg/ml (or 300 ng/well) as evaluated on the basis of the detection limit and the slope of the standard curve. After washing, the coated plates could be stored at 4 °C under dry conditions for up to 4 weeks. The best binding of NE, as assessed by the signal intensity, was obtained with an overnight incubation time (18 h) at +4 °C, and was used in further studies. The concentration of the secondary AP-conjugated antibody (guinea pig anti-NE IgG) was chosen to saturate the captured NE at the highest standard point, and was 3 μg/ml, i.e. 300 ng/well. The calibration curve was a classical one representing the linear part of a sigmoid function (Fig. 2A). For NE concentrations higher

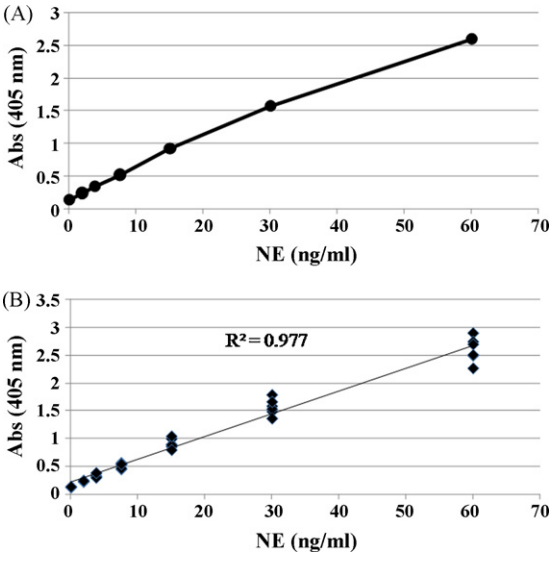

Fig. 2. Calibration curve for equine NE ELISA (A) and linear regression representation with correlation coefficient ($n = 6$) (B).

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Table 1
Intra- and inter-assay values of reference equine NE curve and reproducibility of equine NE measurements in EDTA-plasma samples.

Purified NE (ng/ml)	Intra-assay			Inter-assay		
	Abs ($n = 5$)	SD	CV (%)	Abs ($n = 6$)	SD	CV (%)
60	2.69	0.04	1.7	2.60	0.22	8.59
30	1.58	0.08	5.3	1.57	0.15	9.29
15	0.9	0.03	3.5	0.92	0.09	9.92
7.5	0.54	0.01	2.2	0.52	0.05	8.84
3.75	0.33	0.01	2	0.34	0.04	10.59
1.875	0.24	0.01	3.6	0.24	0.01	3.24
0	0.15	0.01	3.9	0.14	0.01	4.55

Plasmatic NE (ng/ml)	Intra-assay			Inter-assay			
n[a]	Mean NE value	SD	CV (%)	n[a]	Mean NE value	SD	CV (%)
3	7.39	0.49	6.68	4	8.23	0.68	8.26
3	10.06	0.37	3.69	4	9.8	0.72	7.39
3	81.66	3.29	4.03	3	11.92	0.13	1.08
4	143.4	9.19	6.4				
4	252.66	3.5	1.38				

[a] Number of measurements per sample.

than 60 ng/ml the curve reached the classical plateau. The linear regression performed on the calibration curve indicated a highly significant correlation ($p < 0.0001$) (Fig. 2B). The intra-assay and inter-assay CV of the calibration curve did not exceed 5.30 and 10.59%, respectively, indicating the good reproducibility of the test (Table 1). The detection limit of the test was 0.56 ng NE/ml. The NE values measured by ELISA in 3 successive dilutions of a sample were quiet similar: 30.1, 30.5 and 33.7 ng/ml for sample 1, 23.2, 20.9 and 22.8 ng/ml for sample 2, 10.6, 10.5 and 11.3 ng/ml for sample 3, and 6.8, 5.9, and 5.9 ng/ml for sample 4. With the good recovery obtained from the addition of known NE amounts to the plasma samples, the results were consistent with a high accuracy of our immunoassay.

The influence of anticoagulant and delay between blood sampling and centrifugation on the measured NE values are summarized in Fig. 3. The high levels of NE measured in the serum samples could be explained by the activation of the neutrophils during the coagulation. With lithium–heparin, we also measured high levels of NE, but there was no evidence of increased values with time. Studies showed the ability of heparin compounds to remove some proteins such as myeloperoxidase and lipoprotein lipase from the cell surface (Chevreuil et al., 1993; Baldus et al., 2006), and it was reported that human neutrophils express persistently active cell surface bound NE (Owen et al., 1995). The high NE value that we observed for samples drawn on lithium–heparin could perhaps be explained by such a releasing effect of heparin. The lowest concentrations of NE were observed with EDTA and citrate as anticoagulants and these concentrations did not increase over time, demonstrating the stability of the neutrophils. We thus decided to use EDTA-anticoagulated plasma samples in further investigations.

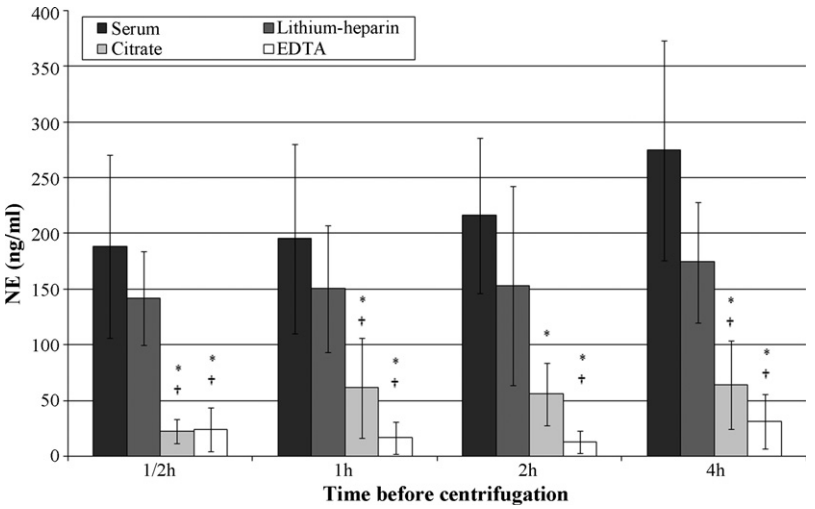

Fig. 3. Effect of the delay of centrifugation after sampling and the type of anticoagulant on the measured NE value (mean ± SD, $n = 5$). $^{*}p < 0.05$ between serum and citrate or EDTA. $^{\dagger}p < 0.05$ between lithium–heparin and citrate or EDTA.

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The mean NE value ± SEM measured in 37 healthy horse plasmas was 32.53 ± 4.6 ng/ml (range: 4.7–101.53 ng/ml). Interestingly, this value is similar to human normal value (22 ± 20 ng/ml) (Gach et al., 2006). The NE concentration from healthy horses was statistically different from the mean NE values (141.96 ± 26.42 ng/ml; range: 11.42–824.19 ng/ml) obtained for all the pathological horses ($n = 49$). No significant difference was detected between the mean NE values of the subgroups of pathological horses: 121.03 ± 41.93 ng/ml (range: 11.42–824.19 ng/ml) and 169.45 ± 51.98 ng/ml (range: 27.03–728.87 ng/ml) in strangulated small ($n = 20$) and large ($n = 16$) intestinal disorders, respectively, and 140.33 ± 44.17 ng/ml (range: 12.27–516.81 ng/ml) in horses with medical diseases ($n = 13$). The optimal cutoff point to distinguish between "health" and "pathology" was 35.9 ng NE/ml of plasma (sensitivity 81.6%, specificity 73%). The area under the ROC curve was 0.810 (95% confidence interval: 0.711–0.886). The intra- and inter-assay CV in plasma are gathered in Table 1 and did not exceed 10%.

Our ELISA thus measures NE in plasma with sufficient specificity, sensitivity and accuracy to allow clinical studies, and preliminary results display the interest of a better knowledge of the role of NE in the pathophysiology of shock in colic or inflammatory processes. To our knowledge, this is the first report about the characterization of a test for the measurement of NE in plasma of horses. In a near future, the measurement of NE will be extended to other biological fluids such as peritoneal, synovial and bronchoalveolar fluids.

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