

The negative acute phase response of serum transthyretin following *Streptococcus suis* infection in the pig

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Abstract – Transthyretin (TTR) is a serum protein which is a negative acute phase reactant in humans and levels of TTR are routinely measured as an indicator of health status. Such tests have yet to be established for the pig. In order to measure serum TTR in the pig during an acute phase response an assay was developed using anti-human TTR antibodies which cross reacted with porcine TTR. The assay had a detection limit of 32 µg/mL while the mean concentration of transthyretin measured in healthy pig serum was 302 ± 8 µg/mL ($n = 63$). There was no significant difference in the serum concentration of TTR in three different age groups from 10 to 25 weeks. Following *Streptococcus suis* type 2 infection transthyretin showed a negative acute phase response with serum concentrations reaching a significantly lower level at two days following infection.

acute phase proteins / pigs / infection *Streptococcus suis*

1. INTRODUCTION

Acute phase proteins (APP) are blood proteins, which alter concentration dramatically following infection, inflammation and trauma [10]. The serum level of these proteins either increases in the case of positive acute phase proteins such as haptoglobin, C-reactive protein and serum amyloid A [5] or decreases in the case of negative acute phase proteins such as transthyretin (TTR) [13, 14]. The level of positive APP in porcine sera has been shown to

provide an objective measure of animal health status and is becoming widely used as a gauge of animal welfare which is understood as freedom of disease and, to a certain extent stress [8, 12, 17–19, 21]. In addition, concentration of the positive acute phase proteins has been shown to be proportional to the severity of the condition so they can be used both to monitor the presence and extent of a disease in pigs [3, 7] and may also be used to measure the efficacy of antibiotic treatment [12, 15, 16]. In human medicine the CRP/TTR ratio has

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been used for the early diagnosis of post operative bacterial infection [9]. Monitoring the levels of negative acute phase reactants in porcine sera in addition to the positive ones should bring additional value to these measurements, for example by constituting an internal control for dilution and evaporation artefacts.

TTR, also known as thyroxin-binding prealbumin, is a serum protein with a molecular mass of ~ 55 kDa made up of four identical subunits. It is one of the three major thyroxin binding proteins and forms a complex with retinol binding protein to aid the transport of vitamin A in plasma [14, 20]. The porcine isoform shares 85% sequence homology with the human form but has three additional amino acids at the C-terminus (Gly-Ala-Leu) giving it a subunit length of 130 amino acids [2, 6]. In man TTR serum levels fall due to decreased synthesis in inflammation and stress [13]. Human serum levels of TTR have been measured for hospital patients as an indicator of health status [4, 11, 22] and a number of commercial assays are available for this purpose. However, whether transthyretin is indeed a negative acute phase reactant has yet to be established for the pig. Therefore the aim of this study was to develop and validate an assay to measure TTR in porcine serum and to use this assay to determine whether TTR levels in porcine sera fall during an acute phase response. An enzyme linked immuno sorbent assay (ELISA) was established for this purpose using an antiserum to human TTR. The specificity of this antiserum for porcine TTR was confirmed by western blotting.

2. MATERIALS AND METHODS

2.1. Animals and collection of samples

Blood samples were collected from two different groups of animals. The first group was made up of 63 conventionally housed healthy pigs. Animals were defined as

healthy on the basis of a clinical examination in the herd by a trained technician looking at the moving animal and at the immobilised animal that was palpated (limbs and umbilicus). Three pigs from each of three age groups, 10–14, 15–19 and 20–25 weeks from each of 7 different herds were used. Additional blood samples were collected from a second group of five pigs (approx. 6 weeks of age at time of infection) from days -8, 0, 1, 2, 5, 8, 12 and 14 after infection with a *Streptococcus suis* type 2 isolate. The experimental animals were infected by subcutaneous injection of approx. 10^{11} CFU of *S. suis* strain SS02-0119 as previously described [1]. Blood was allowed to clot and serum aspirated and stored at -20°C until analysed. Establishment of infection was confirmed in all five pigs by culture of *S. suis* type 2 from blood samples on day 1 and 8 post inoculation (p.i.). The pigs were monitored by recording clinical signs of disease and body temperature daily throughout the experiment. Four of the five pigs developed transient signs of arthritis during days 2–7 p.i. Arthritis was confirmed by histopathological examination. These four pigs also developed fever, defined as a rise in temperature $\geq 40^{\circ}\text{C}$. One pig did not develop any clinical signs of disease or fever. All animal experiments were performed in accordance with a licence from the Danish Animal Experiments Inspectorate.

2.2. Two dimensional gel electrophoresis

Porcine serum was prepared in rehydration buffer (8 M urea, 2% w/v CHAPS, 0.5% Biolyte 3–10 IPG Buffer and 2.8 % w/v dithiothreitol) at a protein concentration of 1 mg/mL. The sample (125 μL) was then applied to 7 cm immobilised pH gradient gel (IPG) ready strip pH 3–10 (Bio-Rad, Hemel Hemsted, UK) which was then rehydrated in a Bio-Rad Protean IEF cell for 600 Vhrs followed by isoelectric focusing for 24 000 Vhrs.

Immediately following IEF the strip was equilibrated with 6 M urea, 0.375 M Tris

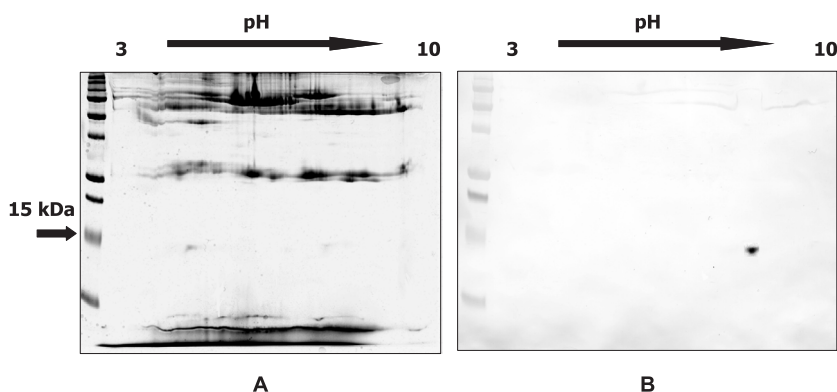


Figure 1. (A) 2-Dimensional electrophoresis gel of porcine serum stained with Coomassie Brilliant Blue. (B) Immunoblot of 2DE gel showing crossreactivity with sheep anti-human prealbumin anti-serum.

pH 8.8, 1% v/w, SDS, 20% (v/v) glycerol and 2% (w/v) dithiothreitol for 10 min and then for a further 10 min in the same buffer with the dithiothreitol replaced by 2.5% (w/v) iodoacetamide. After equilibration the strip was applied to a 15% polyacrylamide gel which was run on Bio-Rad Mini Protean 3 system according to the manufacturer's instructions. Gels were then stained with Coomassie Brilliant Blue R-250.

2.3. Immunoblotting

Proteins were transferred from acrylamide gels onto nitrocellulose membranes at 100 V for 1 h using the mini Trans-Blot Cell (Bio-Rad, Hemel Hempsted, UK). Transfer buffer was 25 mM Tris, 192 mM glycine, pH 8.3, 20% (v/v) methanol. Following protein transfer the membranes were blocked with 5% (w/v) non fat dry milk, 0.1% Tween-20 in Tris buffered saline (TBS), (100 mM Tris-HCl, 150 mM NaCl) pH 7.4 (TTBS). The membranes were then incubated for 1 h with sheep anti-human prealbumin (TTR) (IgG fraction) (ICN Bio-medicals, Basingstoke UK) diluted 1/1000 in TTBS containing 0.5% non fat dried milk, washed with TTBS and then incubated with HRP conjugated anti-sheep IgG (Sigma, Poole, UK) 1:2000 in TTBS for

30 min. The membrane was washed with TTBS three times and then developed using the Opti-4CN Substrate Kit (Bio-Rad, Hemel Hempsted, UK).

2.4. Mass spectrometry

Mass spectrometry (MS) was carried out at The Sir Henry Wellcome Functional Genomics Facility at The University of Glasgow. Spots from Coomassie Blue stained gels were excised and subjected to digestion with trypsin. Extracted peptides were desalted manually (ZipTip tips, Millipore, UK) before using Q-Star PulsarTM LC/MS/MS (Applied Biosystems, UK) for sequence analysis of the peptides. The resulting MS/MS data were then searched in-house using MascotTM software (<http://www.matrixscience.com/>) equipped with translated databases for protein identification.

2.5. Confirmation of antibody specificity

To confirm the specificity of the sheep anti-human prealbumin raised to human TTR it was tested for cross reactivity with porcine TTR. Porcine serum was run on 2D PAGE and the resulting gel stained with Coomassie Blue (Fig. 1A) an identical gel

was run and transferred to nitrocellulose, immuno blotting was carried out using anti human TTR (Fig. 1B). The protein spot on the Coomassie Blue stained gel which showed cross reactivity with anti TTR was cut from the gel and confirmed as porcine transthyretin after MS of the peptides generated following digestion by trypsin. The MS fingerprint data was examined using Mascot™, MS/MS Ions Search Analysis and showed protein identity matches to accession number gil47523508 (similar to transthyretin (prealbumin), amyloidosis type I) [*Sus scrofa*] and to accession number gil975233 (putative transthyretin (prealbumin)) [*Sus scrofa*] [6]. The antibody was therefore specific for porcine transthyretin and could be used to measure transthyretin levels in porcine sera.

2.6. TTR assay development

Microtiterplates (96 well, Corning Costar, Cambridge UK) were coated with serum samples or purified human prealbumin (TTR) (Sigma-Aldrich, Poole, UK) diluted in 50 mM NaHCO₃ (pH 9.6), 100 µL/well for 20 hours at 4 °C. The samples were then decanted and unbound sites blocked by adding 250 µL of 5% (w/v) non fat dried milk in assay buffer, (0.12 M NaCl, 0.02 M Na₂HPO₄, 0.1% (v/v) Tween 20, pH 7.4) at room temperature for 30 min. The plates were washed with assay buffer and then 100 µL of sheep anti-human TTR diluted 1/1000 in assay buffer was added to each well and the plates incubated for 1 h at 37 °C. The wells were then decanted and washed three times in assay buffer. Then 100 µL of HRP conjugated anti-sheep IgG (Sigma, Poole, UK) 1:2000 in assay buffer was added to each well and incubated for 30 min at 37 °C. After washing three times with assay buffer the wells were filled with 100 µL freshly prepared TMB substrate solution (KPL, Guildford, UK) the reaction was stopped after 30 min by the addition of 50 µL of 1 M HCl and then the absorbance was read at 450 nm. The TTR concentration in the serum samples after a 1:400 dilution

was compared to a standard curve of human TTR over a range of 0.03–2.00 µg/mL. Detection limits were determined by measuring 15 replicates of saline and replicates of the lowest concentration standard and calculating the mean concentration of saline +2 SD of the lowest concentration using human TTR as standard.

2.7. Acute phase protein measurements

Serum amyloid A (SAA) in porcine serum was assayed using the Phase SAA Assay kit (Tridelta Development plc, Ireland) according to the manufacturer's instructions.

2.8. Statistical analysis

Data are expressed as means ± SEM. Comparisons were performed using paired *t*-test. Significance was set at $P < 0.05$.

3. RESULTS

Initially a standard curve was established by binding increasing amounts of purified human TTR to a microtitre plate. Figure 2A shows a representative standard curve and parallel curve for doubling dilutions of pooled porcine sera starting with a 1 in 400 dilution. The lower detection limit for porcine serum TTR (1:1000 dilution) was determined as 0.032 µg/mL. Linearity under dilution was demonstrated by a plot of measured versus calculated values of TTR in porcine serum which had a regression line with equation $y = 1.0269x + 3.543$ and a correlation coefficient of 0.997. The interassay precision determined by calculation of coefficient of variance (CV) of 8.4% at 129.4 µg/mL and 13.8% at 83.6 µg/mL was obtained by measuring the same two porcine serum samples in 18 separate assays. The intraassay CV was ascertained to be 2.2% being the mean of the CVs of 28 duplicate samples in the same assay at a concentration range of 117–292 µg/mL.

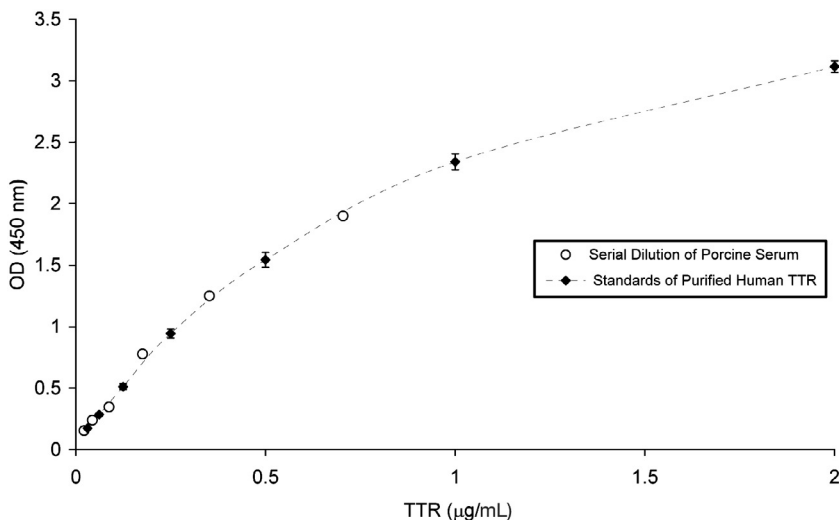


Figure 2. Mean standard curve of purified human TTR ($n = 18$) compared to doubling dilutions of porcine serum starting with a 1 in 400 dilution.

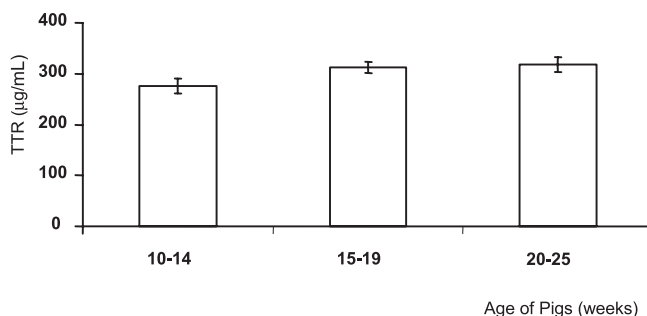


Figure 3. Serum transthyretin concentrations in samples from healthy pigs, 63 conventionally housed pigs (3 pigs from each of three age groups from each of 7 different herds).

Serum samples taken from individual healthy pigs, in three different age groups ($n = 21$ per group) were assayed for TTR (Fig. 3) and showed there were no differences in TTR concentrations between the age groups. The mean value for all of the samples was $302 \pm 8 \mu\text{g/mL}$ ($n = 63$). In order to assess the acute phase reaction of transthyretin in pigs following infection TTR was measured in serum samples from five pigs at time intervals following infec-

tion with a *S. suis* type 2, ribotype I isolate administered by subcutaneous injection. These samples were tested in duplicate in the same analytical run. The mean values for all five pigs at each time interval is shown in Figure 4. A drop in transthyretin levels was observed with the mean TTR concentration falling to 81% of preinfection concentration by day 2 following injection. This fall was found to be significantly different ($P = 0.004$ two tailed paired *t* test)

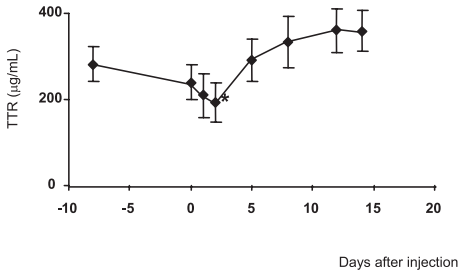


Figure 4. Transthyretin concentrations in pigs after infection (s.c. injection) with a *S. suis* type 2, ribotype I isolate. Samples from five pigs (approx. 6 weeks of age at time of infection) from days -8, 0, 1, 2, 5, 8, 12 and 14. * $P < 0.05$ compared to day 0.

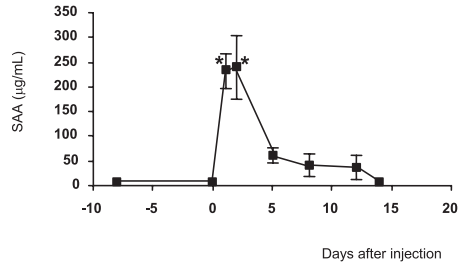


Figure 5. SAA concentrations after infection (s.c. injection) with a *S. suis* type 2, ribotype I isolate. Samples from five pigs (approx. 6 weeks of age at time of infection) from days -8, 0, 1, 2, 5, 8, 12 and 14. * $P < 0.05$ compared to day 0.

from the values prior to infection (day 0). The serum level of the positive acute phase protein SAA was also measured in these samples (Fig. 5) and serum levels of SAA increased significantly by day 1 and day 2 reaching a mean value of $232 \pm 34.8 \mu\text{g/mL}$ following infection before returning to base line levels.

4. DISCUSSION

For the first time a method to measure the levels of transthyretin in porcine serum has been developed which shows that TTR is a negative acute phase reactant in pig.

Immunoblotting and mass spectrometry demonstrated that commercially available sheep polyclonal antiserum raised to human transthyretin cross reacted with porcine TTR. This cross reactivity is probably due to the 85% amino acid sequence homology between porcine and human transthyretin protein sequences and the lack of glycosylation of TTR [14, 20]. The specific antibody was used to develop an assay for measuring transthyretin in pig serum. Accuracy of the assay was demonstrated by the parallel curve obtained by serial dilution of a porcine serum sample compared to the standard curve. The CV calculations ($< 130 \mu\text{g/mL}$)

fall below the normal range ($300 \mu\text{g/mL}$) and the range during *S. suis* infection ($> 200 \mu\text{g/mL}$) and it is possible that the inter-assay CV may increase with increasing concentrations of transthyretin. However, no significant differences were found in values obtained from repeat assays of samples containing $> 250 \mu\text{g/mL}$ of TTR. Using this assay TTR in porcine sera can be measured in a consistent and reproducible manner with acceptable precision.

Following *S. suis* infection transthyretin showed a negative acute phase response with serum levels falling significantly two days following treatment then returning to pretreatment values after 5 days. In contrast serum levels of SAA which is an established acute phase indicator in pigs [12] rose one day after infection, remained high for two days and then fell back to baseline levels after 5 days. The fall in levels of TTR during the acute phase response was relatively small compared to the increases found in SAA which increased by 39 fold. However, it is consistent with drops in human transthyretin levels following infection reported previously [9].

The assay we have developed for TTR may have the disadvantage of relying on passively adsorbing the antigen to a surface in competition with all the other serum

proteins. This, on the other hand may be less of a drawback considering that TTR concentrations vary only slightly. However the detection limit might be lowered significantly by using a sandwich ELISA with antibodies raised to purified porcine transthyretin to trap the TTR.

It is possible therefore that porcine TTR could be a valuable negative acute phase protein for monitoring the health of pigs. Indeed even if the acute phase change of TTR in pigs adds only marginally to the diagnostic information on the acute phase its measurement may be more useful for monitoring nutritional or growth related phenomena as it is in man [14].

In conclusion we have developed a means to measure TTR in pig serum and demonstrated that the concentration of this protein in serum falls in response to *S. suis* type 2 infection. Measuring the levels of TTR in serum in addition to other acute phase proteins to monitor pig health status may provide extra information and warrants further investigation.

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