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Research paper

Development and validation of an ELISA for the quantification of bovine ITIH4 in serum and milk

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ABSTRACT

Inter alpha trypsin inhibitor heavy chain 4 (ITIH4) is a serum protein belonging to the Inter alpha trypsin inhibitor (ITI) family, which was previously characterized by our group as a new APP in cattle. This protein was firstly described in pigs where is known to be a major acute phase protein, also denominated Pig-MAP. Increases of ITIH4 of up to 12 times the pre-infection values were previously reported in the serum of heifers with experimentally induced summer mastitis. ITIH4 was detected in the milk of cows with mastitis by western blot, but the method previously used to quantify this protein, radial immunodiffusion, was not sensitive enough to quantify it in milk samples. In this study we developed an ELISA method which allows the quantification of bovine ITIH4 in serum and milk samples. Previously developed antibodies were used to perform the assay, including anti bovine ITIH4 polyclonal antibodies and a monoclonal antibody against pig ITIH4 that also recognizes the bovine homologous protein. The ELISA developed showed an adequate precision, with inter and intra- assay coefficients of variation lower than 10% for serum and milk samples. The assay keeps linearity under dilution for both serum and milk samples. A good agreement was observed between the values measured by ELISA and radial immunodiffusion in serum samples.

1. Introduction

The acute phase response belongs to the first barrier of the immune system against infection or trauma. It is a systemic, non-specific reaction that occurs before specific immunity. Activation of the acute phase response is mediated by pro-inflammatory cytokines such as IL-6, IL-1 and TNFα. It includes fever, catabolism of muscle protein, alterations in sleep and appetite as well as changes in the concentration of some plasma proteins. Those proteins whose concentrations in serum change at least 25% during the acute phase response are denominated acute phase proteins (APPs). The APPs can be synthesized by both liver and peripheral tissues (Skovgaard et al., 2009), and according to their change in concentration are classified as positive or negative (Murata et al., 2004).

APPs have been extensively studied in humans and different animal species, including farm animals such as pigs or cattle. In human and veterinary medicine APPs are used to monitor systemic inflammation (Dayer et al., 2007; Petersen et al., 2004). The importance of these proteins as inflammatory markers in veterinary medicine has increased in the last decades, and they have been proposed biomarkers for monitoring the health status and welfare of animals during farm production and at slaughter (Petersen et al., 2004; Eckersall and Bell, 2010). The APP response varies with the species. In cattle positive APP include haptoglobin (Hp), serum amyloid A (SAA), alpha-1-acid glycoprotein, and inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4). Hp, and in less extent SAA, are the most studied APP in cattle. They have demonstrated efficacy as biomarkers of inflammation in the diagnosis and prognosis of diseases such as mastitis, enteritis, peritonitis, pneumonia, endocarditis and endometritis (Murata et al., 2004; Petersen et al., 2004; Eckersall and Bell, 2010). Interestingly these APPs are increased not only in serum but also in the milk of cows with mastitis, and a number of studies have focused on their quantification in milk for the detection of mastitis, particularly subclinical mastitis (Eckersall et al., 2001; Gronlund et al., 2003; Nielsen et al., 2004; Miglio et al., 2013; Bochniarz et al., 2017).

Less information is available about ITIH4, characterized as a new APP in cattle by our group (Piñeiro et al., 2004). Increases of up to 12 times the pre-infection values were reported in the serum of heifers

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with experimentally induced summer mastitis. ITIH4 also increased in calves experimentally infected with BRSV (Piñeiro et al., 2004).

ITIH4 belongs to the inter-alpha-trypsin inhibitor (ITI) family. However, differently to other members of the ITI family, it lacks the binding sequence for bikunin (which contains two Kunitz-type protease inhibitor domains) and does not possess inhibitory activity (Gonzalez-Ramon et al., 1995; Hashimoto et al., 1996; Soury et al., 1998). ITIH4 is up-regulated by IL-6 in hepatocarcinome HepG2 cells (Piñeiro et al., 1999) and in porcine primary cultured hepatocytes (Gonzalez-Ramon et al., 2000). Although its biological function still remains unknown, it has been shown to be involved in the inhibition of actin polymerization and inflammatory cells activity (Choi-Miura et al., 2000) and it has been associated with liver formation and regeneration (Bhanumathy et al., 2002). As in cattle, ITIH4 has been reported to be an acute phase protein in other species such as swine (Lampreave et al., 1994; Carpintero et al., 2007), human (Piñeiro et al., 1999), canine (Soler et al., 2016), mice (Duan et al., 2005) and rats (Daveau et al., 1998). In our previous studies bovine ITIH4 was measured by single radial immunodiffusion (SRID), a technique not sensitive enough to quantify ITIH4 in milk samples. However, in more recent studies using proteomic techniques, ITIH4 has been shown to increase in the milk and whey of cows with mastitis (Alonso-Fauste et al., 2012).

The aim of this study was to develop an immunoassay capable of measuring ITIH4 in serum and milk samples. A sandwich ELISA was developed using a previously available monoclonal antibody as capture antibody, and rabbit polyclonal antibodies as detection antibody. The assay was validated for the measurement of serum and milk samples.

2. Materials and methods

2.1. Serum and milk samples

Serum samples from cows with different clinical status were collected to be used in the validation studies. 20 sera were from healthy cows, and another 20 from cows with inflammatory conditions, including lameness (two), metritis (four), mastitis (eight), combined mastitis and metritis (one) and abomasal displacement (five). After clotting of blood, serum was separated by centrifugation, and stored at -80 °C until use.

Quarter milk samples were obtained from cows without any clinical signs of mastitis and without abnormalities in the udder or milk. The samples selected for method validation included samples with SCC < 200,000 cells/mL (healthy samples) and samples with SCC > 500,000 cells/mL, in which *Streptococcus sp* has been isolated. Milk samples were frozen at -80 °C until use.

2.2. ITIH4 purification

Bovine ITIH4 was purified from bovine serum that had been previously used to purify IgG by affinity chromatography with Protein G (serum excluded from the column and depleted of IgG). Bovine ITIH4 was obtained by affinity chromatography using a mAb (M1) previously developed (Piñeiro et al., 2009), covalently linked to CNBr-activated Sepharose 4 Fast Flow (GE healthcare). 10 mL of the bovine serum were applied to the affinity column previously equilibrated with PBS containing 0.5 M NaCl. After extensively washing, ITIH4 was eluted with 0.5 M NaCl, 0.1 M glycine – HCl buffer pH 2.8. Fractions were collected in tubes containing 0.2 M K_2HPO_4, pH 8.0 to neutralize the mixture. Eluted ITIH4 was dialyzed against PBS and concentrated by ultrafiltration.

2.3. Antibodies production

Antiserum against bovine ITIH4 was raised in rabbits by s.c. injection of the purified protein, as previously described (Piñeiro et al., 2004). Anti ITIH4 antibodies were purified from the specific antisera by affinity chromatography with immobilized bovine ITIH4. To prepare the affinity column, 3 mg of purified ITIH4 were fixed to 1 mL HiTrap NHS-activated HP column (GE Healthcare) according to the manufacturer´s instructions. Antibodies eluted were labelled with horseradish peroxidase (Type VI-A, activity 1100 U/mg, Sigma) by glutaraldehyde covalent linkage (Avrameas, 1969).

2.4. Approval of the ethic committee

The animal care and experimental procedure used in this study conformed to regulations and guidelines of the ethic committee about the protection of animals used for scientific research from University of Life Sciences, Lublin, Poland (num of approval 40/2014) and from University of Zaragoza, Spain (num of approval PI25/16).

2.5. Immunochemical methods

Western-blot. Serum samples were subjected to SDS-PAGE (10%), and transferred to nitrocellulose membranes using a semi-dry transfer cell (Bio Rad). After blocking with 3% ovalbumin the membrane was incubated first with the anti ITIH4 polyclonal Ab (diluted 1:5000) or mAb (1 μg/mL) and second with a horseradish peroxidase (HRP)-labelled sheep-anti rabbit IgG Ab or an anti mouse IgG Ab, respectively (Sigma, dilution 1:20,000). The immunocomplex formed were revealed with an enhanced chemiluminescent substrate for detection of HRP (Pierce™ ECL Western Blotting Substrate, Thermo).

SRID was performed in 1% agarose gels containing rabbit anti bovine ITIH4 specific antiserum, as previously described (Piñeiro et al., 2004).

ELISA. 96 wells microtiter plates (Thermo Scientific) were coated with mAb (M1) (Piñeiro et al., 2009). Wells were filled with 100 μL of 4 μg/mL AbM1 solution in PBS. After incubating overnight at 4 °C, wells were washed three times with PBS, and blocked for three hours at room temperature (RT) with a solution consisting in 3% ovalbumin in PBS. After incubation, plates were washed three times with PBS, dried out at RT, and stored at 4 °C in a sealed bag until their use.

100 μL of bovine sera samples, diluted 1/5000 in dilution buffer (PBS containing 0.05% ovalbumin), were added to the wells and incubated for 60 min at RT. The ELISA was calibrated using a bovine serum of known ITIH4 concentration. The concentration of ITIH4 in this standard serum was determined by SRID using purified ITIH4 as primary standard (Piñeiro et al., 2004). Serial dilutions of the sera were prepared (1:5000 to 1:160,000), and added to the plate in each assay to construct the calibration curve (14–460 ng/mL of ITIH4). After washing three times with PBS, wells were incubated with 100 μL of HRP-conjugated rabbit anti-bovine ITIH4 diluted 1/20,000 in PBS containing 0.1% ovalbumin. After the final washing, 100 μL of TMB solution (Prestained TMB Plus-2, KEM EN TEK Diagnostics) was added to the wells and incubated for 30 min at RT. Reaction was stopped by the addition of 100 μL/well of stop solution (0.5 N sulphuric acid). Absorbance was determined at 450 nm using a microplate reader (Sunrise, Tecan)

Milk samples were analysed in the same way as sera samples, with the only difference being the dilution previously performed to the sample (1/100 instead of 1/5000).

2.6. Validation studies in serum and milk samples

Precision studies were carried out for three serum samples of low, medium and high ITIH4 concentration, and two milk samples (high and low ITIH4 concentration). Intra-assay coefficients of variation (CV) were calculated for 10 replicates of the sample in the same plate. Inter-assays CV were calculated with the results obtained in 10 different assays performed at different days using different plates. Samples were added in duplicates, and calculations made with the mean value of duplicates obtained in each individual assay.

In order to evaluate the accuracy of the assay a serum sample with

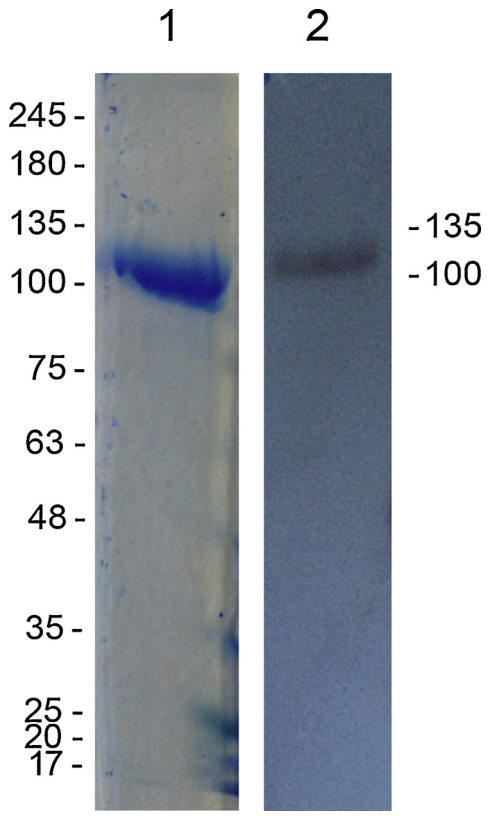

Fig. 1. Analysis by SDS-PAGE and Western blot of purified bovine ITIH4. 1) SDS-PAGE (10% polyacrylamide gel, 3 mg/mL of ITIH4, coomasie blue stained). 2) Western-blot of purified bovine ITIH4 (3 mg/mL diluted 1/120) using first the monoclonal antibody (1 µg/mL) and second an HRP-labelled sheep anti mouse IgG antibody (Sigma, dilution 1:20.000). The position of the molecular weight markers (BlueStar Plus Prestained Protein Marker, Nippogenetics) is indicated (KDa).

high ITIH4 concentration (1.77 mg/mL) and a milk sample with high ITIH4 concentration (28 µg/mL) were tested at different dilutions covering the working range of the assay. Results obtained were compared with the expected value considering the dilution factor.

The limit of the detection (LoD) of the assay was determined by analysing 16 times a blank sample composed of ovalbumin 0.05% in PBS. LoD was calculated as mean concentration of blank samples + 3SD.

ELISA was compared with SRID, method previously used for ITIH4 measurements (Piñeiro et al., 2004). A total of 40 bovine serum samples were analysed by the two methods and the results obtained compared by linear regression analysis.

3. Results

A previously developed mAb against swine ITIH4 (M1) also recognized the bovine homologous protein, and was successfully used to obtain a preparation of ITIH4 of high purity by affinity chromatography (Fig. 1). Purified protein was confirmed as bovine ITIH4 after analysis by MALDI-TOF mass spectrometry (data not shown). The purified protein was used for the immunization of rabbits in order to obtain a polyclonal antibody that could be used in the development of a sandwich type ELISA. The specificity of the polyclonal Ab obtained was evaluated by Western-blot (Fig. 2). Purified ITIH4, and ITIH4 present in serum and milk, were recognized by the Ab, whereas no cross reactivity with other serum or milk proteins was detected.

A sandwich-type ELISA was developed using mAb M1 as capture antibody and the polyclonal Ab coupled to horseradish peroxidase as

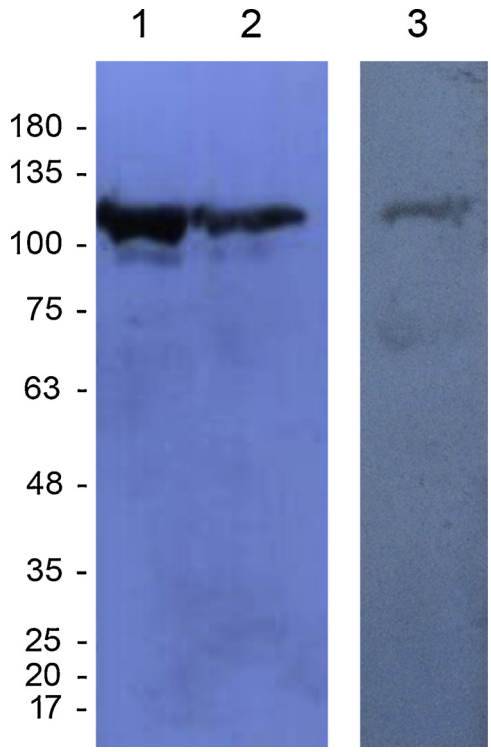

Fig. 2. Western-blot with the polyclonal antibodies anti bovine ITIH4. 1: Purified bovine ITIH4 (3 mg/mL) diluted 1/120. 2: Bovine serum diluted 1/120. 3: Milk from a cow with mastitis, diluted 1/2. The membrane was incubated first with the polyclonal anti ITIH4 antibody diluted 1:5000 and second with an HRP-labelled sheep anti rabbit IgG antibody (Sigma, dilution 1:20,000). Blue Star Plus Prestained Protein Marker (Nippogenetics) was used as molecular weight standards.

detection Ab. With the assay conditions selected the LoD was 0.013 µg/mL, and the dynamic range expanded until 0.46 µg/mL. Taking into account the different level of concentration of ITIH4 in serum and milk, different pre-dilutions of the sample were used to obtain a concentration falling into the working range of the assay. Serum samples were diluted 1:5000, and milk samples 1:100. According to this it was possible to quantify serum samples in the range 0.07–2.3 mg/mL and milk samples in the range 1.4–46 µg/mL.

Linearity under dilution was evaluated for both, serum and milk samples (Fig. 3). No deviation from linearity was observed with any of the assayed matrixes, being the correlation between the expected and observed values very good.

The results of the study of precision are shown in Tables 1 and 2. The assay showed an adequate precision with intra assay CV of variation in the range 3–7%, and inter assay CV of variation lower than 10% and 11% respectively for serum and milk samples.

ITIH4 values obtained by ELISA were compared with values obtained by SRID, a method previously developed in the laboratory for bovine ITIH4 quantification. 40 samples, including sera from apparently healthy animals and animals with inflammatory conditions, and ranging from 0.27 mg/L to 1.96 mg/mL, were measured (Table 3). The values (mean of duplicates) obtained with both techniques are compared in Fig. 4. The analysis by linear regression showed a good agreement between ELISA and SRID, as the slope was not different from one and the x-intercept not different from zero:

ITIH4 ELISA (mg/mL) = 1.033(0.958–1.109) x ITIH4 SRID – 0.0375 (-0.105 to 0.029) (mg/mL; 95% confidence interval shown in parenthesis).

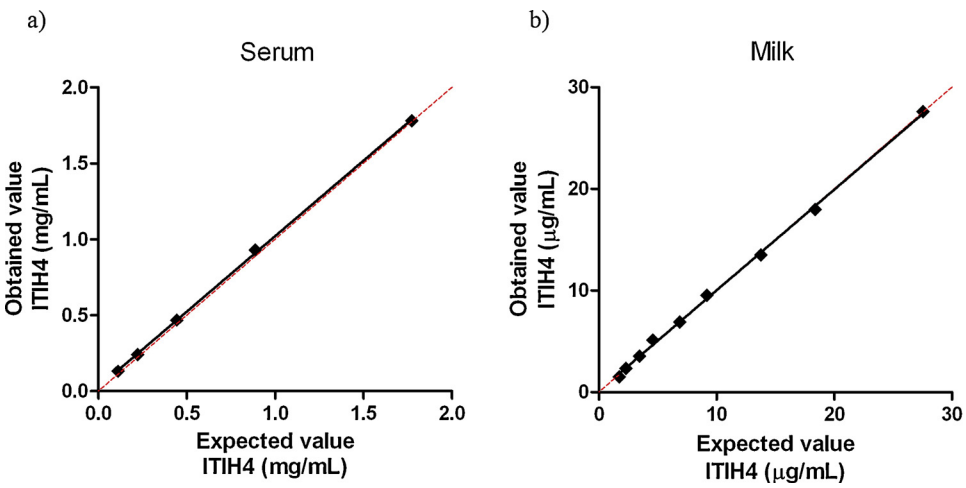

Fig. 3. Study of linearity under dilution. A) serum samples and B) milk samples. Measured concentration obtained for each of the dilutions were plotted against the expected value and adjusted to a line by minimal square fitting (black line). Discontinue red line shows y = x (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Intra-assay coefficients of variation (n = 10) for sera and milk samples. Range: minimum and maximum values. SD: standard deviation. CV: coefficient of variation.

Sample	mean	range	SD	CV
Serum 1	0.28 mg/mL	0.28 – 0.31	0.020	7.1 %
Serum 2	0.51 mg/mL	0.49 – 0.53	0.014	2.9 %
Serum 3	1.37 mg/mL	1.30 – 1.50	0.080	5.8 %
Milk 1	2.8 µg/mL	2.80 – 3.00	0.07	2.4 %
Milk 2	25.6 µg/mL	23.1 – 28.2	1.68	6.6 %

Table 2
Inter-assay coefficients of variation (n = 10) for serum and milk samples. Range: minimum and maximum values. SD: standard deviation. CV: coefficient of variation.

Sample	mean	range	SD	CV
Serum 1	0.23 mg/mL	0.20 – 0.25	0.021	9.5 %
Serum 2	0.51 mg/mL	0.45 – 0.57	0.042	8.3 %
Serum 3	1.34 mg/mL	1. 21 – 1.47	0.084	6.8 %
Milk 3	1.9 µg/mL	1.60 – 2.20	0.21	10.9 %
Milk 4	22.0 µg/mL	19.2 – 25.3	1.93	8.8 %

Table 3
Descriptive statistics for ITIH4 concentration (mg/mL) in serum samples from healthy cows and cows with inflammatory conditions (Inflammation). Data are presented as mean, standard deviation (SD), median, minimum (Min) and maximum (Max) values.

Health status	Mean	SD	Median	Min	Max
Healthy (n = 20)	0.48	0.11	0.47	0.27	0.72
Inflammation (all, n = 20)	1.04	0.42	0.93	0.48	1.96
Abomasal displacement (n = 5)	1.24	0.43	1.04	0.93	1.96
Lameness (n = 2)	0.74	0.08	0.74	0.68	0.80
Mastitis (n = 8)	1.11	0.49	1.01	0.48	1.71
Metritis (n = 4)	0.88	0.27	0.89	0.58	1.17
Mastitis and metritis (n = 1)	1.43	—	—	—	—

4. Discussion

The purification procedure described herein, based on affinity chromatography with a mAb, is simple, fast and facilitates the obtaining of bovine ITIH4 preparations of high purity. Moreover, proteolytic fragments of ITIH4 were not apparent in the preparation, as had been described with the isolation procedure previously reported by our group (Piñeiro et al., 2004), that includes precipitation with ammonium sulphate and several chromatographic steps. The same mAb, together

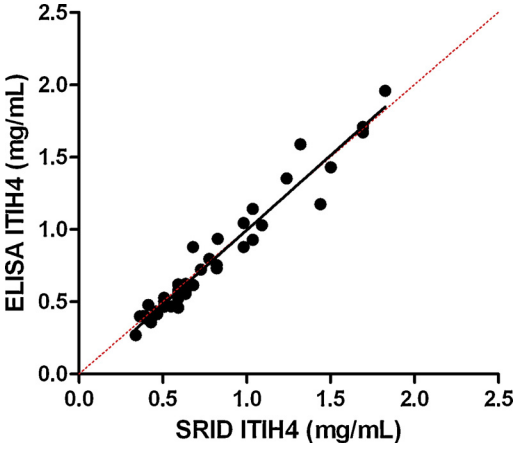

Fig. 4. Comparison of the bovine ITIH4 concentration values obtained by ELISA (abscisas) and radial immunodifussion (ordenades). Black line shows linear regression and discontinued red line shows y = x (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with polyclonal Ab against bovine ITIH4 raised in rabbits were subsequently used in the development of a sandwich ELISA which allowed the quantification of ITIH4 not only in serum but also in matrixes such as milk were the concentration is expected to be much lower. ITIH4 had been detected in the milk of cows with mastitis by western blot, suggesting a potential use as biomarker of the disease, as has been reported for other APPs such as haptoglobin or SAA (Eckersall et al., 2001; Nielsen et al., 2004). However, the method previously used for ITIH4 measurements, SRID, was not sensitive enough to explore this possibility, and had the additional disadvantage of being a time consuming method that requires more than 48 h to obtain the results. These inconveniences can be overcome with the ELISA method described in this study.

The ELISA method developed is a fast, precise and accurate method that allows the quantification of ITIH4 in both serum and milk samples. The same calibrator and procedure were used with serum and milk samples, only adapting the pre-dilution of the sample that in the case of milk samples was 1:100 instead of 1:5000. The measuring range of the assay was 0.07 to 2.3 mg/mL for serum samples (referred to the concentration in the undiluted sample), that would include the concentration values observed in healthy cows, as well as in many pathological samples. ITIH4 values found in this study for healthy animals ranged from 0.27 to 0.72 mg/mL, with 0.48 mg/mL as mean value. These values are similar to those reported for healthy calves and heifers in a previous study (Piñeiro et al., 2004) and also is in accordance with

the range of values reported for healthy cows by Soler et al (2018) (0.27 to 0.90, median value of 0.52 mg/mL). ITIH4 concentration measured in this study in cows with naturally occurring inflammatory processes were lower than 2 mg/mL, although higher values were reported in heifers with experimentally induced summer mastitis (Piñeiro et al., 2004). Thus, to obtain an accurate value, samples with an ITIH4 concentration equal to or higher than the upper limit of quantification of the assay should be re-analysed at a higher dilution.

The assay was sensitive enough to allow the measurement of milk samples with concentrations in the range of 1.4–46 µg/mL of ITIH4, using a 1:100 pre-dilution of the sample. The analytical range for milk samples was optimized to include the concentration values observed in milk samples from healthy cows (1.4–9 µg/mL) according to our preliminary data, reported in another study (Soler et al., 2018).

The analysis by Western blot did not reveal any unspecific reaction of the detection Ab with bovine serum or milk proteins. Accordingly, the study of linearity under dilution showed that the assay quantified proportionally in the analytical range both, serum and milk samples. Although milk is a matrix with different composition than serum, no interference or matrix effect was observed with the analytical conditions used.

The accuracy of the ELISA was further evaluated by performing a method comparison study with the SRID. The results of the study showed a good correlation between both methods, with no proportional or constant errors detected, which would allow a direct comparison of analytical results obtained with the two methods.

The ELISA showed an adequate precision. Intra-assay CVs were lower than 7%, and the results were similar for serum and milk samples (Table 1). Inter-assay CVs were lower than 10% for all the samples evaluated except the milk sample with low ITIH4 concentration, which showed a CV slightly higher than 10%. However, this sample was close to the LoD of the assay. The precision of the assay was similar to the observed with the ELISA developed for the homologous pig protein, which uses the same mAb as capture Ab (Piñeiro et al., 2009).

ITIH4 is acknowledge as a major acute phase protein in pigs, which has been extensively studied in different pathological conditions including viral, bacterial and parasite infections (Heegaard et al., 1998; Carpintero et al., 2007; Heegaard et al., 2011), as well as in pigs affected by stressors compromising animal welfare (Piñeiro et al., 2007a, b; Marco-Ramell et al., 2011). A moderate increase of human ITIH4 (from 1.4 to 3 times) was observed in patients undergoing different acute phase processes such as myocardial infarction, unstable angina or surgery (Piñeiro et al., 1999). In humans changes of concentration of ITIH4 or its proteolytic fragments have been associated with different types of cancer, including gastric adenocarcinoma (Subbannayya et al., 2015), ovarian cancer, breast cancer (Mohamed et al., 2008), or hepatocellular carcinoma (Li et al., 2018), among others. Moreover, ITIH4 has been suggested as a potential diagnosis and prognosis marker of human diseases such as amyotrophic lateral sclerosis (Tanaka et al., 2013), or ischemic stroke (Nayak et al., 2016).

In conclusion, an ELISA method with sensitivity to quantify bovine ITIH4 in milk samples has been developed and successfully validated. The method shows good precision, keeps linearity under dilution and shows equivalence with the SRID. The availability of this ELISA will facilitate the study of ITIH4 under different pathological conditions in bovine, in order to evaluate its potential use as biomarker, alone, or in conjunction with other major APPs such as haptoglobin or SAA.

Declaration of Competing Interest

LS and MP work for Acuvet Biotech a company with interests in animal acute phase proteins tests. MP is among the funders of Acuvet Biotech.

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