“Meat Juice” Is Not a Homogeneous Serological Matrix

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Abstract

Meat juice samples are used in serological assays to monitor infectious diseases within the food chain. However, evidence of inferior sensitivity, presumably due to low levels of antibodies in the meat juice compared to serum, has been presented, and it has been suggested that adjusting the dilution factor of meat juice in proportion to its blood content could improve sensitivity. In the present study, the agreement between *Toxoplasma gondii*-specific immunoglobulin G (IgG) levels in meat juice and serum was evaluated, and whether the level of immunoglobulins in meat juice was dependent on its blood content. Serum and meat juice from diaphragm, heart, tongue, *Musculus triceps brachii* and *M. semitendinosus* were collected from 20 pigs experimentally infected with *T. gondii*. Analysis of total IgG, heme-containing proteins (hematin), and hemoglobin (Hb) revealed significant differences between samples from different muscles, with the highest levels in samples from heart and tongue, and the lowest in samples from leg muscles. Comparison of *T. gondii*-specific antibody titers in meat juice and serum revealed a strong positive correlation for meat juice from heart ($r_s=0.87$; $p<0.001$), while it was lower for *M. semitendinosus* ($r_s=0.71$; $p<0.001$) and diaphragm ($r_s=0.54$; $p=0.02$). Meanwhile, the correlation between total IgG and *T. gondii* titer ratio (meat juice/serum) was highest in diaphragm ($r_s=0.77$; $p<0.001$) followed by *M. semitendinosus* ($r_s=0.64$; $p=0.005$) and heart ($r_s=0.50$; $p=0.051$). The correlation between Hb and *T. gondii* titer ratio was only significant for diaphragm ($r_s=0.65$; $p=0.008$), and for hematin no significant correlation was recorded. In conclusion, the specific IgG titers in meat juice appeared to depend on the total IgG level, but the correlation to blood (Hb or hematin) was poor. Importantly, large significant differences in total IgG levels as well as in specific antibody titers were recorded, depending on the muscle the meat juice had been extracted from.

Introduction

MEAT JUICE CONSISTS OF intracellular and extracellular fluid, blood, and lymph. It is used as a serological matrix for surveys of foodborne pathogens such as *Salmonella* and *Toxoplasma gondii* in livestock (Lundén *et al.*, 2002; Berger-Schoch *et al.*, 2011; Wacheck *et al.*, 2012). It is also used for large-scale serosurveillance of *Salmonella* in fattening pigs, as regulated in control programs in several European countries (Mousing *et al.*, 1997; Alban *et al.*, 2002; BPEX, 2002; Merle *et al.*, 2011). Meat samples from pigs are easily accessible at slaughter since sampling may be performed at almost any slaughter-line position, preferably one already used for the legislated testing of *Trichinella* spp. (Commission Regulation (EC) No 2075/2005 of December 5, 2005).

In 2010, the European Food Safety Authority (EFSA) was requested to give an opinion on the modernization of meat inspection toward a risk-based system. Differentiation of pig

batches into high or low risk based on serological monitoring for selected pathogens was proposed for improving meat inspection (EFSA, 2011). Meat juice has been suggested as an appropriate matrix for such screening (Meemken *et al.*, 2014), which would generate a significant role for meat juice testing. However, it has been shown that serology using meat juice might not be reliable to detect animals with low-grade infections and/or low antibody levels (Gamble *et al.*, 2005; Wilhelm *et al.*, 2007; Vico and Mainar-Jaime, 2011; Forbes *et al.*, 2012; Ranucci *et al.*, 2012). The levels of specific antibodies are lower in meat juice than in serum, and to compensate for this, meat juice is usually tested at lower dilutions, often around 10 times less than serum (Wingstrand *et al.*, 1997; Nielsen *et al.*, 1998). However, specific antibody levels in meat juice depend on the muscle chosen for sampling (Nielsen *et al.*, 1998; Wallgren and Persson, 2000; Forbes *et al.*, 2012), probably due to the degree of vascularization. Furthermore, the efficiency of exsanguination at

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TABLE 1. NUMBER OF PIGS ANALYZED FOR EACH PARAMETER

Sample type ^a	Toxoplasma gondii IgG ^b	Toxoplasma gondii titer ^c	Total immunoglobulin G	Hematin	Hemoglobin	Myoglobin
Serum	23	19	23	NA	NA	NA
Heart	19	16	23	22	19	19
Tongue	21	0	23	23	19	19
Diaphragm	22	18	22	22	20	20
Semitendinosus	23	18	23	23	23	23
Triceps	23	0	23	23	22	22
Total number of samples	131	71	137	113	103	103

^aSerum or meat juice from the indicated muscles.

^bS/P value $[(OD_{\text{sample}} - OD_{\text{negative control}})/(OD_{\text{positive}} - OD_{\text{negative control}})] \times 100$ measured at dilution 1:2 (meat juice) or 1:10 (serum).

^cSamples tested in 2-fold dilutions from 1:2 (meat juice) or 1:10 (serum) and results expressed as titer at 50% S/P.

NA, not applicable; OD, optical density.

(continued)

FIG. 1. The distribution of (A) *Toxoplasma gondii* titer in meat juice from diaphragm (Dia), heart, semitendinosus (Sem), and in serum, and of (B) total IgG, (C) hematin, (D) Hb, and (E) myoglobin in meat juice from five different muscles from pigs.

FIG. 1. (Continued).

slaughter likely affects antibody levels in meat juice. Consequently, it has been proposed that the lower sensitivity of meat juice serology could be improved by adjusting the dilution of meat juice samples according to their blood content (Mecca *et al.*, 2011).

Meat juice contains different heme-containing proteins, of which hemoglobin (Hb) and myoglobin constitute the greater part (Ledward, 1992). Hb originates from blood and myoglobin is released from muscle cells upon the damage caused by freezing and thawing, a process often used to collect meat juice. Total heme protein content can be measured using the spectrophotometric alkaline hematin detergent method (AHD), first described by Zander *et al.* (1984) and Wolf *et al.* (1984). Adding the AHD reagent to the meat juice sample lyses any remaining erythrocytes and releases the Hb, after which all heme species (including myoglobin) are converted to hematin. The AHD method is advantageous because nontoxic reagents are used and the measured end product is stable even when samples are turbid (containing debris, lipids, bilirubin, etc.) (Zander *et al.*, 1989).

Standardization of meat juice extraction and processing is crucial for quality assurance, and evaluation of factors influencing test sensitivity and specificity are essential. In the present study, the agreement between specific antibody levels in serum and meat juice was investigated using pigs experimentally infected with *T. gondii* as a model. Meat juice from cardiac muscle and different striated muscles were used to test the hypothesis that higher blood content in meat juice samples leads to higher antibody levels. The distribution of

antibodies to meat juice was measured as total IgG, and the amount of total heme protein (hematin) or Hb was used to estimate the blood content.

Methods

Animals

The samples originated from a trial primarily designed to determine the protective effect of immunization with live *T. gondii* tachyzoites on parasite dissemination to different muscles following oral infection with *T. gondii* tissue cysts or oocysts, the two most likely sources of infection for pigs (A. Burrells *et al.*, unpublished data). The study comprised 23 Large White/Landrace crossbred pigs. Four groups ($n=5$) were inoculated either with 1000 oocysts (strain M4), 1000 M4 tissue cysts, S48 tachyzoites (subcutaneously) + 1000 M4 oocysts after 4 weeks, or tachyzoites only. Three pigs served as uninfected controls. Six weeks postinfection, all pigs were culled by electrical stunning and bled out while lying down. The experiment was performed at the Moredun Research Institute, Scotland, UK, in accordance with UK Home Office regulations and was approved by Moredun Research Institute's experiment committee.

Samples

Immediately after culling, blood and tissue samples ($2.5 \times 2.5 \times 2.5$ cm) from heart, tongue, diaphragm, foreleg (*Musculus triceps brachii*; triceps) and hind leg (*M.*

semitendinosus; semitendinosus) were collected into separate containers. The same part of each muscle was sampled from all pigs, and visible fat and connective tissue was avoided. Samples were frozen and stored at -20°C until shipped on ice to our laboratory. At arrival, the tissue samples were transferred frozen to special containers for meat juice collection (CC Plast A/S, Hillerød, Denmark) (Nielsen *et al.*, 1998) and thawed for 12 h at $+20^{\circ}\text{C}$ to release the meat juice, which trickled into a collecting tube. Meat juice and sera were stored at -20°C until analyzed. Due to limited recovery of fluid from some samples, all analyses were not performed on all samples (Table 1).

T. gondii titer

T. gondii-specific antibodies were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (ID Screen Toxoplasmosis Indirect Multi-species; IDvet Innovative Diagnostics, Montpellier, France) according to the manufacturer's instructions, with results expressed as sample/positive control (S/P) percentage $\{SP = [(OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive}} - OD_{\text{negative control}})] \times 100\}$, where OD is optical density. Initially, serum and meat juice samples were tested at the recommended dilutions 1:10 and 1:2, respectively. Based on the results, serum and meat juice from heart, diaphragm, and semitendinosus (representing muscles with high, intermediate, and low S/P) were tested in 2-fold dilutions from 1:10 (serum) or 1:2 (meat juice). Titers were calculated from interpolation of the linear part of the titration curves and expressed as reciprocals of the dilution with S/P=50%. To evaluate the association between *T. gondii* titer and different blood parameters in samples from each muscle, a *T. gondii* titer ratio (meat juice titer/serum titer) was used to adjust for differences in antibody responses of individual pigs.

Total IgG

Total IgG was measured by a capture ELISA as previously described (Magnusson and Fossum, 1992) but with goat anti-swine immunoglobulin ($5 \mu\text{g/mL}$) (Jackson Immuno-research, Suffolk, UK) as capture antibody, Polysorp microtitration plates (PolySorp, Nunc Immuno Plate, Thermo Fisher Scientific, Waltham, MA), and peroxidase conjugated goat anti-swine immunoglobulin (diluted 1:150,000) (Jackson Immuno-research) as detection antibody. Two blank wells and a standard (Swine immunoglobulin; Jackson Immuno-research) in 2-fold dilutions from 1200 to 9.38 ng/mL were included on each plate. Standards and samples were tested in duplicates, and each sample was analyzed in four 2-fold dilutions from 1:1600 to assure that measurements were done within the linear part of the titration curve.

Blood content

Hematin. The AHD method was performed as described by Frenchik *et al.* (2004) with $2 \mu\text{L}$ of meat juice added to $198 \mu\text{L}$ AHD reagent. Porcine chlorohemin powder ($>98\%$ pure; Sigma-Aldrich, Stockholm, Sweden) was used as standard and a series of dilutions from 0.0015 to 0.1534 mmol/L was added to each plate. Samples and standards were analyzed in duplicates. After incubation on a plate shaker for 30 min (340 rpm) at room temperature, the OD at 570 nm was measured in a plate reader (Multiscan FC version 2.5, Thermo

Fisher Scientific) and the concentration of hematin (mmol/L) in the samples was calculated.

Hb and myoglobin. To estimate the contribution of Hb and myoglobin to the total amount of heme proteins, myoglobin concentrations were analyzed by a Porcine Myoglobin ELISA (Innovative Research, Peary Court Novi, MI) according to the manufacturer's instructions. Meat juice was diluted 1:100,000 or more when necessary to retrieve readings within the standard curve. For each sample, the myoglobin content (expressed as millimoles per liter) was subtracted from the hematin content to yield the concentration of Hb, which was presented as grams per liter.

Statistics

All statistical analyses were performed in R version 3.0.3 (R Development Core Team, 2012). Kruskal-Wallis chi-square test was used to compare results for the different groups of pigs. Differences between muscles were evaluated with Bonferroni correction for multiple comparisons using the R function "pairwise.wilcox.test". Spearman correlation test was used to estimate the correlation between (1) different parameters for samples (serum and meat juice from heart, diaphragm, and semitendinosus) from each individual pig and (2) different parameters for samples from each of these muscles ($n=16$ –18 per muscle; Table 1). Results were considered significant when $p < 0.05$.

Results

Significant differences between meat juice from different muscles

Serum and meat juice samples from all infected pigs tested positive in the *T. gondii* ELISA, and all samples from uninfected control pigs were negative. Because there were no significant differences in *T. gondii* titer, total IgG, Hb, myoglobin, or hematin levels between experimental groups, all inoculated pigs were treated as one group in all further comparisons. Significant differences were recorded for *T. gondii*-specific IgG (S/P value and titer), total IgG, hematin, and Hb between meat juice from different muscles (Fig. 1A–D). Heart samples had significantly higher levels regarding all parameters, compared with the other muscles ($p < 0.05$) and also the greatest variation. Samples from diaphragm and tongue generally had intermediate levels, and the levels for each muscle were more homogeneous. These levels were in turn significantly different from those in semitendinosus and triceps ($p < 0.05$), which mostly showed low levels with very little variation. In contrast, for myoglobin (Fig. 1E), there were no significant differences between muscles.

Agreement between *T. gondii* titers in serum and meat juice

The highest correlation with titers in serum was seen for meat juice from heart ($r_s=0.87$; $p < 0.001$), followed by semitendinosus ($r_s=0.71$; $p < 0.001$) (Fig. 2A and B) and diaphragm ($r_s=0.54$; $p=0.02$) (Fig. 2C). The ratios between serum and meat juice titers are shown in Figure 3.

FIG. 2. Correlation between *Toxoplasma gondii* titers in serum and in meat juice from (A) heart ($n=16$), (B) semi-tendinosus ($n=18$), and (C) diaphragm ($n=18$).

FIG. 3. Distribution of *Toxoplasma gondii* titer ratio (serum/meat juice) for diaphragm ($n=18$), heart ($n=16$), and semitendinosus ($n=18$). The whiskers extend to the most extreme values. The plus indicates the mean value and the line indicates the median.

T. gondii titers in relation to the content of total IgG and blood

Comparison of total IgG and *T. gondii* titer in the different samples from each pig revealed a very high correlation, although not significant ($r_s = 1$; $p = 0.08$). The correlation between total IgG and *T. gondii* titer ratio in samples from diaphragm, semitendinosus, and heart muscle from different pigs was $r_s = 0.77$ ($p < 0.001$), $r_s = 0.64$ ($p = 0.005$), and $r_s = 0.50$ ($p = 0.051$), respectively (Fig. 4A–C).

Analysis of hematin/Hb and *T. gondii* titer in different samples from each pig revealed a very high correlation (similar to that of total IgG and *T. gondii* titer), although not significant. Within muscle, however, a significant correlation between Hb and *T. gondii* titer ratio was only seen in samples from diaphragm ($r_s = 0.65$; $p = 0.008$) (Fig. 5). In samples from heart, the correlation was low and not significant ($r_s = -0.19$; $p = 0.53$), while samples from semitendinosus showed a negative correlation ($r_s = -0.66$; $p = 0.003$) due to many Hb values close to zero. For hematin, the correlation with *T. gondii* titer ratio was low and not significant for all the muscles tested (diaphragm: $r_s = 0.35$; $p = 0.16$; heart: $r_s = -0.1$; $p = 0.7$; semitendinosus: $r_s = 0.17$; $p = 0.5$).

Discussion

The main objective of this study was to test whether detection of specific antibodies is dependent on the level of blood in the sample. We found significant differences in specific antibody levels in meat juice depending on the muscle source, which agrees well with previous findings by others (Nielsen *et al.*, 1998; Forbes *et al.*, 2012; Ranucci *et al.*, 2012). The highest levels were recorded in meat juice from heart, followed by diaphragm and semitendinosus. The same pattern was recorded for total IgG, hematin, and Hb, with the highest levels in samples from heart followed by diaphragm, tongue, semitendinosus, and triceps in decreasing order. Furthermore, a linear relationship was seen between each of these parameters and the *T. gondii* titer in samples from the same pig, implicating that blood supply to the

muscle (and to the sample) is an important factor and that correction for blood could be useful in meat juice serology. However, analysis of the relationship between “blood” parameters and *T. gondii* antibody levels (titer ratio) in each type of sample from different pigs revealed a complicated pattern. While the correlation between *T. gondii* titers in serum and meat juice was strong for heart and semitendinosus, it was less for diaphragm. On the other hand, for diaphragm samples the *T. gondii* titer appeared to be dependent on the Hb concentration. Thus, it seems as variations in “blood content” can affect the results of meat juice serology, but this effect could be dependent on the muscle used for meat juice extraction.

It is difficult to give any obvious explanation why variations in Hb content would affect immunoglobulin levels only in meat juice from some muscles. Possibly, the myocardium contains such high blood levels that small variations are not important. Wingstrand *et al.* (1997) showed that washing the heart muscle before comparing meat juice and serum titers made no difference from when the heart was used unwashed, leading to the conclusion that antibodies originated mainly from the myocardium and not from contaminating blood. Regarding meat juice from semitendinosus, we recorded very low levels of heme-containing proteins, indicating that the contribution of IgG to the sample from peripheral blood was minimal. Furthermore, for a few semitendinosus samples the calculated Hb value was negative, probably due to calculations being based on measurements of hematin (spectrophotometry) and myoglobin (ELISA), which may give measurement errors that are bigger than the hematin or Hb levels themselves in low-level muscles. Thus, the calculated Hb values would have a very low correlation to the *T. gondii* titer ratio.

Another factor that may influence the antibody concentration in meat juice is the water-holding capacity (WHC) of muscles (Nielsen *et al.*, 1998). After slaughter the muscle pH drops, which reduces the WHC and increases the amount of meat juice, which most likely results in diluted antibody levels. These postmortem changes can vary greatly between animals, muscles, and parts of muscles, and are influenced by genetic factors, animal handling before slaughter, and meat

FIG. 4. Correlation between *Toxoplasma gondii* titer ratio (meat juice/serum) and total immunoglobulin G (IgG) in meat juice from (A) diaphragm ($n=18$), (B) semitendinosus ($n=18$), and (C) heart ($n=16$).

FIG. 5. Correlation between *Toxoplasma gondii* titer ratio (meat juice/serum) and hemoglobin (Hb) in meat juice from diaphragm ($n=16$).

processing. In the present study, the animals were kept, culled, and sampled in a standardized way, which would reduce variations in muscle WHC. However, differences in WHC could still have caused some variation in our data. Samples collected during meat inspection are probably more affected by this factor, which adds to the complexity of meat juice serology.

The majority of epidemiological studies are performed on diaphragm meat juice (Richomme *et al.*, 2010; Berger-Schoch *et al.*, 2011; Methner *et al.*, 2011; Wacheck *et al.*, 2012; Deksne and Kirjušina, 2013). The current study reports a relatively low correlation ($r_s=0.54$) between serum titers and titers in diaphragm meat juice. In contrast, *T. gondii* titers in meat juice originating from heart muscle had a strong agreement with titers in matched serum samples ($r_s=0.87$). This is consistent with previous findings that meat juice from heart is in best agreement with serum (Wingstrand *et al.*, 1997; Forbes *et al.*, 2012). Consequently, the heart might be a more suitable source of meat juice for serology than the diaphragm. However, in many countries the heart is used as a delicacy, so using it to extract meat juice is not appropriate within the food industry.

For a number of serological assays it has been shown that meat juice should be diluted to approximately a tenth of the serum dilution (Wingstrand *et al.*, 1997; Nielsen *et al.*, 1998; Nöckler *et al.*, 2005). However, it is often assumed that this relationship is constant, and assays designed for analysis of serum are adapted to meat juice simply by using a dilution factor that is one tenth of the one recommended for serum. In the current study, we found that approximately 70% of the diaphragm samples had *T. gondii* titers less than one tenth of the serum titer. This indicates that the use of meat juice from diaphragm may lead to underestimation of the number of positive samples if serological tests are adapted to meat juice simply by lowering the dilution factor. Furthermore, in the present study all semitendinosus samples had titers at least 30 times lower than in serum. In contrast, meat juice from heart had titers that were on average one fifth of those in serum. It is thus evident that consideration to the sample source is necessary when meat juice is tested. In this context it is noteworthy that some

meat juice-based monitoring programs are open to the possibility of choosing between different muscles for sampling (Mousing *et al.*, 1997; BPEX, 2009).

As mentioned above, the sensitivity of meat juice serology is a concern, but this has not been directly addressed in the current study because it was based on samples from experimentally infected pigs with high antibody titers. Nevertheless, we believe that the results demonstrating large and significant differences in immunoglobulin levels as well as in specific antibody titers, dependent on which muscle the meat juice has been extracted from, are of great interest and could be useful for further considerations of the use of meat juice as a serological matrix.

Conclusions

The concentration of antibodies in meat juice depends on which muscle is used for extraction. The specific IgG titers correlated well with the total IgG level. Also, the Hb content appeared to influence specific antibody levels in meat juice from diaphragm but not from heart or semitendinosus. Thus, to increase the sensitivity of meat juice analysis, the muscle source should be standardized, and antibody distribution to the sample needs to be considered. For seroepidemiological studies and to identify farms with high prevalence of food-borne infections, the cost for adding another test to adjust for variation in antibody distribution is probably disproportionate to the gain. However, if meat juice analysis is to be implemented to distinguish between infected and noninfected animals at an individual level, samples with low antibody levels are unreliable.

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Disclosure Statement

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