Development of a Radioimmunoassay for Bovine Chymosin B

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Summary

The present study was conducted to develop and validate a specific radioimmunoassay system for measurement of bovine chymosin B (bChyB) concentrations in plasma samples. Bovine ChyB was used for immunization of rabbits and as standard and tracer. Chymosin B concentrations were measured in plasma samples from two groups of calves (Group 1: calves sampled from birth to 24 hours; Group 2: calves sampled from Day 1 to 21 after birth) and from one cow during the peri-partum period. Detection limit of the assay was 9.0 ng/ml. Recovery was higher than 89.3%. Repeatability and reproducibility ranged from 1.52% to 5.23% and from 1.52% to 12.57% respectively. No cross-reaction was found with pepsinogen A from bovine, porcine or human origins. In Group 1, bChyB concentrations increased from 47.3 ± 45.1 ng/ml (5 min after birth) to 325.5 ± 161.2 ng/ml (12 hours after birth), then no significant change was observed till 24 hours after birth (293.0 ± 161.5 ng/ml). In Group 2, concentrations decreased from Day 1 (455.3 ± 191.1 ng/ml) to Day 21 (117.9 ± 85.1 ng/ml). In adult cow, mean concentration was 136.0 ± 32.3 ng/ml. In conclusion, bChyB is able to cross the stomach basal membrane and to reach the blood circulation at detectable levels in both young calves and adult cows.

Résumé

Développement d'un dosage radioimmunologique de la chymosine B bovine

La présente étude a été menée pour développer et valider un système de dosage radioimmunologique spécifique pour la détection de la chymosine B (bChyB) dans des échantillons de plasma. De la chymosine B a été utilisée pour l'immunisation des lapins et la production de standard et de traceur. Les concentrations de bChyB ont été mesurées dans le plasma chez deux groupes de veaux (Groupe 1: de la naissance à 24 heures, Groupe 2: du jour 1 au jour 21 après la naissance) et chez une vache gestante durant la période péri partum. La limite de détection du dosage était de 9 ng/ml et l'exactitude était supérieure à 89,3%. La répétabilité et la reproductibilité étaient comprises entre 1,52% et 5,23%, et entre 1,52% et 12,57% respectivement. Aucune réaction croisée n'a été détectée avec les pepsinogènes A d'origine bovine, porcine ou humaine. Chez le Groupe 1, les concentrations de bChyB augmentent de 47,3 ± 45,1 ng/ml (5 min après naissance) à 325,5 ± 161,2 ng/ml (12 heures après naissance), puis aucun changement notable n'est observé jusqu'à 24 heures après naissance (293,0 ± 161,5 ng/ml). Chez le Groupe 2, les valeurs diminuent du jour 1 (455,3 ± 191,1 ng/ml) au jour 21 (117,9 ± 85,1 ng/ml). Chez l'adulte, la concentration moyenne était de 136,0 ± 32,3 ng/ml. En conclusion, le système a permis de doser spécifiquement la bChyB dans des échantillons provenant de veaux et de vache adulte, démontrant ainsi le passage de cette enzyme de la paroi stomacale vers la circulation sanguine.

Introduction

The measurement of blood pepsinogen is considered to reflect the morphological and functional status of the gastric mucosa. Pepsinogen assay is used for the indirect diagnosis of gastrointestinal diseases by *Ostertagia* or *Haemonchus* infestation in bovines. These worms, in particular *Haemonchus,* are widespread in both temperate and tropical area. They are responsible of important losses in animal husbandry by mortality or decrease of milk and meet production. Especially in tropical area, *Haemonchus* was shown being developed high capacity to resist and survive in these arid or sub-arid countries. These helminthosis need a better control by indirect diagnosis before the first clinical signs of disease. The digestive enzymes such as the different forms of pepsinogens and prochymosines are good candidate to serve as clinical markers.

Recently, we purified and developed the radioimmunoassay of bovine pepsinogen A (18). Pepsinogen belongs to the aspartic proteases family. In this group pepsinogen A is coexisting with pepsinogen C (progastricsine) and prochymosins A, B and C which are in general considered to be specifically found in neonate animals. These molecules are constituted by one single polypeptide chain containing 372 and 381 residues respectively for bovine pepsinogen A and bovine prochymosin B. These zymogens are specialized in the digestion of dietary proteins and the optimum pH of their activation is around 2 and 4.5 respectively. The C form of pepsinogen have been detected in bovine, however, probably due to its relative low gastric expression when compared to pepsinogen A, it was not fully characterized in term of amino acid sequence.

In human, pepsinogens were also studied for individual diagnonis of gastrointestinal pathologies, in 1989, Biemond *et al*. (5) showed that the specific and simultaneous measurement of two different forms of pepsinogen (A and C), and the use of the ratio A/C, gave more accurate informations for the differential diagnosis of gastric ulcers and cancers. After this, the scientific literature underlined repeatedly the greater interest of this simultaneous measurement of different zymogens in peripheral blood. So, after the development of the RIA of bovine pepsinogen A as indicated above, we complete our approach in order to develop an indirect marker for bovine helminthiasis, by the radioimmunoassay for a second molecule belonging to the family of the aspartic proteases. Because of the difficulty to obtain the purified bovine pepsinogen C, bovine chymosin B was considered in this work. This protein being available from commercial origin (> 90% purity). The RIA was validated in plasma, in buffer and then used to measure

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plasmatic concentrations in newborn and young calves, and to investigate the presence of these zymogens in an adult cow around parturition.

Materials and methods

Antisera production

Work on animals was approved by the ethical committee from University of Liège (Authorisation n. 297). Antisera against bChyB were raised in New Zealand white rabbits according to the method of Vaitukaitis *et al.* (22). During two months, four rabbits (R#814, R#815, R#816 and R#817) aged between 3 and 5 months received intradermal injections (15 days interval) of 500 µg of bChyB (Sigma-Aldrich Co., St. Louis, MO, USA; Ref. R-4879) dissolved in 500 µL of phosphate buffer (0.05 M, pH 7.5), and emulsified in an equal volume of Freund's complete adjuvant (Sigma-Aldrich Co.). Afterwards, rabbits received monthly booster doses of bChyB (500 µg) emulsified in Freund's incomplete adjuvant (Difco Laboratories, Detroit 1, USA) over a period of six months. The animals were bled two months after the first injection and then monthly. The blood samples were allowed to clot overnight at room temperature. The next day, the serum was transferred and centrifuged at 2,500 x g for 20 min, then aliquoted and stored at -20 °C till used.

Radiolabeled bChyB (tracer)

The antigen was labeled according to the chloramine T method (13). For the radio-iodination, 10 µl of bChyB (1 mg/ml), 10 µL of phosphate buffer (0.5 M, pH 7.5), 10 µL of ^{125}I-Na (1 mCi) (Amersham Biosciences, Uppsala, Sweden), buffered by 10 µl of phosphate buffer (0.5 M, pH 7.5) and the oxidation is induced by 10 µL of chloramine T (5.0 mg/ml) (Sigma-Aldrich Co.). After 1 min of gentle stirring, 10 µL metabisulfite (30.0 mg/ml) (Sigma-Aldrich Co.) was added to stop the reaction.

^{125}I-bChyB was purified by gel filtration on a Sephadex G-75 column (1 x 30 cm) (Amersham Biosciences) equilibrated and eluted with the 0.05 M phosphate buffer. Eluted aliquots of 1.0 ml were collected, submitted onto a test with the antiserum, and selected according to the non-specific binding value (NSB) and binding/total (B/T) ratio. The fractions exhibiting the highest performances in term of specific binding were diluted, aliquoted (1.0 ml) and stored at -20 °C until used in the RIA procedure.

Second antibody precipitant system

The second antibody was prepared by mixing sheep anti-rabbit IgG serum with normal rabbit serum (5:1; v:v). This solution was incubated for 16 h at 4 °C before used at 1% in the precipitation system, which constituents were 0.05 M phosphate buffer (pH 7.5), 0.4% BSA, 4% PEG 6000 (VWR, Leuven, Belgium) and 0.05% microcristalline cellulose (Merck, Darmstadt, Germany).

Antisera test

The four obtained antisera were serially diluted in the RIA buffer (1/10,000, 1/20,000, 1/40,000, 1/80,000, 1/160,000 and 1/320,000) in order to obtain a tracer-binding ratio in the zero standard of approximately 30-50% (B_0/T) and non-specific binding below 2%. For the binding test, 100 µL of the initially diluted antiserum, 100 µL of the ^{125}I-bChyB and 300 µL of the RIA buffer were incubated overnight before addition of the second antibody precipitation system, centrifugation and count of the pellet radioactivity.

Standard curves

Bovine chymosin B (lyophilized powder) was diluted with assay buffer (Phosphate buffer: 0.05 M, pH 7.5, containing 1 g/L BSA and 5.0 ml of Tween 20 was used throughout the procedure) to give standard curves ranging from 4.75 to 2,500 ng/ml. The RIA was performed at 4 °C in polystyrene tubes (75 x 12 mm). Each tube contained 100 µL of standard dilution or 100 µL of unknown sample, 100 µL of antiserum (R#817) diluted at 1/50,000 (1/250,000 final dilution), and 100 µL of labeled ^{125}I-bChyB (20,000 cpm). The incubation volume was made up to 500 µL by addition of 200 µL of RIA buffer. In the zero standard tubes (B_0), the standard dilution was replaced by assay buffer. The non-specific binding (NSB) tubes contained 400 µL of buffer and 100 µL of the tracer. All standard and unknown tubes were set up in duplicate. The mixture was incubated at 4 °C from 18 to 24 h. After addition of 1.0 ml of the second antibody precipitation system, the mixture was allowed to react at 4 °C for 1 h. Free and bound bChyB were then separated by centrifugation at 3,000 x g for 20 min. The supernatant was discarded and the radioactivity of the pellet was determined in an automatic gamma counter with a counting efficiency of 75% (1261 Multigamma, Wallac, Turku, Finland).

Characteristics of the RIA

To determine the specificity, available members of the aspartic protease family such porcine pepsinogen A (Sigma-Aldrich Co.) and human pepsinogen A (Sigma-Aldrich Co.) were tested in the range from 19.5 to 10,000 ng/ml. Purified bovine pepsinogen A (18) was tested in the range from 19.5 to 10,000 ng/ml. In addition, different dilutions of a plasma sample containing detectable concentrations of bChyB were set up in buffer (1/1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64). The accuracy test was carried out by adding defined concentrations of bChyB (9.5, 39.0, 156.0, 625.0 and 2,500 ng) to three bovine plasmas containing known concentrations. The percentage of recovery was calculated as following [observed value (ng/ml)/expected value (ng/ml)] x 100. The precision of the RIA was determined by estimating the intra- and inter-assay coefficients of variation (CV). To determine the intra-assay CV, the same serum was assayed 10 times using the same assay. The inter-assay CV was assessed by analyzing the concentrations of different sera in five consecutive assays. The sensitivity was determined by measuring the least detectable dose (LDD) of bChyB in the developed RIA (20). A set of 20 zero binding value were assayed in the same test. The mean and the standard deviation (SD) of precipitate counts at zero bChyB concentration were calculated. The bChyB value that corresponded to the mean count minus two standard deviations transposed onto the standard curve was defined as the sensitivity of the RIA.

Animals and plasma samples

Two groups (Group 1 and 2) of ten newborn calves each were followed. The first group (n= 10) was serially sampled at 5 minutes, 1 hour, 6 hours, 12 hours and 24 hours after birth. The second group (n= 10) was housed in the experimental farm of the Faculty of Veterinary Medicine (University of Liège, Belgium) and sampled when calves were 1, 3, 5, 7, 14 and 21 days old. In addition, one cow was serially sampled during peri-partum period (from day 33 before parturition till day 3 after calving). Blood samples were taken from the jugular vein. Plasma was separated by centrifugation at 2,000 x g and stored at –20 °C until used.

Statistical analysis

The data obtained were analyzed with commercially available Microsoft Excel program. The inter- and intra-assay CV were calculated as the SD divided by the mean value. Mean recoveries at each concentration were calculated as a percentage of the expected value.

Results

Antisera dilution

The dilution curves of the four raised antisera are presented

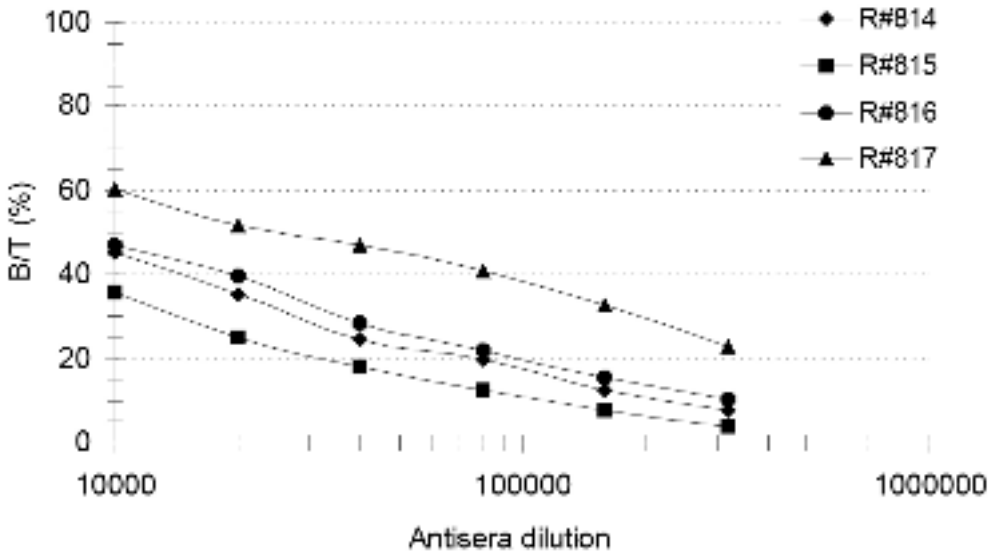

Figure 1: Binding ratio after serial dilutions of four antisera (R#814, R#815, R#816 and R#817) raised against a bovine chymosin B preparation.

in figure 1. In the presence of excess of antiserum R#817 (1/10,000), 60.4% of labeled bChyB was bound and the non-specific binding was below 2%. At the same antisera excess conditions (1/10,000), the binding ratios of R#814, R#815 and R#816 were 45.25%, 35.71% and 47.06%, respectively. These binding levels point out the fact that the performed protocol of immunization had a good efficacy.

Standard curve and bChyB-RIA validation

The ^{125}I-bChyB tracer bound antiserum R#817 at a high final dilution (1:250,000). At this dilution, it bounds 40% of tracer with a non-specific bound below 2%. The standard inhibition curve ranged from 97.7% to 10.3% binding when serial dilutions of bChyB (ranging from 4.75 to 2,500 ng/ml) were assayed. The calculated detection limit was 0.9 ng/tube or 9 ng/ml of plasma. The displacement of the standard curve of the RIA is presented in figure 2.

As shown in figure 3, no cross-reaction was observed in bChyB assay with either bovine, porcine or human pepsinogen A. Serial dilution of a calf plasma sample showed a dose–response curve parallel to the standard curve (Figure 2). The recovery of the assay ranged from 89.3% to 112.4% (Table 1). The coefficients of variation are presented in table 2. The intra-assay CV ranged from 1.52% to 5.23%, and the inter-assay CV from 1.52% to 12.57%.

Plasma bChyB concentrations

In the first group, bChyB was detected at low concentrations just after birth (5 minutes) (47.3 ± 45.1 ng/ml). Thereafter, the concentrations increased progressively and the highest value was observed at 12 hours after birth (325.5 ± 161.2 ng/ml) (Figure 4a). In the second group, plasma bChyB concentrations showed a gradual decrease with increasing age from day 1 (455.3 ± 191.1 ng/ml) to 21 days of age (117.9 ± 85.1 ng/ml) (Figure 4b).

Interestingly, bChyB concentrations were also detected in an adult cow. In this animal, concentrations ranged from 85 to 222 ng/ml (136 ± 32.3 ng/ml, for the whole period) indicating the presence of a basal detectable bChyB concentrations in adult animals.

Discussion

The present study was performed to develop a specific RIA system for bovine chymosin B, as well as to measure its concentration in plasma samples from calves and adult animals. The protocol of immunization gave four antisera with high titers, allowing the choice of antiserum R#817 for our RIA system. When used at 1:250,000 final dilution, this antiserum yielded 40% of specific binding. We recently described the development of a bovine pepsinogen A RIA

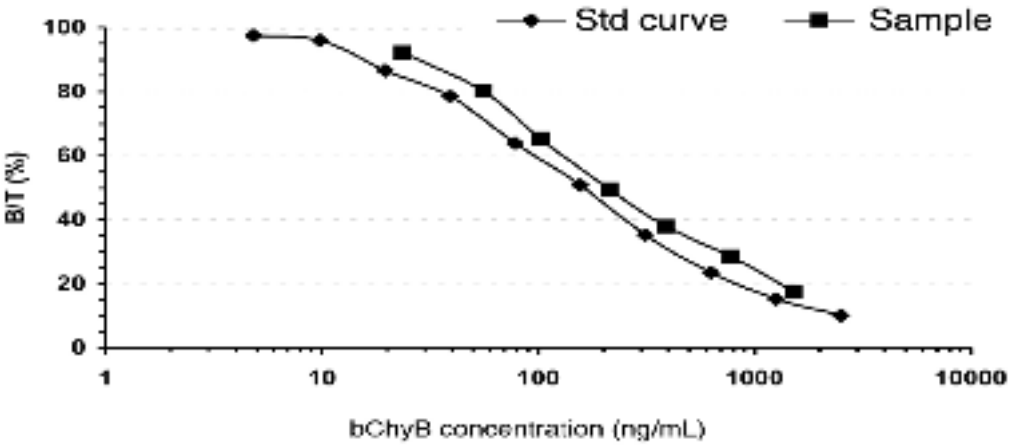

Figure 2: Parallelism between bovine chymosin B (bChyB) standard curve and serial dilution of a calf serum sample. The standard curve was calculated by the linear scale of B/B_0 ratio versus decadic logarithmic of the standard concentration using radiolabeled bChyB. B/B_0 means tracer bound / tracer bound in zero standard. The serum sample (1/1) was serially diluted at 1/2, 1/4, 1/8; 1/16, 1/32 and 1/64.

Table 1
Recovery of bovine chymosin B (bChyB) added to three samples containing different concentrations of bChy

Initial serum sample bChyB concentration	Amount of bChyB (ng) added	Expected bChyB concentration (ng/ml)	Observed bChyB concentration (ng/ml)	Recovery* (%)
1.7 ng/ml	9.5	11.2	10.0	89.3
	39.0	40.7	37.8	92.9
	156.0	157.7	160.1	101.5
	625.0	626.7	570.0	91.0
	2,500.0	2,501.7	2,252.9	90.0
25 ng/ml	9.5	34.5	34.7	100.7
	39.0	64.0	69.1	108.0
	156.0	181.0	162.0	89.5
	625.0	650.0	605.3	93.1
	2,500.0	2,525.0	2,667.7	105.6
125 ng/ml	9.5	134.5	133.4	99.2
	39.0	164.0	162.8	99.3
	156.0	281.0	271.1	96.5
	625.0	750.0	677.0	90.3
	2,500.0	2,625.0	2,951.0	112.4

*(Observed value/Expected value) x 100.

Table 2
Coefficients of variation intra-assay and inter-assay of bovine chymosin B (bChyB) RIA

Bovine sample	Intra-assay		Inter-assay	
	bChyB concentration[a]	CV (%)	bChyB concentration[a]	CV (%)
Sample 1	126.62 ± 1.93 ng/ml	1.52	126.30 ± 1.92 ng/ml	1.52
Sample 2	30.61 ± 1.52 ng/ml	5.23	25.16 ± 3.16 ng/ml	12.57

[a]Mean ± SD.

system (19), the dilution of the antiserum being closely related to that of chymosin B (1:250,000). It is a general observation that valuable RIA systems are developed with antiserum used at high dilution. This assumption is sustained by the RIA systems developed for human pepsinogens A and C (5), in which antisera were diluted at 1:200,000 and 1:300,000, respectively. Similar high antiserum titer was also reported by Banga-Mboko *et al.* (3) who used a dilution of 1:200,000 for porcine pepsinogen A RIA system. Nevertheless, a number of studies reported lower dilution levels of some pepsinogen antisera. This was the case of studies conducted by Samloff and Liebmann (17) who reported a final dilution of 1:50,000 for human pepsinogen antiserum, and by Nappert *et al.* (16) who reported a final dilution of 1:18,000 for porcine pepsinogen antisera. In the same way, Gomes *et al.* (12) reported the development of an ELISA for bovine pepsinogen in which the antiserum was diluted at 1:2,000. Advantages related to the use of high dilutions of antiserum are the possibility to perform a large number of assays with a small volume of the antiserum, and a gain in sensitivity and specificity.

In our RIA protocol, the assay gave a sensitivity of 0.9 ng/tube (9 ng/ml of plasma) without any pre-incubation step. Considering the specificity, no cross-reaction was noted with bovine, porcine or human pepsinogen A. This lack of cross-reaction point out the high specificity of the present assay. The recovery values calculated in the present work (89.3% or higher) were closely similar to those reported for RIA of porcine pepsinogen A (81.7 to 102.3%), human pepsinogen A (81.3 to 139.5%) or bovine pepsinogen A (85.5 to 103.3%) (3,5,19). The assay showed an intra-assay coefficient of variation of 5.23% or lower, and an inter-assay variation about 12.57% or lower. The general characteristics of the assay in term of sensitivity, specificity, accuracy, intra and inter-assay coefficients of variations were satisfactory; the assay is therefore suitable for the measurement of chymosin in bovine plasma.

To our knowledge, the present study is the first report on bChyB concentrations in both young calves and adult cow plasma samples. So, any comparison of the present concentrations with those of other zymogens or those obtained in other species has to be interpreted with great caution. Just after birth, chymosin concentrations were low, they increased between the first and the fourth samples. This sharp increase correspond also to the period of colostrum consumption. Surprisingly, in our study, high level of chymosin was observed in colostrum whereas no chymosin increase was detected in the maternal blood around

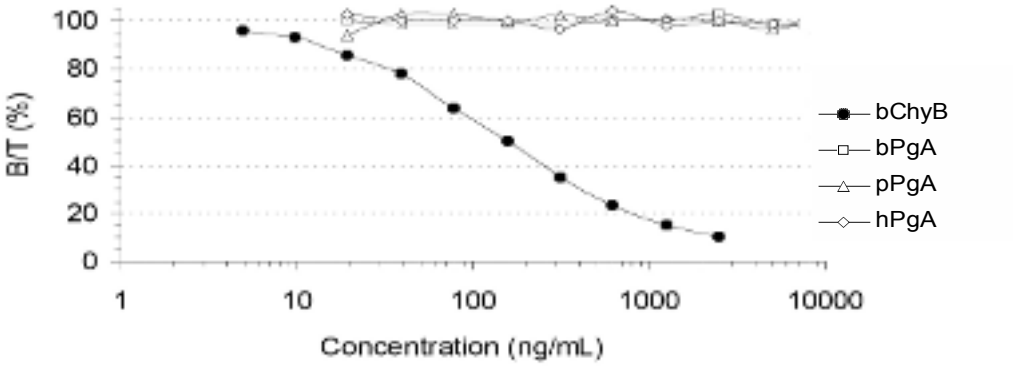

Figure 3: Specificity of the bovine chymosin B radioimmunoassay. The standard curve was calculated by the scale of B/B_0 ratio versus decadic logarithmic of bChyB concentrations (expressed in ng/ml) using radiolabeled bChyB (^{125}I-bChyB). B/B_0 means tracer bound / tracer bound in zero standard.

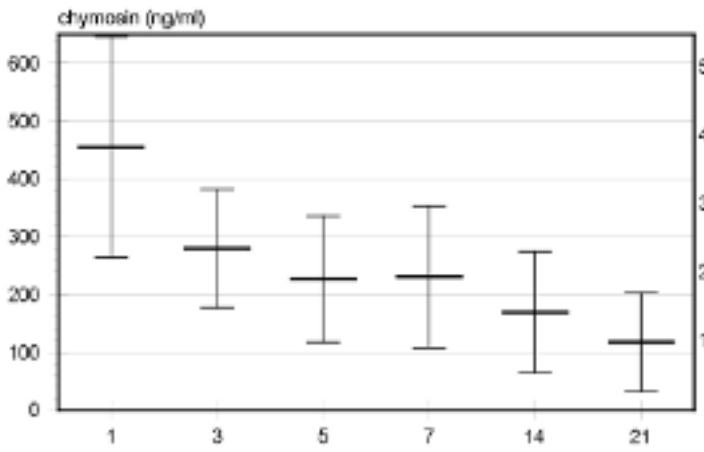

Figure 4a: Evolution of bovine chymosin B concentrations (mean ± SD) in plasma samples of ten young calves sampled from 5 minutes after birth to 24 hours of age.

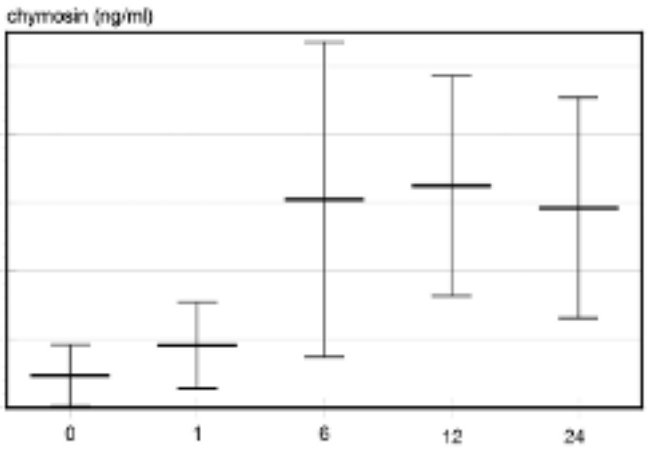

Figure 4b: Evolution of bovine chymosin B concentrations (mean ± SD) in plasma samples of ten calves sampled from day 1 to day 21 after birth.

parturition. As the concentration of chymosin were also high in colostrum (unpublished data), this sharp increase during 12-24 hours of the calves life remain not clearly explain. Is chymosin released in stomach directly reabsorbed in gut like for other macromolecules brought by colostrum?; or do the colostrum bring an additional amount of chymosin that is also reabsorbed? Moreover the possible role of colostrum chymosin remain unexplained. In this group 1, the bChyB plasmatic concentration corresponds to the typical suckling activity of very young calves, increased bChyB concentrations being observed 12 hours after birth. These results are sustained by the study of Andren and Bjorck (1), who demonstrated that in bovine, the mucous neck cells and chief cells in the lower base of the gastric gland are immunoreactive to prochymosin both before and after weaning. The high level of plasma chymosin was observed a few time after the first colostrum feeding, this phenomenon point out the high level of intestinal absorption before the closure occurs 24 hours after birth. From day 1 to 21 after birth, an age dependence was observed, calves from group 2 showing a constant decrease of concentrations from day 1 to 21 after birth. Interestingly, plasma samples from one adult cow also exhibited detectable levels of bChyB. This finding shows that in bovine species, from very young till adult life, bChyB synthesized by the gastric mucosa reach the blood circulation at detectable concentrations.

Conclusion

The RIA showed good sensitivity, specificity, accuracy and reproducibility. By using the assay for blood bChyB determination, we demonstrate that this zymogen is able to cross the basal gastric membrane and to reach the general blood circulation in calves, and still detectable in adults. Furthermore, bChyB-RIA can be associated to the pepsinogen A RIA in the expectation to highlight differential diagnosis of gastrointestinal disorders due to parasites as *Paraphistomum*, *Ostertagia* and *Hoemonchus*. The last being frequently responsible of disease in tropical or sub-tropical area.

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