Received: 7 April 2020 | Revised: 13 August 2020 | Accepted: 2 September 2020

DOI: 10.1111/xen.12646

ORIGINAL ARTICLE

Xenotransplantation WILEY

Dot blots of solubilized extracellular matrix allow quantification of human antibodies bound to epitopes present in decellularized porcine pulmonary heart valves

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: Cluster of Excellence 'REBIRTH' and SFB 127 Project C7; "kinderherzen" - Fördergemeinschaft Deutsche Kinderherzzentren e. V.

Abstract

Background: The present study reports the development of a sensitive dot blot protocol for determining the level of preformed antibodies against porcine heart valve tissue derived from wild-type (WT) and α-Gal-KO (GGTA1-KO) pigs in human sera.

Methods: The assay uses decellularized and solubilized heart valve tissue; antibody binding found in this dot blot assay could be correlated with antibody titers of preformed anti-α-Gal and anti-Neu5Gc antibodies detected by a sensitive ELISA.

Results: The ultimate protocol had an inter-assay variance of 9.5% and an intra-assay variance of 9.2%, showing that the test is reliable and highly reproducible. With the aid of this dot blot assay, we found significant variation with regard to antibody contents among twelve human sera. Binding of preformed antibodies to WT tissue was significantly higher than to GGTA1-KO tissue.

Conclusions: The dot blot assay described herein could be a valuable tool to measure preformed antibody levels in human sera against unknown epitopes on decellularized tissue prior to implantation. Ultimately, this prescreening may allow a matching of the porcine xenograft with the respective human recipients in demand and thus may become an important tool for graft long-term survival similar to current allotransplantation settings.

KEYWORDS

antibody, decellularization, preformed antibodies, solubilized ECM, xenoantigen, xenotransplantation

1 | INTRODUCTION

Decellularized allografts show superior clinical results compared with other available heart valve substitutes, especially with regard to remodeling capacities and growth potential.[1,2] This renders them a particularly suitable transplant for pediatric patients.[2] However, there is a global growing shortage of human donor heart valves that particularly affects children in terms of their need for size matched grafts.[3] The use of porcine-derived xenotransplantation products could cover the demand in all needed numbers and sizes. The main hurdle that is

yet to be overcome is the increased antigenicity of xenogeneic tissue due to so-called xenoantigens.[4] Humans are known to build preformed (natural) antibodies against particular xenoantigens, which may cause a hyperacute or acute rejection of xenogeneic organs and tissues.[5]

The elimination or at least a significant reduction of xeno-antigenicity is a major goal in current xenotransplantation research. But despite significant advances in the past few years, the ultimate solution has yet to be developed. The production of pigs with a genetic knockout of the α-1,3-galactosyltransferase gene (GGTA1) was essential to control the hyperacute rejection response.[6] However, multi-transgenic pigs are considered necessary to obtain long-term survival of the porcine xenograft in primates. Growing evidence suggests that it is indeed possible to prolong survival of the xenografts in the baboon model as recently shown for porcine hearts from triple transgenic pigs, which functionally survived for > 180 days after orthotopic transplantation into baboons.[7] The situation might be different when transplanting isolated cells and tissues rather than solid organs. In the particular case of decellularized heart valves, the elimination of all antigenic surface structures is considered to be essential for long-term function in patients. Presumably, more, yet unknown xenoantigens are to be discovered and would have to be eliminated.[8] Apart from the production of genetically modified donor animals, a promising strategy of antigen reduction is the entire cell removal in order to reduce the antigenic load of the donor tissue without interfering with its remodeling capacity.[9] However, xenoantigens can still be found on the remaining matrix even after decellularization.[9-12]

When testing the success of antigen-reduction treatments, it became apparent that results obtained in vivo cannot necessarily be translated to the pig-to-human situation, as seen for Matrix P™ and Synergraft™ heart valves.[13,14] These porcine heart valves showed promising results in the sheep model, but failed in human patients suggesting species-specific differences of immune compatibility.

The goal of this study was to provide a simple in vitro test system that allows us to quantify antibodies present in human sera binding to decellularized heart valve matrices.

Therefore, we developed a new dot blot method to give insight into the immune reaction of patient sera when exposed to porcine heart valve tissue derived from either wild-type or genetically modified (GGTA1-KO) donor pigs. Our results demonstrate that the dot blot method described here can be used as a control for the reduction of antigens in potential implants in a laboratory setting. Additionally, it can be used for assessing the potential immunogenicity of non-fixed decellularized xenogeneic tissue grafts for human recipients and therefore might be a useful screening tool for future graft-recipient matching purposes.

2 | MATERIALS AND METHODS

2.1 | Serum acquisition

After having received written consent, blood of 12 healthy volunteers was collected in serum Monovettes (Sarstedt). This procedure was approved by the local ethic committee. After centrifugation (2500 ×*g*, 20 minutes), the supernatant was frozen at −80°C until thawed for use.

2.2 | Heart valve acquisition

Porcine wild-type pulmonary heart valves (German Landrace) were collected from a local slaughterhouse, porcine GGTA1-KO pulmonary heart valves (German Landrace as genetic background) were obtained from Friedrich Loeffler Institute, Institute of Farm Animal Genetics in Mariensee, Germany. They were collected from pigs slaughtered in specific animal experiments. Parts of the human pulmonary artery were recovered after lung transplantation at the Hannover Medical School.

2.3 | Decellularization

After removal of fat and loose connective tissue, porcine pulmonary heart valves (pPHV) were disinfected in Braunol® (B. Braun) for 5 minutes and washed in PBS for 20 minutes. For decellularization, the pPHVs were incubated in turbulence bottles on an orbital shaker, running at 200 rpm at room temperature in 150 mL of 0.5% of sodium dodecyl sulfate (SDS) (Carl Roth) and 0.5 sodium deoxycholate (SD) (Sigma-Aldrich) for 2 × 12 hours, followed by washing twice in AMPUWA® (Fresenius Kabi) and 12 times in PBS for 12 hours each. All steps were conducted under sterile conditions.

2.4 | Solubilization

The decellularized tissue was mechanically minced and enzymatically digested using 20 μL collagenase Type II (Worthington; 260 U/mg; 5 mg/mL) in 80 μL Tris-buffered saline (TBS) at 37°C at 1000 rpm o/n. The non-digested debris was sedimented by centrifugation. The concentration of solubilized matrix proteins in the supernatant was measured by NanoDrop Spectrometer (Coleman Technologies) using collagenase solution as blank.

2.5 | Dot blot method

A dot blot device (Minifold I, GE Healthcare) was assembled with a filter (Bio-Dot SF filter paper) and a nitrocellulose blotting membrane (GE Healthcare, size: 11 × 7 cm, 0.2 μm pore size) that had been preincubated in TBS. The membrane was loaded with 10 μg solubilized heart valve tissue in 200 μL TBS per well by applying vacuum to the device. The following incubation steps were conducted on an orbital shaker at 2 rpm at RT. Loaded membranes were placed in an incubation dish and 150 mL liquid were used throughout the remainder of the experiments, if not stated otherwise. Loaded proteins were fixed on the membranes using 0.5% glutaraldehyde (Merck) for 20 minutes followed by three washing steps in TBS and one incubation step with 50 mmol/L glycine/PBS for 5 minutes each. After another

washing step in TBS, the membranes were incubated in Animal-Free™ (BIOZOL) blocking solution for 1h. For exposure, human serum as primary antibody was diluted in Animal-Free™ (BIOZOL) in a ratio of 1:47 to a total volume of 42 mL, added to the membranes in a closed container and incubated o/n at 4°C on an orbital shaker at 2 rpm. Afterward, the membranes were washed three times in Tris-buffered saline with 0.05% Tween® 20 (TBST) for 5 minutes each at RT, followed by incubation with goat anti-human IgA, IgG, IgM-HRP (antikoerper-online.de, ABIN 117312) as secondary antibody against all three Ig-isoforms in a dilution of 1:80 000 in TBST for 1h at RT. The following washing steps included three iterations with TBST for 5 minutes each and two with TBS for 10 minute each. In order to visualize antibody binding, membranes were exposed to 2 mL of a mix of solution A and B of the enhanced chemiluminescence (ECL) kit (Perkin Elmer), dried with Whatman™ paper (GE Healthcare) and imaged by the use of ChemiDoc Imager (Bio-Rad).

2.6 | Standard for signal normalization

On each membrane, beside matrix proteins to be tested, a defined dilution row of human serum type AB (Lonza) (starting from 1:16 000 in TBS) was loaded in duplicate in order to normalize each membrane for comparison.

2.7 | Quantification of human anti-α-Gal- and anti-Neu5Gc-antibodies

The ELISAs for α-Gal and Neu5Gc were conducted as described by Ramm et al. (2015). Briefly, MaxiSorp 96-well plates (Thermo Fisher Scientific) were pre-coated o/n by either α-Gal conjugated BSA (MoBiTec) for detection of anti-α-Gal antibodies, or by Neu5Gc-conjugated PAA (polyacrylamide) on one half of the plate and Neu5Ac-conjugated PAA on the other half of the plate for the detection of anti-Neu5Gc-antibodies. These coated plates were loaded in a duplicated dilution series with equivalent human serum samples that were used for the dot blot experiments. TMB ELISA substrate (Thermo Fisher Scientific) was used for color development and visualized by PARADIGM plate reader (Beckman Coulter). For normalization, one particular serum (blood donor No. 10 for α-Gal and blood donor No. 5 for Neu5Gc), which served as internal standard, was used on all measured plates in order to calculate arbitrary units. For detection of anti-Neu5Gc-antibodies, the reactivity to Neu5Ac was subtracted from the value of Neu5Gc.

2.8 | Statistical analysis

In all experiments, samples were dotted in technical triplicates to evaluate the mean ± standard deviation (SD). As mentioned above, respective standard was loaded in duplicate and used for normalization. Two-way ANOVA followed by Bonferroni post-test was performed in order to calculate statistical significances. The intra- and inter-assay variances were calculated by coefficient of variation (CV) which is the relative standard deviation expressed in percent. Pearson correlation was performed to compare dot blot results with antibody titers obtained by ELISA. For normal distribution, the logarithm (ln) of Neu5Gc titers was used. *P* values of ≤.05 were considered as significant.

3 | RESULTS

3.1 | The dot blot method

Decellularized heart valves were mechanically minced and solubilized using collagenase, dotted on nitrocellulose membranes and exposed to human serum. Serum antibodies that bound to epitopes on the membrane were visualized via horseradish peroxidase (HRP) coupled secondary anti-human IgA, IgG and IgM antibodies and adding enhanced chemiluminescence (ECL). Dots on imager generated pictures allowed to correlate arbitrary signal intensity and antibody binding. The more antibodies bound to the tissue, the darker a dot appeared and signal intensity increased concomitantly (Figure 1A).

Dots from decellularized WT tissue showed higher signal intensity than those of decellularized α-Gal-KO tissue. Interestingly, signal intensity for dots from decellularized human tissue was generally higher than for porcine knockout tissue (Figure 1B). We observed that when only secondary antibodies (anti-human IgG, IgA and IgM) were exposed to membranes loaded with solubilized tissue of different origin (WT, KO, H) and human serum as standard, it bound to human pulmonary artery and the human standard, but not to porcine material (Figure 1B). Since this signal appeared without exposure to primary antibodies (in the sera), but solely to exposure to the secondary antibody, its intensity had to be subtracted from the signal intensity generated by exposure to sera in human samples, in order to obtain values for the binding of antibodies in sera to human tissue.

3.2 | Evaluation of dot blot reproducibility

The reproducibility of the dot blot technique developed for this study was investigated by exposing serum of one healthy blood donor (No. 10 in Figure 3) to different identically decellularized heart valve tissues (WT n = 3, KO n = 3, H n = 1) in six separate experiments (n = 6) (Figure 2). All samples were loaded in technical triplicates, and an intra-assay variance of 9.2% was determined by calculating the coefficient of variation (CV) between the triplicates. The CV of values obtained by all six experiments led to an inter-assay variance of 9.5%, indicating a high reproducibility of this method.

3.3 | Antibody binding is dependent on individual human sera and on the type of heart valve

Decellularized matrix tissue samples from three porcine wild-type and three porcine GGTA1-KO pulmonary heart valves and one

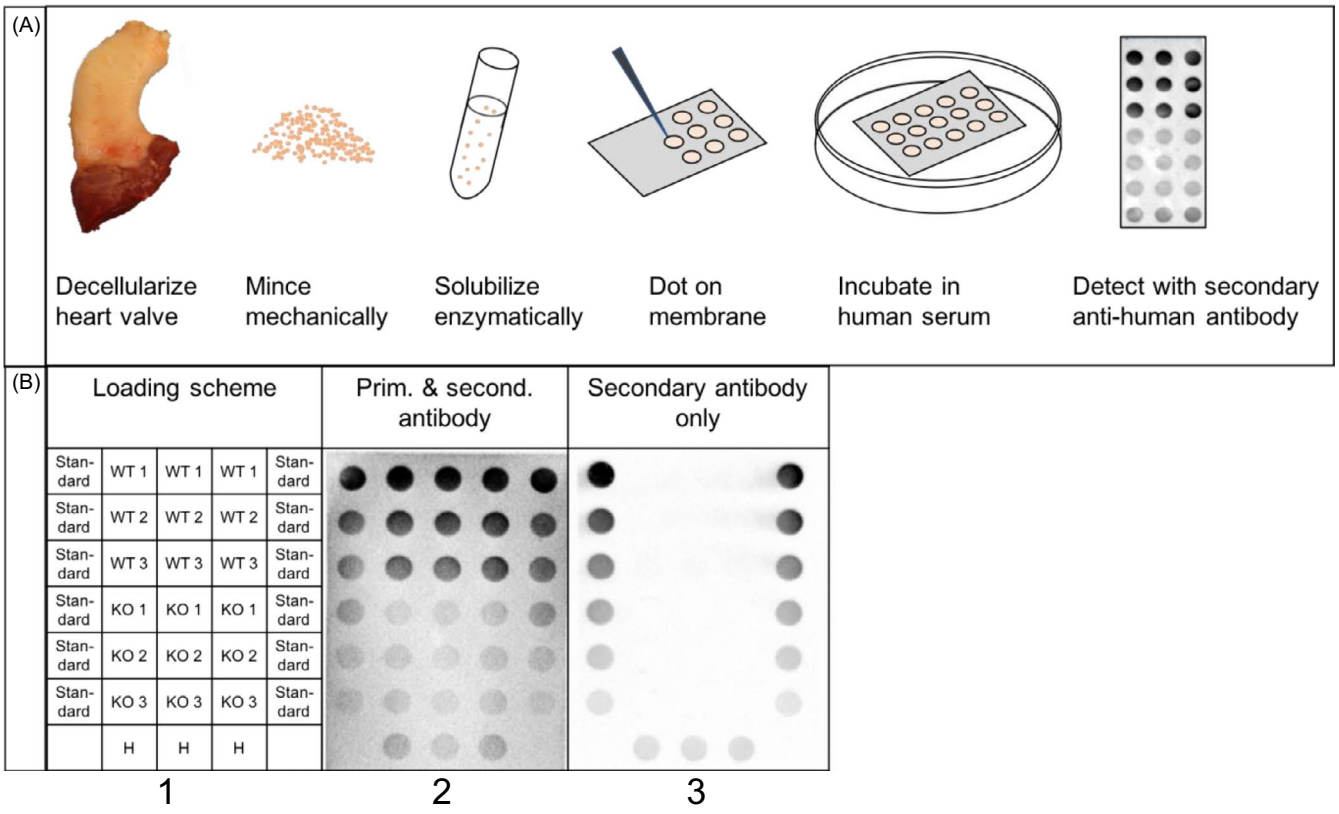

FIGURE 1 A, Scheme illustrating the individual steps of the dot blot method. B, Blotting and signal detection after incubation w/o sera exposure 1) Loading scheme: the standard is loaded in a duplicated dilution series, porcine heart valve tissue is loaded in technical and biological triplicates, human pulmonary artery in technical triplicates, 2) Signals after membrane exposure to primary (human serum) and secondary (anti-human IgG, IgA, IgM) antibody, 3) Signals after membrane exposure to secondary antibody only, WT = porcine wild-type, KO = porcine GGTA1 knockout, H = human

human pulmonary artery were exposed to sera of twelve healthy blood donors (Figure 3, Figure S1). For each serum, the binding of preformed antibodies to WT tissue was significantly ($P < .001$) higher than to GGTA1-KO tissue. The individual signal intensities to WT varied by 74.6% between sera, whereas the individual signal intensities to KO tissue varied with a coefficient of variation of 51.4%. Even though the variance between the sera was smaller for KO tissue, serum-dependent significant differences were determined both for the reaction to WT ($P < .0001$) and to KO ($P < .0001$) tissue.

When related to the AB0 blood groups, sera of blood group AB revealed significantly ($P < .01$-$.001$) lower signal intensity toward porcine WT tissue samples compared to sera of the other three blood groups (Figure 3).

3.4 | Correlation of dot blot results with antibody titers detected by ELISA

As shown in Table 1, a significant positive correlation between dot blot results for WT pig tissue and anti-α-Gal antibody titer as measured by ELISA for the same sera ($P < .01$) was calculated, whereas dot blot results for GGTA1-KO pig tissue did not correlate with anti-α-Gal antibody titers. The logarithm of antibody titers for Neu5Gc obtained by ELISA correlated significantly ($P < .05$) with dot blot results for KO and WT tissue for the same sera (Figure 4).

4 | DISCUSSION

Decellularized allografts provide superior clinical function compared to conventional mechanical[15] or other biological[1] heart valve protheses. However, the current growing shortage of human donor organs prevents an unlimited supply of acellular allogeneic heart valve matrices. Porcine-derived decellularized xenografts constitute a promising alternative, provided that the xenoantigen-related immunogenicity of such tissues and matrices can be abolished reliably prior to clinical use.[4] Thus, a simple straightforward testing system that can discriminate patient individual incompatibilities with the respective porcine xenograft to be implanted would be of great value. Equally important for future research perspectives, a valid detection tool is required for screening human sera for binding patterns to unknown xenoantigens, since no animal model has the capacity to sufficiently recover all relevant

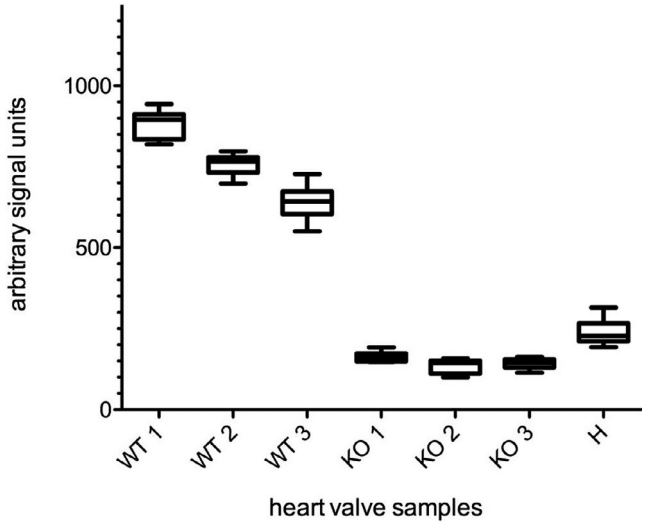

FIGURE 2 Reproducibility test: binding of preformed antibodies of one human blood donor to three porcine wild-type (WT 1-3), three porcine GGTA1 knockout (KO 1-3) and one human (H) decellularized pulmonary heart valve matrices in 6 individual repetitions (n = 6). Box and Whiskers presentation: Box = interquartile range, Whiskers = min and max, line = median

TABLE 1 Results of Pearson product-momentum correlation coefficient (PPMCC) for antibody titers of human sera tested with dot blot method and ELISA

Correlation of	Significance *P*-valued	Pearson correlation coefficient (*r*)
Dot blot (WT) vs α-Gal ELISA	.0056	0.7430
Dot blot (KO) vs α-Gal ELISA	.5591	0.1877
Dot blot (WT) vs Neu5Gc ELISA	.0427	0.5917
Dot blot (KO) vs Neu5Gc ELISA	.0242	0.6428

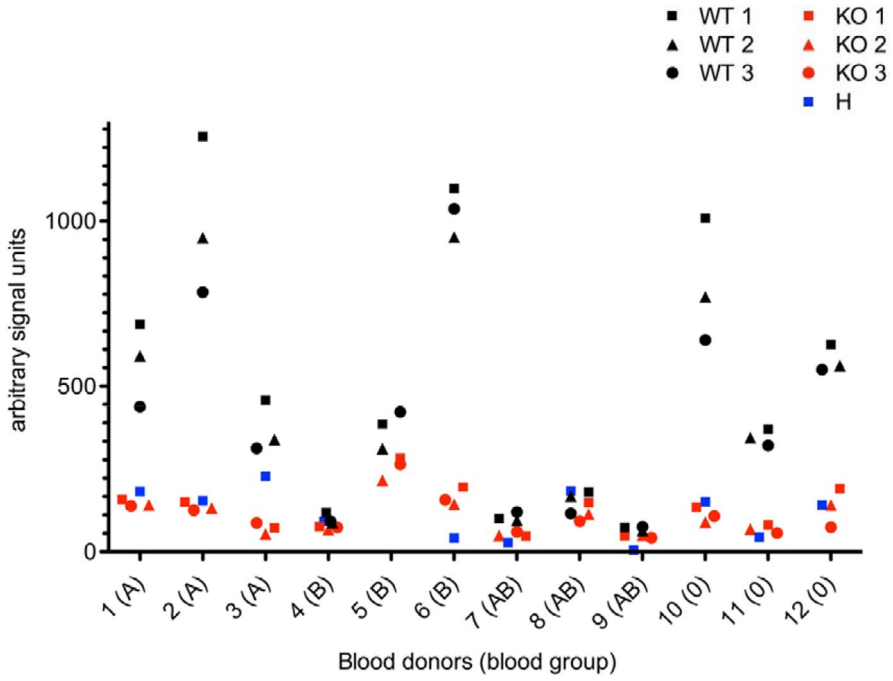

FIGURE 3 Antibody binding of three A, three B, three AB and three O blood group donors to three WT (WT 1-3), three KO (KO 1-3) and one human (H) heart valve matrices. WT = porcine wild-type, KO = porcine GGTA1-knockout, H = human

incompatibilities between a human immune system and a xenogeneic implant entirely independent from its donor origin.

In the current study, we developed a reliable in vitro method that enables antigenicity assessment of insoluble extracellular matrix protein components of non-fixed, decellularized heart valve matrices solubilized by collagenase digestion. As an endoprotease, collagenase has the advantage of cutting only specific parts of the exposed protein structures and, as extensively tested during the course of this work, generates only light background signals when applied for respective blotting methods. The complete peptic digestion of extracellular matrix compounds allows the testing of water-insoluble and water-soluble matrix components in parallel. Antibodies within the applied sera individually bind to the respective blotted solubilized matrix components and can be visualized afterward by a suitable secondary antibody, which is species-specific for the immunoglobulin subclasses that are intended to be detected. The developed assay here was highly reproducible in terms of a semi-quantitative determination of preformed antibody titers in human sera against components of porcine decellularized heart pulmonary valves matrix proteins, with an intra-assay variance of 9.2% and an inter-assay variance of 9.5%.

Interestingly, also the direct application of antibodies directed against human immunoglobulins IgG, IgA and IgM without any prior serum exposure to target membranes, containing blotted solubilized protein constituents of decellularized human pulmonary artery

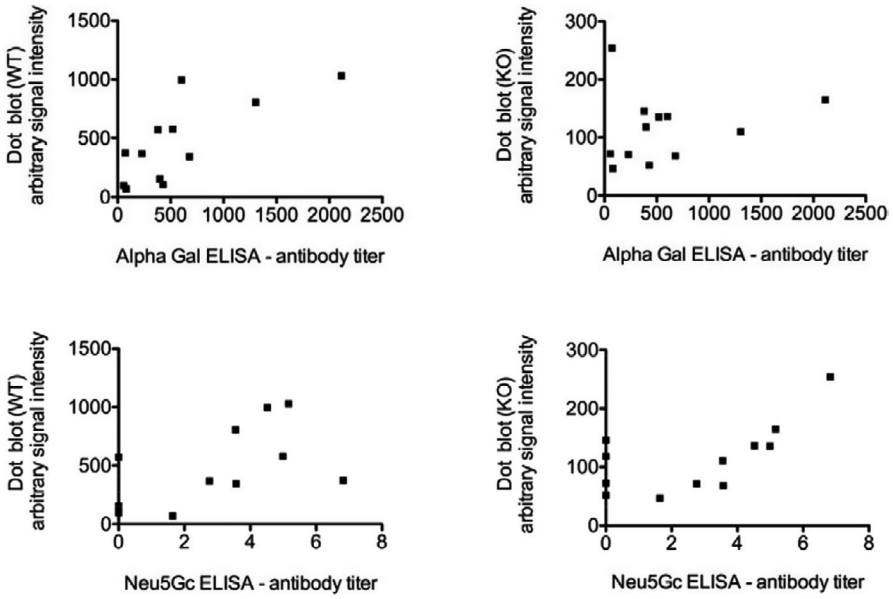

FIGURE 4 Correlation between results obtained by dot blot method and ELISA for 12 human sera: A, Significant correlation between dot blot results for WT and α-Gal antibody titers (ELISA) ($P < .01$), B, No correlation between dot blot results for GGTA1-KO tissue and α-Gal antibody titers (ELISA) ($P > .05$), C, Significant correlation between dot blot results for WT and logarithm of Neu5Gc antibody titers (ELISA) ($P < .05$), D, Significant correlation between dot blot results for GGTA1-KO tissue compared to logarithm of Neu5Gc antibody titers (ELISA) ($P < .05$). Each square represents the result of one human serum in both experiments

matrix tissue resulted in detectable signals. This observation might be explained by residual donor-derived antibodies retained within the pulmonary artery vessel matrix even after decellularization. Thus, our analytical strategy was to routinely include control groups in all conducted experiments in order to generate a respective baseline in terms of the specific antibody binding behavior of the applied secondary antibodies to human tissue samples.

Previous studies focused on attempts to establish in vitro methods of measuring antigenicity, but so far in these tests a preselection of either antibodies by using affinity purification[11] or antigens by using extracts of matrix tissue where utilized,[16,17] which is in all probability associated with a loss of relevant information. A dot blot method has previously been used with extracts of heart valve tissues,[17] whereby antibody binding was detected in native α-Gal-positive (WT) valve tissue, but not in respective decellularized tissue samples. In contrast, the assay developed and introduced here is capable to detect levels of preformed antibody populations in human sera directed not only against porcine decellularized α-Gal-positive (WT) heart valve tissue components, but moreover against respective porcine decellularized α-Gal-negative KO tissue samples as well, indicating a high degree of sensitivity of this method.

Consistently in this study, we could demonstrate the existence of significant interindividual differences between antibody titers of the different tested human sera directed against both porcine decellularized WT and GGTA1-KO extracellular heart valve matrix protein components.

These findings can be correlated with previous data showing that both humans[18] and non-human primates[19,20] express preformed α-Gal and non-Gal antibodies which are essentially involved in porcine xenograft rejection and whose titers appeared to be highly individual not only in terms of their quantity but also with respect to the quality of their acting and effecting relevance.

To confirm the introduced dot blot assay, we determined anti-α-Gal antibody titers in all tested sera by performing a well-investigated and established ELISA technique and found a significant correlation of the results with those of the dot blot method utilizing porcine α-Gal-positive WT tissues. Results suggest that α-Gal-specific antibodies are preformed binding antibodies even for decellularized porcine WT tissues. This confirms previous results identifying α-Gal epitopes on decellularized porcine WT matrices.[11] The dot blot-related results for all porcine heart valve tissues could be correlated with anti-Neu5Gc antibody titers even though anti-Neu5Gc ELISA can only detect a limited subset of Neu5Gc antibodies. Interestingly, we had one serum with relatively high antibody titers to Neu5Gc. This is why we had to calculate the logarithm of the titers in order to obtain a normal distribution of the titer values. Nevertheless, all sera exhibiting high antibody titers in the ELISA showed relatively strong signal intensities in the dot blot evaluation as well, suggesting that our dot blot method is sensitive also for detection of Neu5Gc antibody levels. Interestingly, some tested sera revealed high antibody levels directed toward porcine GGTA1-KO valve tissue samples determined by the dot blot technique, but did not show high Neu5Gc titers in the corresponding ELISA, suggesting that the optimized dot blot method might be capable of detecting preformed antibodies to yet unknown antigens. A probable explanation might be the fact that the applied dot blot method does not preselect antibodies or antigens and works without any animal derived material other than the tested matrices.

Conclusively, the dot blot method developed here enables semi-quantitative measurement of preformed antibody populations in human sera directed against unknown epitopes on decellularized xenogeneic extracellular matrix protein components. Thus, the effectiveness of new strategies to reduce the antigen load can be evaluated in an in vitro setting prior to potential in vivo testing. However, due to its basis on preformed antibodies, long-term predictions

about graft rejection patterns and mechanisms toward a potential implant cannot be made by the implementation of our method since it cannot provide any information about the adaptive immune response in a prolonged rejection setting. Moreover, no assertions of a possible antigenicity threshold for instant rejection of implants can be achieved by exploitation of this method. The decellularized human pulmonary artery tissue samples, utilized for this study, are representative for acellular human allografts, which evidentially are not rejected in a clinical transplantation setting. Nevertheless, the determined values of these samples during the course of this study cannot be used for the estimation of a threshold from which relevant mechanisms of graft rejection have to be expected, since our test revealed false-positive signals concerning human tissue samples, possibly due to residual human antibodies retained within the decellularized matrix compounds.

Furthermore, the fixation of membrane bound proteins and protein fragments with 0.5% glutaraldehyde may have a negative impact on antibody binding by structural changing of potential epitopes and reducing access ability by new covalent bonds and cross-links between proteins. However, based on the observation that non-glutaraldehyde-treated protein dots attract less serum born antibodies than glutaraldehyde-treated ones, the loss of antigenic epitopes by washing off small and/or uncharged proteins or protein fragments makes a stabilization on the membrane by glutaraldehyde fixation beneficial.

Nevertheless, when conducted with animal derived sera, this method could be applied as a pre-transplantation selection tool for future in vivo studies in order to refine animal experiments by matching the later recipient and the donor graft in order to exclude high responders in advance. The particular value of our dot blot method is the prediction of potential antigenicity based on preformed antibody levels. However, antibody populations evolving after a potential implantation into a living organism could still be monitored by our novel tool during future in vivo studies by repeatedly performing the test at certain time points after implantation. Thus, in direct comparison with the status prior to implantation, conclusions can be drawn in terms of the dynamic of the occurrences, as well as the increases and decreases of different antibody populations of interest.

Moreover, the optimized dot blot protocol described here could also be used to monitor potential antibody binding of human sera constituents to human allografts to be implanted into clinical patients. Thus, this work might facilitate determination of an immunogenic threshold, which would be useful in terms of translational approaches for the establishment of decellularized non-fixed xenografts for future clinical transplantation settings.

ACKNOWLEDGEMENTS

We are very thankful to PD Dr. med. Dietmar Böthig for his excellent help with statistical questions. This work was financed by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) via the Cluster of Excellence "From regenerative biology to reconstructive therapy" (REBIRTH) and via project C7 of TRR127 (Biology of xenogeneic cell and organ transplantation—from bench to bedside) and the foundation "kinderherzen—Fördergemeinschaft Deutsche Kinderherzzentren e.V." Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Smart I, Goecke T, Ramm R, et al. Dot blots of solubilized extracellular matrix allow quantification of human antibodies bound to epitopes present in decellularized porcine pulmonary heart valves. *Xenotransplantation*. 2021;28:e12646. https://doi.org/10.1111/xen.12646