Improvement of swine oral fluid specimens for antibody-based diagnostics

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

To my mother and main teacher,

Lic. Margarita Diaz Cruz.

To my major professor,

Dr. Jeffrey J. Zimmerman.

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ABSTRACT

Improvements in swine diagnostic medicine is a never-ending pursuit. However, whereas most researchers focus on assay improvements, this research focused on developing improvements to the sample itself.

The thesis begins with a review of the IgG molecule and the mechanisms and external factors that affect its stability in aqueous solutions. Chapter 2 summarizes the key discoveries that led to an understanding of the structure and functionality of antibodies, the main physicochemical mechanisms and external factors that affect the stability of IgG in aqueous solutions, the implications of IgG stability for diagnostic medicine, and practical recommendations for preserving IgG in diagnostic specimens.

Chapter 3 describes an experiment that evaluated the immediate and temporal effects of chitosan-based clarification treatments on PRRSV ELISA-detectable IgG in swine oral fluids. Serum and OF samples of known status were generated by vaccinating pigs (n = 17) with a PRRSV MLV vaccine. Individual pig oral fluid samples were collected from day post vaccination -7 to 42 and then subdivided into 4 aliquots. Each aliquot was treated with one formulation (A, B, C) with the 4th aliquot serving as untreated control. All samples were tested by PRRSV OF ELISA immediately after treatment (day post-treatment DPT 0). Thereafter, samples were held at 4°C and re-tested on DPTs 2, 4, 6, and 14. Both immediate and temporal treatment effects were evaluated for their quantitative (sample-to-positive ratio) and qualitative (positive vs negative) effects on the PRRSV oral fluid ELISA results. Analysis of results showed that the chitosan-based swine oral fluid clarification formulations evaluated in the study did not affect the stability or diagnostic functionality of PRRSV IgG either immediately after treatment or up to 14 days post-treatment, as measured using PRRSV ELISA S/P results.

CHAPTER 1. THESIS ORGANIZATION

This thesis is organized in three chapters. Chapter 1 contains a general introduction to the thesis organization. Chapter 2 is a literature review titled “Stability of IgG in diagnostic specimens – Considerations for veterinary diagnosticians” for submission to the Journal of Veterinary Diagnostic Investigation. Chapter 3 is a scientific research paper titled “Effect of chemical clarification of oral fluids on the detection of PRRSV IgG” which has been submitted to the Journal of Porcine Health Management.

CHAPTER 2. STABILITY OF IgG IN DIAGNOSTIC SPECIMENS – CONSIDERATIONS FOR VETERINARY DIAGNOSTICIANS

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Abstract

The 1890's saw the introduction of agglutination and/or precipitation diagnostic assays for a variety of bacterial pathogens of animals and humans, but the foundations of antibody-based diagnostic medicine were firmly laid by early 20th century researchers whose work revealed the structure and function of the antibody molecule. In mammals, the IgG is the most important antibody in diagnostic medicine because it is highly abundant in serum and is found in nearly every conceivable diagnostic specimen, e.g., colostrum, milk, oral fluids, urine, feces, meat exudate ("meat juice"), and others. However, this specialized glycoprotein is susceptible to physicochemical and enzymatic processes that affect its structural integrity and diagnostic

functionality. The purpose of this review is to summarize the key events that led to an understanding of the structure of antibodies, the mechanisms and external factors that affect the stability of antibodies in aqueous solutions (especially IgG), and issues for antibody stability in diagnostic specimens.

Key words: IgG; Antibodies; Stability; Functionality; Diagnostic;

Introduction

The purpose of this review is to summarize the key events that led to an understanding of the structure of antibodies, the mechanisms and external factors that affect the stability of antibodies in aqueous solutions (especially IgG), and the implications of antibody stability for diagnostic medicine.

In 1796, Edward Jenner observed that cowpox, a mild disease in humans caused by infection with vaccinia virus, seemed to confer protection against smallpox. This idea was confirmed by "vaccination" (Jenner's term) experiments, i.e., intentional exposure of people to vaccinia virus (Jenner, 1800). Nearly a century later, in 1890, Behring and Kitasato (MacNalty, 1954) furthered the idea of protection by intentional exposure when they showed that animals became resistant to tetanus or diphtheria after vaccination with these inactivated pathogens. They hypothesized that the immunized animal produced “antitoxins” that countered the effect of these pathogens, thus causing the vaccinated animal to become resistant to doses fatal to unvaccinated animals. Furthermore, they showed that serum from vaccinates protected non-vaccinates from clinical disease, i.e., the protective substances were present in the serum of immunized animals (Macnalty, 1954). The foundations of humoral immunity theory were definitively established in 1906 when these “antitoxins” were termed “antibody” by Paul Ehrlich in his book Collected Studies on Immunity (Ehrlich et al., 1906). Subsequently, working with immune precipitates from horse and rabbit serum, Heidelberger and collaborators determined that antibodies came in two principal forms with distinct mass and sedimentation rates, i.e., high (19S) and low (7S)

molecular weights. These same forms are today known as immunoglobulins (Ig) IgM and IgG, respectively (Eisen, 2001; Heidelberger and Pedersen, 1937).

As reviewed by Merchant and Packer (1956), the ability of antibodies to agglutinate and/or precipitate bacteria was recognized in 1889 and subsequently used to develop laboratory diagnostic assays for a variety of bacterial pathogens of humans and animals, e.g., *Salmonella* spp., *Brucella* spp., *Burkholderia mallei*, *Erysipelothrix rhusiopathiae*, and others. In time, portable kits were developed, such as the "rapid agglutination test for the diagnosis of swine erysipelas", described by Schoening and Creech in 1935.

Research on the development of antibody-based assays for viral pathogens was delayed by the requirement for reliable cell culture techniques (Shope, 1931), but in 1941, the serendipitous observation of red blood cell agglutination by allantoic fluid from chick embryos infected with influenza A virus led to the development of the hemagglutination inhibition assay (Hirst, 1941, 1942). At about the same time, Coons (1942) used fluorescein-labelled antibody and fluorescence microscopy to visualize pneumococcal antigen in tissue sections.

After World War II, antibody-based technology broadened to new formats and expanded beyond infectious diseases. For example, radioimmunoassay (RIA) was described in 1956 and was later applied to the quantitation of various targets, e.g., hormones, vitamins, enzymes and others (Berson et al., 1956; Yalow and Berson, 1960). Wide and Porath (1966) subsequently used the principles of RIA to develop the enzyme-linked immunosorbent assay (ELISA). New doors in research and diagnostics were opened when Kohler and Milstein (1975) found that monoclonal antibodies, i.e., antibodies intentionally created for a pre-defined purpose, could be produced by fusing cultured myeloma cells and spleen cells from an immunized mouse. The first therapeutic monoclonal antibody was approved by the U.S. FDA in 1986. OKT3, a murine monoclonal antibody, was able to neutralize mature T-lymphocyte by binding CD3 and was used to treat acute organ transplant rejection (Sgro, 1995). In the following 30 years, new techniques for creating monoclonal antibodies were developed, concurrently with the number of FDA-approved

therapeutic monoclonal antibodies, particularly in the areas of oncologic and autoimmune diseases (An, 2010; Clementi et al., 2012; Liu, 2014).

Antibody structure and function

Marrack (1938) hypothesized a common antibody polypeptide structure on the basis of earlier work showing similar protein composition in IgG antibodies with different responses to antigens. Work by Smith et al. (1955) demonstrating an identical polypeptide sequence among rabbit immunoglobulins with different immunological specificities plus later improvements in technology, e.g., the automatic amino acids analyzer, ion-exchange chromatography, etc., confirmed this hypothesis (Moore et al., 1958). Workers in the 1960s established methods to fragment IgG, described the amino acid composition of antibody structures, and explored their functions (Nisonoff et al., 1960; Yamashita et al., 1968). Finally, in 1977, an understanding of the three-dimensional structure of the intact glycoproteic structure of the IgG molecule was achieved through X-ray crystallography (Padlan, 1994; Silverton et al., 1977).

As shown in Figure 1, the mammalian antibody is a Y-shaped glycoprotein (Butler et al., 2017; Padlan, 1994; Wang et al., 2007). The end of each arm of the "Y" (Fab or fragment antigen binding region) contains the hypervariable region (HV), which defines the specificity of the antibody's antigen binding properties (Padlan, 1994). The base of the "Y" (Fc or fragment crystallizable region) in involved in various functions: activation of complement and interactions with specific Fc receptors (FcRs) associated with phagocytosis, cell-mediated cytotoxicity, transplacental transport of antibody, and antibody catabolism, (Butler et al., 2017; Mimura et al., 2001; Ravetch and Kinet, 1991; Vidarsson et al., 2014). The Fab and Fc portions of the antibody structure are connected by a hinge that allows the molecule to twist and flex during interactions with antigens, FcRs, and complement (Cervenak et al., 2009; Mimura et al., 2001). The hinge region varies in length among isotypes and species, with increasing in length corresponding to an increasing number of amino acids and disulfide bridges (Padlan, 1994).

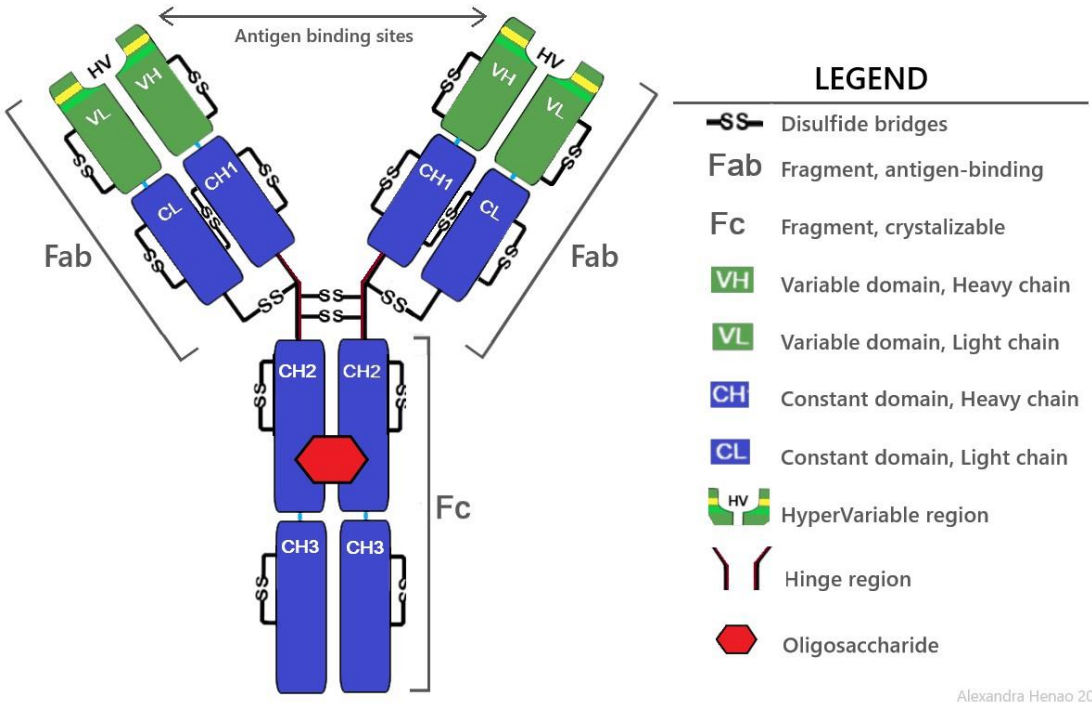

Figure 2.1. Primary or monomeric antibody (IgG) structure. The heavy chain is formed by VH+CH1+CH2+CH3 domains, the light chain is formed by VL+CL domains. All domains include intra-chain disulfide bridges. Inter-chain disulfide bridges connect the light with the heavy chains. The Fabs and Fc functional portions assemble the IgG structure through the hinge region and disulfide bridges.

Greater length results in higher molecular weight, but also a more susceptibility to degradation (Lipman, 2005; Wang et al., 2007).

Structurally, the antibody monomer is composed of four chains: two identical light chains, each comprised of two compact globular subdivisions or domains (VL and CL), and two identical heavy chains with four domains (VH and CH1, 2, 3) (Butler et al., 2009). Intra-chain and inter-chain disulfide bridges hold the molecule together and provide stability (Wang et al., 2007). Intra-chain disulfide bridges link two amino acids within the same domain and inter-chain disulfide bridges link two amino acids on different heavy or light chains (Liu et al., 2008). An oligosaccharide located between the heavy chains functions in modulating antibody interaction with complement molecules and FcRs and also reduces the susceptibility of the antibody to

degradation by proteases (Arnold et al., 2007; Mimura et al., 2001; Raju and Scallon, 2006; Wang et al., 2007).

This general antibody model holds for nearly all mammalian species; camelids (camels, llamas, alpacas, guanacos, etc.) are one exception. In these species, antibodies differ from the general model most notably by the fact that they are heavy-chain-only antibodies (HCAbs) with functional single-domain fragments (VHH or Nanobody®) (De los Rios et al., 2015; Harmsen and Haard, 2007). In mammalian species in which the general model applies, five isotypes are recognized (IgG, IgM, IgA, IgD, IgE). Isotypes are differentiated on the basis of the heavy chain constant regions characteristics: 1) non-cross-reacting antigenic determinants; 2) amino acid sequence; and 3) number and location of oligosaccharides (Kenneth, 2012; Shade and Robert, 2013). Antibodies can be either monomeric (IgG, IgE, IgD) or polymeric (IgM, IgA). In IgM and IgA, a 'tailpiece' in the heavy chain constant region connects multiple monomers via a polypeptide ("J chain") produced by the B cell. This connection results in the creation of new disulfide bridges that stabilize the polymer structure (Butler et al., 2017; Shade and Robert, 2013).

Antibody stability is the tendency of the molecule to maintain its structural conformation and functionality (Jacob et al., 2006). This discussion will focus on IgG because it is the most important immunoglobulin in diagnostic medicine (Chelius et al., 2005; Vidarsson et al., 2014). This reflects the fact that it is the most abundant immunoglobulin in serum, e.g., IgG constitutes ~88% of the total serum antibody in cattle and swine (Butler et al., 2017; El-Loly, 2007; Hurley and Theil, 2011). However, diagnostically-useful levels of IgG are also found in nearly every conceivable diagnostic specimen, e.g., colostrum, milk, oral fluids, urine, feces, and meat exudate ("meat juice") (Butler et al., 2017; El-Loly, 2007; German et al., 1998; Mortensen et al., 2001; Pricket et al., 2008). IgG is present in nearly every compartment and fluid because neonatal Fc receptors (FcRn) widely distributed in epithelial tissues selectively bind IgG and actively facilitate its bidirectional transport across tissue barriers (Roopenian and Akilesh, 2007; Ward et al., 2015; Yoshida et al., 2004).

IgG trends to be more stable *in vitro* than other proteins because of its globular rigid structure and multiple cross-linked bonds (Lumry and Eyring, 1954). However physicochemical processes can affect to its stability and functionality, as discussed in the following sections (Ishikawa et al., 2010b; Jacob et al., 2006; Lumry and Eyring, 1954). Specific limitations in this discussion should be recognized: 1) the majority of the information is based on studies of human antibodies, 2) the mechanisms involved are not always fully understood, and 3) the high degree of homology in the mammalian IgG primary structure suggests a similar pattern of susceptibility among antibodies within the isotype, but minor variation in structure or composition likewise infers some variability in stability (An, 2010; Butler et al., 2017; Esteves and Binaghi, 1972; Ishikawa et al., 2010b; Jacob et al., 2006; Vidarsson et al., 2014).

Physicochemical processes that affect IgG stability

Protein denaturation

At its simplest, four levels of protein structure are recognized: primary (amino acid sequences), secondary (alpha helices and beta sheet formation), tertiary (three-dimensional folded structure), and quaternary (assemblies of more than one amino acid chain). Denaturation of proteins is thus defined as changes in conformation (unfolding) that affect structure and/or functionality (Lumry and Eyring, 1954). Changes in the tertiary or quaternary structures may be reversible; changes in the primary or secondary structures are irreversible. Antibodies fulfill their biological activity only when they are correctly folded; unfolding leads to conformational instability, permanent denaturation, and changes in binding activity, including inter-antibody interactions (Lumry and Eyring, 1954; Rocco et al., 2008).

Protein aggregation

First described by Lumry and Eyring (1954), aggregation or "oligomerisation" is protein self-association (Jacob et al., 2006; Kiese et al., 2008; Rouet et al., 2014). Proteins may aggregate by physical association with one another and without any changes in primary structure (physical aggregation) or by formation of new covalent bond(s) (chemical aggregation). Physical

aggregation occurs after the tertiary structure unfolds sufficiently so as to expose hydrophobic zones and change the antibody polarity, thereby triggering aggregation (Fincke et al., 2014; Wang, 2005). Chemical aggregation results from the formation of disulfide bonds among antibodies or antibody fragments (Fincke et al., 2014).

Polymerization

Polymerization is the combination of ≥ 2 small and similar molecules (monomers) to form a new macromolecule (polymer) (Heck et al., 2013; Yamaguchi et al., 1998). Antibody polymerization is result of the formation of new covalent bonds between two or more similar protein structures (protein crosslinking), with IgM and IgA being examples of naturally-occurring polymers (Chapuis and Koshland, 1974; Heck et al., 2013). Polymerization can be produced by chemicals that generate covalent bonds between lysine amino acids in the heavy chains of the IgG structure. For example, glutaraldehyde, generates larger, insoluble IgG polymers under pH 7 or smaller soluble IgG polymers under pH 9 (Yamaguchi et al., 1998). In contrast with other antibody physicochemical processes, polymerization is generally not temperature-dependent (Yamaguchi et al., 1998).

Isomerization

Isomerization is the transformation of one molecule into another as a result of a rearrangement in the atoms (Wakankar et al., 2007). Isomerization occurs spontaneously under normal physiological temperatures (Geiger et al., 1987). Aspartate, an important amino acid component of antibodies, is highly susceptible to isomerization when it is followed by small amino acids, e.g., serine, alanine, cysteine or glycine, and/or when it is in a slightly acidic environment (Sydow et al., 2014; Wakankar et al., 2007; Yan et al., 2016). Antibody isomerization usually occurs in the Fab hypervariable region (HV) region, thereby increasing its flexibility and unpredictably affecting binding affinity (Yan et al., 2016). The conditions under which aspartate isomerization occurs are not well understood and possibly differ among subclasses of IgG (Sydow et al., 2014; Yan et al., 2016).

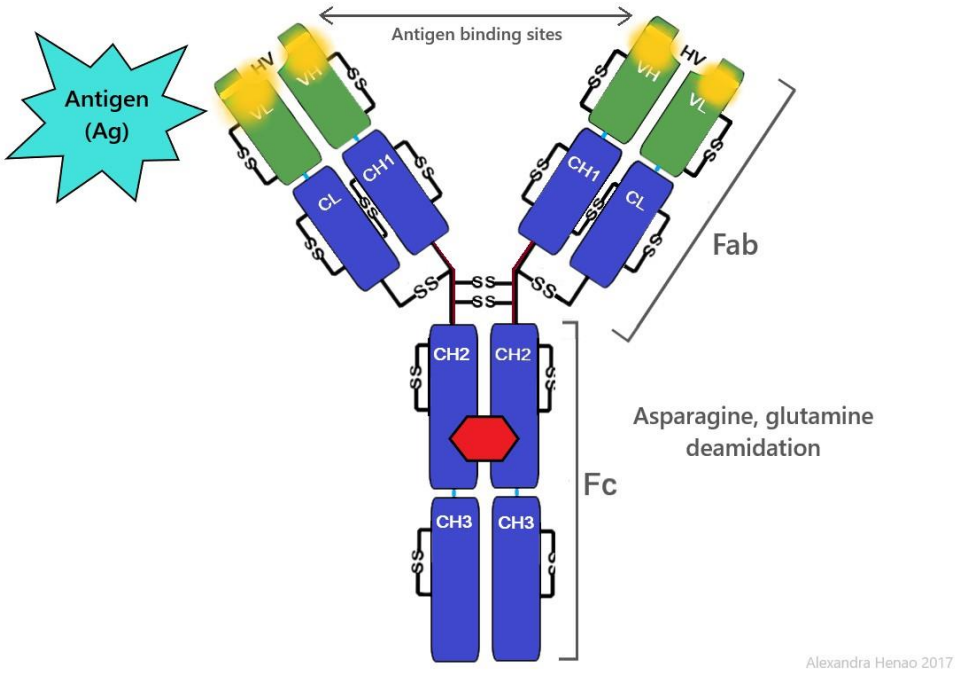

Figure 2.2. Deamidation of asparagine or glutamine (amino acids involved in antigen-antibody interactions) may affect antigen binding.

Deamidation

Deamidation is a spontaneous reaction in which an amino acid, either asparagine or glutamine, is changed or removed, thereby affecting the primary structure of the protein (Chelius et al., 2005; Haberger et al., 2014; Jacob et al., 2006). Deamidation had been studied, often in conjunction with isomerization, since the 1980's as a means of altering protein structure and function (Geiger et al., 1987). Since asparagine and glutamine are primarily located in the light chain (L) of the Fab and hypervariable (HV) regions, deamidation directly affects antibody biding affinity (Sydow et al., 2014) (Figure 2). Deamidation of IgG can occur under temperature and/or pH stress (Chelius et al., 2005; Haberger et al., 2014; Sydow et al., 2014). Susceptible regions of the molecule have been identified, but the mechanism(s) of deamidation are poorly understood (Sydow et al., 2014).

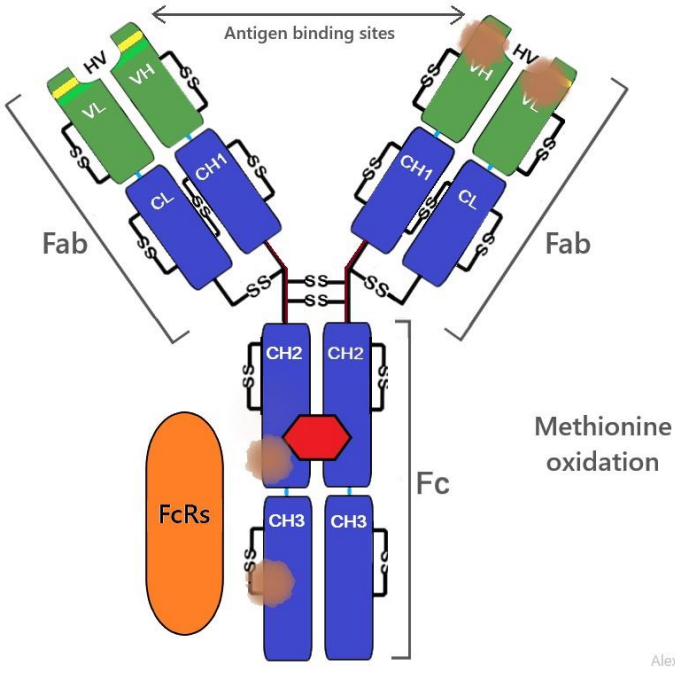

Figure 2.3. Methionine oxidation. Oxidation in heavy chain region may affect FcRs-IgG interactions. Oxidation in the hypervariable region may affect antigen-IgG interactions.

Oxidation

Oxidation is a chemical process in which a molecule loses electrons (Chumsae et al., 2007; Folzer et al., 2015). In antibody molecules, oxidation modifies interactions between amino acids and may disrupt peptide bonds, thereby affecting the primary structure of the protein (Lam et al., 2008; Lewis et al., 2017; Lumry and Eyring, 1954). Methionine, an important antibody constituent, is highly susceptible to oxidation (Folzer et al., 2015; Lam et al., 2008). Methionine is distributed throughout the IgG molecule, but oxidation of methionine in the Fc region affects the antibody's capacity to interact with FcRs and complement (Mo et al., 2016; Smith et al., 1954; Yamashita et al., 1968; Yan et al., 20160) (Figure 3). Others amino acids are likewise susceptible to oxidation, e.g., tryptophan, phenylalanine, tyrosine, cysteine, and histidine, but their interior location in the molecule restricts their exposure to oxidizers (Chumsae et al., 2007; Folzer et al., 2015; Jacob et al., 2006).

Enzymatic processes that affect IgG

Enzymatic degradation of hemoglobin by papain and pepsin was reported in the 1930's (Anson and Mirsky, 1932; Anson, 1937). Using the same approach, Porter (1950) demonstrated a reduction in antigen-antibody interactions after enzymatic antibody fragmentation. Papain and pepsin are non-specific proteases capable of fragmenting all antibody isotypes under physiological conditions (pH 7, 37°C). However, papain has higher activity on phenylalanine, lysine, and glycine, whereas pepsin has higher activity on phenylalanine, tryptophan, and tyrosine (Bennett et al., 1997). Papain preferentially cleaves IgG at the hinge region, dividing the antibody into an Fc portion and linked or separated Fab portions (Bennet et al., 1997). Pepsin cleaves the IgG molecule below the hinge region, dividing the antibody into a linked Fab portion and an Fc portion, which is often further fragmented (Bennet et al., 1997; Lipman et al., 2005) (Figure 4).

Enzymatic fragmentation at the hinge region is also used by bacteria to evade the phagocytosis, e.g., the family of *Streptococcus suis* Ig-degrading enzymes (IdeS) (Pawel-Rammingen et al., 2002; Seele et al., 2015; Spoerry et al., 2016). Some IdeS enzymes have highly specific proteolytic activity, e.g., the *S. suis* IgdE enzyme specifically cleaves the porcine IgG hinge region and the *S. suis* IdeSsuis enzyme exclusively cleaves porcine IgM, but not IgM from other species (Seele et al., 2013; Spoerry et al., 2016). IgG Fab and Fc fragments retain specific binding functions, but the separate components are unable to trigger a complete immunological response (agglutination, precipitation, opsonization, etc.) and do not perform adequately in diagnostic assays.

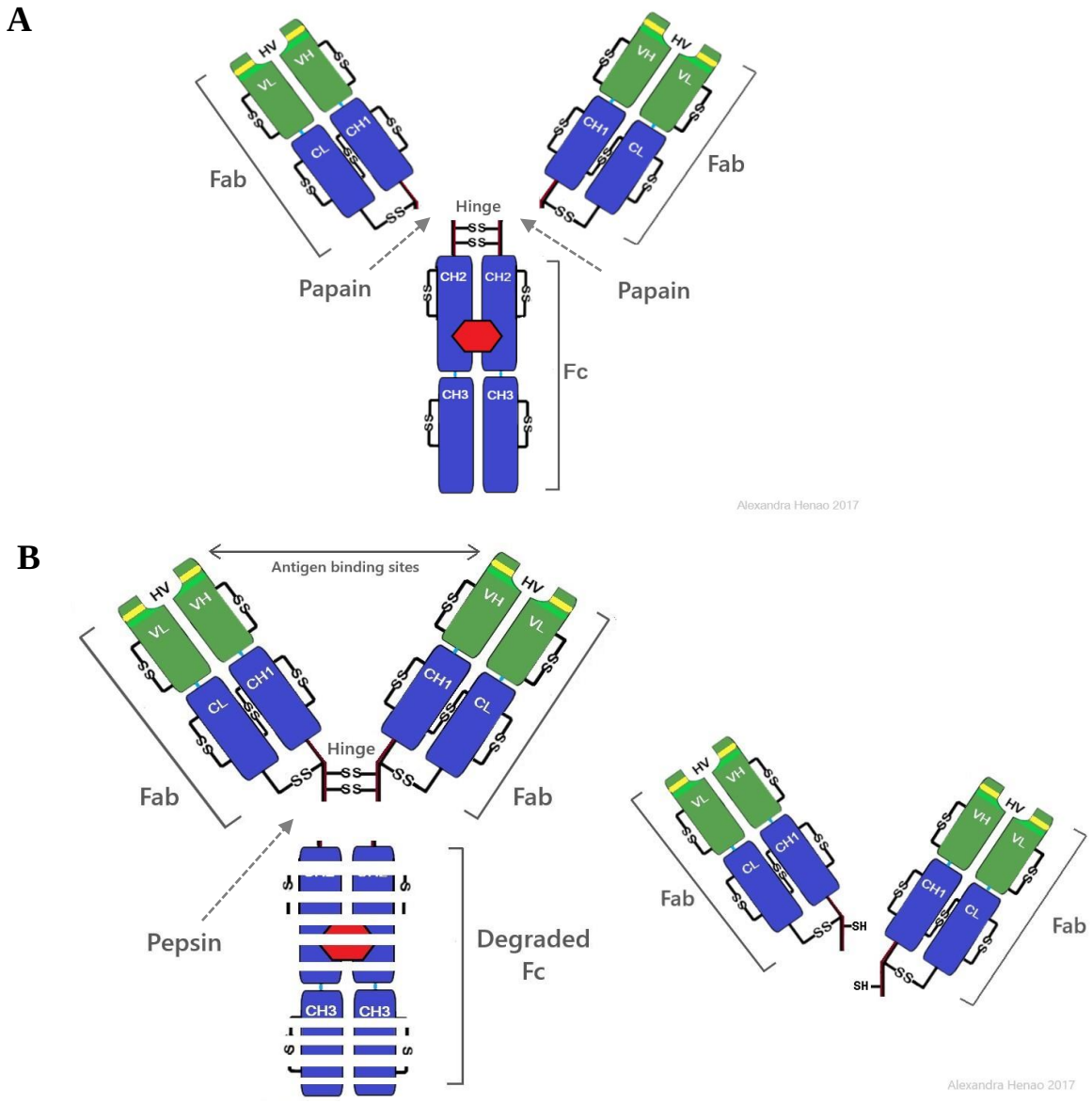

Figure 2.4. Antibody fragmentation by enzymatic fragmentation. Protease papain (A) cleaves the hinge region, dividing the IgG molecule into an Fc portion and linked or separated Fab portions. Protease pepsin (B) cleaves the IgG molecule below the hinge region, dividing the antibody into a linked Fab portion and an Fc portion, which is often further fragmented

Considerations for diagnostic specimens

The intent of this section is to discuss the physicochemical and enzymatic processes described above in the context of specimen handling and storage in the laboratory (Jacob et al., 2006; Wang et al., 2007).

The temperature-by-time-dependent denaturation of IgG is the single most important consideration in the handling and storage of diagnostic samples (Fincke et al., 2014; Jacob et al., 2006; Underwood et al., 1985; Wang et al., 2007). That said, a "unified formula" describing this relationship has not been described, perhaps because the reports in the peer-reviewed literature differ considerably in: 1) antibody evaluated, 2) specimen or matrix, 3) temperature and time evaluated, 4) assay(s) used to measure the effect, and 5) strength of the experimental design.

Regardless, it is recognized that sufficient temperature over time causes unfolding of the secondary and tertiary structures of IgG, i.e., non-reversible and reversible changes, respectively (Vermeer and Norde, 2000). Vermeer and Norde (2000) found that the combination of 20 minutes at 70°C was sufficient to completely denature IgG in phosphate buffered solution (PBS, pH 8.1). Heat-induced changes in protein structure can lead to antibody aggregation. Thus, Hawe el al. (2009) reported that 12% of IgG aggregated in PBS (pH 7.2) after 15 minutes at 77°C. Fincke et al. (2014) found that 29.5% of IgG aggregated in an IgG formulation (pH 7.5) after 72 hours at 60°C and 10% after 5 weeks at 40°C. These conditions reduced the concentration of monomeric IgG from 97.5% to 64.5% and from 100% to 90.3% respectively (t-test, $p < 0.01$). In addition to aggregation, thermal stress can also facilitate other physicochemical processes that affect IgG functionality (Jacob et al., 2006). Xanthe et al. (1997) reported the oxidation of IgG in a buffered solution containing sodium acetate, NaCl, and polysorbate (pH 5.0). Two weeks of exposure at 5°C resulted in specific oxidation of 10% of the methionine in the Fc region, 17% at 30°C, and 52% at 40°C. However, even in the worst case, this resulted in less than a 10% reduction in antibody binding activity (Xanthe et al., 1997). Deamidation of IgG can also be triggered by prolonged exposure (2 to 4 weeks) at 40°C (pH

6.0). This can result in specific deamidation in the HV regions and a decrease of 10% of IgG binding activity (Haberger et al., 2014; Sydow et al., 2014).

In contrast with the effect of thermal stress on antibody, the freeze-thaw cycle specimens commonly undergo in the laboratory setting has little effect on IgG (Hawe et al., 2009). Hawe et al. (2009) found that five freeze-thaw cycles (-80°C, 20-25°C) reduced the concentration of IgG in PBS (pH 7.2) by 0.15% (Hawe et al., 2009). However, one cycle consisting of snap freezing in liquid nitrogen followed by thawing at room temperature aggregated of 32.7% of bovine IgG in PBS (pH 7.2) (Sarciaux et al., 1999).

In addition to temperature and time effects, an additional consideration for the stability of IgG in diagnostic specimens is the presence of bacterial contaminants. For example, ELISA-detectable IgG declined after 2 days in swine oral fluids held at 30°C, but was extended to 12 days in samples held at 10°C, 14 days at 4°C, and months or more in samples stored -20°C/-80°C (Henao-Diaz et al., 2017; Pricket et al., 2010). Lower temperatures were associated both with increased IgG stability and with less bacterial proliferation in the specimen. A similar effect was observed in samples treated with antimicrobial and/or antiproteolytic agents. Thus, ELISA-detectable IgG was stable for up to one month at room temperature in human saliva collected with a commercial device containing antimicrobial and antiproteolytic compounds (Thwe et al., 1999). Likewise, Pricket (2010) observed similar trends in swine oral fluids treated with a bacteriostat (chlorhexidine digluconate, 0.01% by volume).

Conclusions

Structural differences in mammalian IgG subclasses can manifest themselves as variation in susceptibility to physicochemical and external factors that affect antibody stability and diagnostic functionality. Mammalian species share a common core structure in their IgG, thus general conclusions are broadly applicable to diagnostic laboratory testing. The stability of IgG in the diagnostic setting reflects the intrinsic physicochemical processes that affect IgG structural integrity and the characteristics of the specimen.

The first principle in conserving IgG diagnostic functionality is avoidance of thermal stress. In general, IgG is stable for less than 20 minutes at 70°C, 1 - 2 hours at 60°C, < 2 days at 20°C - 40°C, 1 - 2 weeks at 4°C, and indefinitely -20°C, -80°C. Snap freezing should be avoided because abrupt changes in temperature affect antibody integrity and increases aggregation. IgG is highly resistant to repeated "standard" freeze-thaw cycles, but care should nonetheless be taken. For example, splitting a sample into multiple aliquots is recommended as a means to avoid multiple freeze-thaw cycles. Human IgG is stable at pH 5.0 - 5.5, but pH stability varies among antibody subclasses. For this reason, and particularly when the matrix has been modified by the addition of a buffer, good quality control would include measuring and recording the pH on the sample label. Additional factors, e.g., exposure to light, mechanical stress (shaking, stirring), or hydrogen ion concentration (pH) can be hazardous for antibody in diagnostic specimens. However, these factors typically represent minor threats to IgG stability under typical laboratory scenarios (Kiese et al., 2008; Lumry and Eyring, 1954; Prickett et al., 2010).

The majority of peer-reviewed research has focused on the stability of IgG in serum samples. However, new specimen types are increasingly used in the veterinary diagnostic setting, e.g., mammary secretions, fecal specimens, and oral fluids. These specimens may require additional manipulation, e.g., Pricket et al. (2010), showed that the addition of a bacteriostatic improved the stability of antibody in swine oral fluid over time. Identifying and understanding the processes that affect IgG functionality and stability in these alternative diagnostic specimens will be an important process in assuring diagnostic accuracy.

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CHAPTER 3. EFFECT OF CHEMICAL CLARIFICATION OF ORAL FLUIDS ON THE DETECTION OF PRRSV IgG

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Abstract

Routine collection and testing of oral fluid (OF) samples facilitates PRRSV surveillance in commercial swine herds in a cost-effective, “welfare-friendly” fashion. However, OF often contain feed, feces, and environmental contaminants that may affect liquid handling and test performance in the laboratory. Traditional sample processing methods, e.g., filtration or centrifugation, are not compatible with high-throughput laboratories because of the burden of additional processing costs and time. OF "clarification" using chemical coagulants is an alternative approach not widely explored. Therefore, the objective of this study was to evaluate the effect of chitosan-based clarification treatment on a commercial PRRSV OF ELISA and the temporal stability of ELISA-detectable antibody in samples held at 4°C.

Serum and OF samples of known status were generated by vaccinating pigs (n = 17) with a PRRSV MLV vaccine. Individual pig OF samples were collected from day post vaccination -7 to 42 and subdivided into 4 aliquots. Each aliquot was clarified using one treatment (A, B, C) with the 4th aliquot serving as untreated control. All samples were tested by PRRSV OF ELISA immediately after treatment (day post-treatment DPT 0) and then were held at 4°C to be re-tested

on DPTs 2, 4, 6, and 14. Both immediate and temporal treatment effects were evaluated for their quantitative and qualitative effects on PRRS OF ELISA performance.

A repeated measures multiple comparison analysis with Tukey adjustment found no significant difference in ELISA S/P responses among treatments by DPT. Among all DPTs, no difference was detected in the proportion of positive PRRSV antibody samples among treatments (Cochran's Q, $p > 0.05$).

Treatment of swine oral fluids using chitosan-based formulations did not affect the performance of a commercial PRRSV OF ELISA either immediately after treatment or up to 14 days after treatment. Chitosan (or other coagulants) could improve the OF characteristics and could be adapted for use in the field or in high-throughput laboratories.

Keywords: PRRSV, oral fluid, ELISA, chitosan, clarification, antibody stability.

Introduction

Since its recognition in 1991 (Terpstra et al., 1991), porcine reproductive and respiratory syndrome virus (PRRSV) has continually challenged pork producers and swine veterinarians. Holtkamp et al. (2013) estimated the cost of PRRSV to U.S. producers at more than $660 million per year. De Pax et al. (2015) using prevalence estimates from 11 countries, extrapolated the costs of PRRSV to European producers at €1.5 billion per year. Likewise, in Asia, PRRSV imposes heavy economic consequences due to clinical and subclinical infections plus the cost of prevention and control measures (McOrist et al., 2011; Zhang and Kono, 2012).

The only two options for the control of PRRSV are (1) elimination of the virus from the herd or (2) reduction of clinical signs through enhancement and stabilization of herd immunity via vaccination or intentional exposure (Linhares et al., 2014). In both cases, implementation of biosecurity protocols sufficient to stop the introduction of extraneous viruses is mandatory.

Regardless of the control plan, routine sampling and testing is necessary to verify that the chosen strategy is functioning - because clinical signs are not a reliable or timely indicator of PRRSV infection (Duinhof et al., 2011; Frössling et al., 2009). Depending on the circumstances, the survey objective will be either surveillance (detection of PRRSV) or monitoring (tracking the circulation of the virus in an endemically infected population) (Paskin, 1999).

Routine surveillance or monitoring using serum samples is not practical because of the time and cost associated with collecting a statistically sufficient number of samples on a continuous basis. In contrast, oral fluid-based testing offers the possibility of collecting population infectious disease data easily, quickly, inexpensively, and in a “welfare-friendly” fashion (Ramirez et al., 2012; White et al., 2014). Both PRRSV nucleic acid- and antibody-based assays have been adapted to oral fluid specimens (Kittawornrat et al., 2012; Prickett and Zimmerman, 2010) and studies have shown field studies based on oral fluid specimens equal or exceed PRRSV detection based on serum (Biernacka et al., 2016; Ramirez et al., 2012).

While offering practical advantages for routine PRRSV detection, oral fluid samples commonly contain insoluble particles from the environment, e.g., feed, feces, and inorganic material. These contaminants have not been shown to directly affect test performance, but in the laboratory, these contaminants may affect liquid handling characteristics, e.g., the precision of pipetting. The only options for removing particulates from oral fluids are prolonged, high-speed centrifugation or filtration, but neither is practical in a high throughput laboratory. A third option is "clarification", i.e., the removal of particles suspended in a solution, using chemical coagulants. Coagulants function by destabilizing the charge on the surface of the particles, thereby allowing them to flocculate (aggregate) into larger elements that can be more easily removed.

Among the many options, chitosan (deacetylate chitin) is an abundant, biodegradable, biocompatible, and non-toxic coagulant that has been used in a variety of biological applications (Hirano, 1996), e.g., production of foods (Del Nobile et al., 2009) and beverages (Domingues, 2012; Gassara et al., 2015; Mierczynska-Vasilev, 2015), improved drug delivery systems (Park, 2010), adjuvantation of vaccines (Li et al., 2015; Wen, 2011), and clarification of cell culture

media (Riske et al., 2007). Previously, Poonsuk et al. (2017) showed that chitosan could be used to clarify swine oral fluid specimens and that treatment did not affect porcine epidemic diarrhea virus IgA or IgG ELISA test results. Therefore, the aim of the current study was to evaluate the effect of chitosan-based clarification of oral fluids on the performance of a commercial PRRSV antibody IgG ELISA (IDEXX PRRS OF Ab Test™, IDEXX laboratories, Westbrook, ME, USA).

Methods

Experimental design

This study was conducted with the approval of the Iowa State University Office for Responsible Research. In brief, oral fluid and serum samples of known PRRSV antibody status were generated by vaccinating (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, Duluth, GA, USA) pigs under experimental conditions and then collecting samples over a period of 50 days. Following each collection, oral fluid samples were subdivided into 4 aliquots, each of which was subjected to one of four treatments (Control, A, B, C) and then tested for PRRSV antibody (day post treatment, DPT 0). Thereafter, the treated oral fluid specimens were stored at 4°C and re-tested on DPT 2, 4, 6, and 14. At the end of the study, the oral fluid PRRS ELISA sample-to-positive (S/P) results were analyzed for the effect of treatment, storage time, and storage time-by-treatment interactions.

Animal care

PRRSV-negative pigs (n = 17) were acquired from a commercial swine farm at 14 weeks of age (~40 to 50 kg) and housed in biosafety level 2 (BSL-2) research facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Negative PRRSV status was verified by ELISA testing of serum samples collected 14 and 7 days prior to the arrival of the animals. In addition, the final set of serum samples was pooled (≤ 5 samples per pool) and tested by PRRSV qRT-PCR to verify the absence of acute PRRSV infection.

Upon arrival (13 days prior to vaccination), pigs were randomly assigned ID numbers by blindly pulling ear tags from a bag and then assigned to individual pens consecutively by ear tag number. Pens (1.5 m × 1.8 m) were constructed of solid partitions and gates. All pens had gates on at least two sides to allow interaction between animals in neighboring pens. Each pen was equipped with a nipple drinker and a bracket to hold a rope during oral fluid collection. Pigs were fed a commercial swine diet (Heartland CO-OP, Prairie city, IA, USA) twice per day. Animals were closely observed throughout the study by researchers, animal caretakers, and institutional veterinary staff. Five animals were observed through day post vaccination (DPV) 28 and then removed due to facility space limitations; the remainder (n = 12) were observed through DPV 42.

Vaccination

All animals were vaccinated intramuscularly on DPV 0 with 2 ml of a PRRSV modified-live vaccine (Ingelvac® PRRS MLV) using a single-use syringe and needle (PrecisionGlide™, Becton Dickson, Franklin Lakes, NJ, USA),

Serum and oral fluid sample collection

Pigs were allowed to acclimate for 5 days before initiation of sampling. Oral fluid and serum samples were collected between DPV -7 to DPV 42. The number of oral fluid and serum samples collected and evaluated in this experiment is given in Table 2 by DPV.

Serum samples (n = 187) were collected using a single-use vacutainer system (Corvac™ Integrated Serum Separator Tube, 12.5 ml, Covidien, Minneapolis, MN, USA). No pig was bled on consecutive days; rather, subsets of pigs were rotated through the sampling schedule so as to obtain serum samples for DPV 3, 6, 7, 8, 9, and 10 (Table 2). Blood samples were centrifuged (1,500 × g for 15 min) and then serum was aliquoted into 2 ml tubes (Cryos™, Greiner Bio-One, Monroe, NC, USA) and stored at -80ºC.

Oral fluid samples (n = 221) were collected with three-strand twisted 100% cotton rope (Web Rigging Supply, Lake Barrington, IL, USA) hung from a metal bracket fixed to one side of the pen. Brackets were placed such that samples could be collected without entering the pen. During the acclimation period, pigs were given access to the rope for two 30 minute periods daily. The sampling procedure has been described in detail (González et al., 2017; White et al., 2014). In brief, pigs were allowed to interact with the rope for 30 min, then the wet the end of the rope was cut, inserted into a plastic bag (Seal-Top Bag, Elkay Plastics, Commerce, CA, USA), and passed through a towel wringer (WC38K, Dyna-Jet products, Overland park, KS, USA). The oral fluid that accumulated in the bag was then decanted into a 50 ml polypropylene centrifuge tube (Falcon™, Fisher Scientific, Pittsburgh, PA, USA). To maximize the volume collected, oral fluid samples were collected twice daily (8:00 am and 4:00 pm). Oral fluid samples collected in the morning were placed on ice, pooled with the afternoon sample, and then the composite sample aliquoted into 5ml tubes (Fisher Scientific, Pittsburgh, USA) and stored at -80ºC.

PRRSV serum qRT-PCR

Viral RNA was extracted from 140 µl of serum and eluted to 90 µl of elution buffer using QIAmp® viral RNA mini kit (QIAGEN® GmbH, Hilden, Germany) following the instructions provided by the manufacturer. The eluted RNA, primers, and probe were mixed with commercial reagents (EZ-PRRSV™ MPX 4.0 real time RT-PCR, Tetracore®, Rockville, MD, USA). NA PRRSV, EU PRRSV, and internal controls were included in every reaction. The qRT-PCR reactions were done using the T-COR 8™ thermocycler (Tetracore®): 48°C for 15 min, 95°C for 2 min, 95ºC for 5 s (45 cycles), and 60ºC for 40 s. The results were analyzed using an automatic baseline selected by the T-COR 8™ software. Quantification cycle (Cq) values ≤ 40 were considered positive for PRRSV.

PRRSV serum ELISA

Serum samples were tested for PRRSV antibody using a commercial ELISA (IDEXX PRRS X3 Ab Test™, IDEXX Laboratories Inc.) performed as instructed by the manufacturer. In brief, 5

µl of serum was diluted 1:40 with sample diluent in 96-well polystyrene plates (Nunc™ A/S, Roskilde, Denmark) and then 100 µl of the mixture was transferred to an ELISA plate followed by 30 min incubation (19 - 22°C). Plates were then washed 5 times with 300 µl of 1X wash solution, 100 µl of conjugate was added to each well, and the plates were incubated for another 30 min. The washing cycle was repeated, then 100 µl of tetramethylbenzidine-hydrogen peroxide substrate (TMB) was added to each well and the plates incubated for 15 min to visualize the reaction. Thereafter, 100 µl of stop solution was added to each well and the plate was read (650 nm) using an ELISA plate reader (EMax® Plus microplate reader, Molecular devices, Sunnyvale, CA, USA) operated with the SoftMax® Pro 7 software (Molecular devices). The antibody responses in serum samples were calculated as sample-to-positive (S/P) ratios using equation 1. Serum samples with S/P ratios ≥ 0.40 were considered PRRSV serum antibody positive.

Equation 1:

$$S/P\ ratio = \frac{(sample\ OD - negative\ control\ mean\ OD)}{(positive\ control\ mean\ OD - negative\ control\ mean\ OD)}$$

PRRSV oral fluid ELISA

Oral fluid samples were tested for PRRSV antibody using a commercial ELISA (IDEXX PRRS OF Ab Test™, IDEXX Laboratories Inc.) performed as instructed by the manufacturer. In brief, 100 µl of oral fluid was diluted 1:1 with sample diluent in 96-well polystyrene plates (Nunc™) and then 100 µl of mixture was transferred to the ELISA plate. After 2 h incubation (19 - 22°C), plates were washed 5 times using 350 µl of 1X wash solution, then 100 µl of conjugate was added to each well and the plates incubated for 30 min. The wash cycle was repeated and 100 µl of tetramethylbenzidine-hydrogen peroxide substrate (TMB) were added to each well and the plates incubated for 15 min to visualize the reaction. Thereafter, 100 µl of stop solution was added to stop the reaction and the plates read (450 nm) using an ELISA plate reader (Biotek® Instruments Inc., Winooski, VT, USA) operated with the IDEXX xChek® software (IDEXX

Laboratories Inc). Antibody responses in oral fluid samples were calculated as sample-to-positive (S/P) ratios using Equation 1. Oral fluid samples with S/P ratios ≥ 0.40 were considered positive.

Oral fluid clarification treatments

Three chemical clarification treatments (A, B, C) were prepared, as described elsewhere (Poonsuk et al., 2017). The components and quantities used to prepare 100 ml of each treatment are listed in Table 1. Following preparation, 1 ml was pipetted into 5 ml round-bottom polystyrene tubes (Falcon™), held at -80ºC for 24 h, and then lyophilized (FreeZoneTM, Labconco®, Kansas City, MO, USA) for 15 h. After lyophilization, tubes were closed with polyethylene caps (Falcon™), vacuum sealed in plastic bags, and stored at room temperature (19 - 22°C) until use.

Immediate effect of treatment On day post treatment (DPT) 0, oral fluid samples (n = 221; > 4 ml) were thawed at room temperature (19 - 22°C) and vortexed for 5 sec. Oral fluid (1 ml) was then added to each of the three clarification treatments tubes (A, B, and C) and to an empty 5 ml round-bottom polystyrene tube (negative control, NC). Samples were vortexed to resuspend the chemical components (5 sec), centrifuged at 1200×g for 3 min at 4ºC, randomly ordered (www.random.org), and tested for PRRSV antibody (IDEXX Laboratories Inc).

Temporal effect of treatment After testing on DPT 0, all samples (n = 884; A, B, C, NC) were held at 4ºC in an environmental chamber (Caron®, Marietta, OH, USA) and re-tested on DPT 2, 4, and 6. A subset (n = 352), i.e., oral fluid samples collected on DPV -7, 6, 7, 8, 9, 10, 14 and 42, was held and tested on DPT 14. Oral fluid samples were neither vortexed nor centrifuged prior to testing on DPT 2, 4, 6, and 14.

Data analysis

The effect of clarification treatments on the PRRSV oral fluid ELISA S/P ratios and antibody status (positive or negative) was analyzed using statistical software (MedCalc® 17.6, MedCalc Software bvba, Ostend, Belgium; SAS® 9.4, SAS® Institute Inc., Cary NC, USA). Initially, the effect of oral fluid treatment was analyzed separately for oral fluid samples collected prior to the expected appearance of detectable antibody (≤ DPV 7, "early samples") and oral fluids collected after the expected appearance of antibody (≥ DPV 14, "late samples"). The assumption of normality for the "early" and "late" datasets was rejected (Shapiro-Wilk test) and transformation of the data did not achieve normality. Therefore, a nonparametric approach (Kruskal-Wallis test) was used to evaluate the effect of treatment on S/P values for each dataset within each DPT (0, 2, 4, 6, and 14). Thereafter, the complete dataset was analyzed for the effect of treatment (NC, A, B, C) and storage time (DPT 0, 2, 4, 6, 14) on S/P ratios using a repeated measure multiple comparison test with Tukey adjustment (Proc GLIMMIX). Cochran's Q test was used to evaluate differences in the proportion of antibody positive results (S/P ≥ 0.40) among treatments within DPTs and within treatments among DPTs.

Results

Pigs were determined to be naïve for PRRSV infection at the time of arrival on the basis of negative ELISA and qRT-PCR results on serum samples collected prior to arrival. In addition, all serum and oral fluid samples collected prior to DPV 7 were ELISA negative. No clinical signs or adverse health events were observed over the course of the observation period.

A total of 187 serum samples were collected over the course of the study and tested by PRRS ELISA. The number of samples collected vis-à-vis the number of ELISA positives (S/P ≥ 0.4) is given in Table 2 by DPV. Serum antibody ontogeny (ELISA S/P response) is shown in Figure 1.

A total of 221 oral fluid samples of volume sufficient to make 4 aliquots (each of 1 ml) were collected over the course of the experiment. The lyophilized treatments (A, B, C) were readily

rehydrated by adding 1 ml of oral fluid and vortexing briefly. Untreated samples were readily differentiated from treated samples by the light blue color conferred by the xylene cyanol present in formulations A, B, and C.

PRRSV OF ELISA test results for all oral fluid samples on DPT 0 are given in Figure 1 and Table 2. Among DPTs 0, 2, 4, 6, and 14, no difference was detected in the proportion of positive PRRSV antibody samples among treatments (Cochran's Q test, $p > 0.05$).

The effect of oral fluid treatment on ELISA S/P responses was analyzed separately for oral fluid samples collected prior to the expected appearance of detectable antibody (≤ DPV 7, "early samples") and oral fluids collected after the expected appearance of antibody (≥ DPV 14, "late samples") for DPTs 0, 2, 4, 6, and 14 (Table 3). At DPT 0, 2, 4, and 6 a statistically significant difference in the "early samples" S/P response was detected between treatments A or C versus NC or B (Kruskal-Wallis test, $p < 0.0001$). For “late samples”, no differences were detected between treatments (NC, A, B, C).

An analysis of the complete dataset using a repeated measures multiple comparison test with Tukey adjustment found no significant difference in ELISA response between treatments (NC, A, B, C) at each storage time (DPT 0, 2, 4, 6, 14). Likewise, no interaction was detected between treatment and storage time on ELISA S/P ratios. Analysis within treatments showed differences ($p < 0.05$) in S/P responses among DPTs, but the direction of change was inconsistent and not compatible with antibody degradation or inactivation

Discussion

The diagnostic use of swine oral fluid specimens began with the isolation of PRRSV from buccal swabs (Wills et al., 1997). Thereafter, Prickett et al. (2008a) showed that ELISA-detectable PRRSV antibodies appeared concurrently in serum and oral fluid matrices and explored the use of pen-based oral fluid samples under experimental conditions. Pepin et al. (2015) reported the appearance of ELISA-detectable antibody in oral fluids collected in boars 7 days after

administration of a modified-live vaccine. In the field, Kuiek et al. (2015) found a strong temporal association in the ontogeny of oral fluid and serum antibody, while Kittawornrat et al. (2012, 2013) showed the concurrent appearance of serum and oral fluid PRRSV antibody isotypes (IgM, IgG, IgA) under both experimental and field conditions. Overall, the antibody results of this study were compatible with earlier reports. In particular, the design of the study, i.e., 17 individually-penned pigs sampled over a period of 50 days, allowed for a precise estimate of the earliest appearance of ELISA-detectable antibody because a three-day bleeding rotation schedule provided paired serum samples and oral fluid specimens every day during the expected period of seroconversion (DPV 3 to 11).

The current research differed from previous studies with its focus on the oral fluid specimen itself. As reviewed by Faust and Osman (1998), "clarification" of liquids, i.e., the removal of suspended particulates, has a long historical thread. The ancient Minoans (Angelakis and Zheng, 2015), Egyptians, and early Romans clarified drinking water by sedimentation and/or treatment with crushed almonds or clay containing mineral salts. By 1757, aluminum sulfate (alum) was known to remove particulates from water and, in 1881, this approach was used to treat the municipal water supply of the town of Balton in North West England. The coagulant properties of ferrous compounds were also recognized during this period and, in 1884, a perchloride of iron water coagulation system was implemented by the New Orleans Water Company. Prescient of the future scientific foundation of water treatment, the first published scientific study on chemical clarification (1885) reported that the administration of alum at a rate of ~34 ppm was optimum for the treatment of drinking water (Faust and Osman, 1998).

Drinking water was not the only liquid in need of clarification. As society grappled with the problem of sanitation in the later part of the 19th century, specific inorganic chemicals, including alum, were shown to clarify sewage and facilitate the treatment process (Melosi, 2008). At the opposite end of the spectrum, clarification ("fining") of beer and wine was done by brew masters and vintners to reduce turbidity. As reviewed by Baldwin (1824) and Stuntz (1886), fining can be achieved with inorganic coagulants (bentonite, powdered marble, gypsum), organic coagulants (Isinglass, albumen, gelatin, caseins), or a combination of inorganic and organic

coagulants, e.g., silica with gelatin, with isinglass, or chitosan (Mierczynska-Vasilev and Smith, 2015).

Chitosan is a cationic polymer derived by high alkaline partial deacetylation from chitin, the biopolysaccharide structural component in the exoskeletons of arthropods (Rinaudo, 2006). Chitosan's chemical properties vary depending on the degree of deacetylation, polymer length, and product purity but it is generically considered nontoxic, biocompatible, biodegradable, and amenable to a wide variety of clarification applications (Alves and Mano, 2008; Rinaudo, 2006). For example, chitosan was used at a concentration of 3000 ppm to treat textile wastewater (Mohd et al., 2009), 300 ppm to clarify fruit juice (Domingues et al., 2012), 200 ppm to clarify cell culture mediums for antibody recovery (Riske et al., 2007), 5 ppm to clarify beer (Gassara et al., 2015), and as low as 0.6 ppm when combined with alum for water treatment (Zeng, et al 2008). The objective of this study was to evaluate the effect of chitosan-based clarification of oral fluids on the performance of a commercial PRRSV ELISA, i.e., would chitosan reduce antibody levels through coagulation and/or precipitation, or interfere with the antibody-binding functions required for detection by ELISA?

As described in Table 1, a blue dye (xylene cyanol) was added to all formulations (A, B, C) to allow for convenient visual differentiation of treated vs. untreated samples. BSA (0.5%) was included in all treatments (NC, A, B, C) to block non-specific binding (Pruslin et al., 1991; Steinitz, 2000). Chitosan was used at 100 ppm (0.01%) in formulations A and B, with treatments NC and C used for comparisons. Tween® 20 (1.0 %) was added to formulations A and C to further reduce non-specific binding, with treatments NC and B used for comparisons.

The overall conclusion that may be drawn from an analysis of the complete dataset is that neither treatment nor time significantly affected the ELISA quantitative (S/P) or qualitative (positive/negative) responses. As shown in Table 3, statistically significant differences in mean S/Ps were observed among treatments in "early samples" (samples collected $\leq$ 7 DPV), but these small differences in S/P values had no diagnostic impact (Table 2) and no such effect was observed among treatments in "late samples" (samples collected $\geq$ 14 DPV). Further, the

analysis of the residual effects of treatment, i.e., samples held at 4°C and tested at DPT 2, 4, 6, and 14 found no significant change in ELISA S/P values by treatment or time. Although there are no prior reports of testing after storage at 4ºC after 14 days, the results were compatible with previous reports examining antibody stability in oral fluids (Morris et al., 2002; Prickett et al., 2010; Poonsuk et al., 2017).

This research represents initial attempts at improving the characteristics of oral fluid specimens. Providing a cleaner oral fluid sample may lead to the wider acceptance by users and improve the sample-handling characteristics in high-throughput laboratories. Within the constraints of this study, formulation B would be the preferred method for clarification of swine oral fluids for ELISA because it required the fewest components. As noted in Table 1, the cost of formula B components sufficient for treating one oral fluid sample (1 ml) was €0.026 or USD $0.032. Continuing this line of research, future efforts should include the evaluation of other coagulants and/or combinations of coagulants, e.g., chitosan in combination with others. Likewise, it will be necessary to evaluate the compatibility of oral fluid clarification to other pathogens, antibody assays, and nucleic acid detection technologies.

Conclusions

Clarification of swine oral fluids using chitosan-based formulations was effective, inexpensive, and did not affect the performance of a commercial PRRSV oral fluid antibody ELISA (IDEXX PRRS OF Ab Test™) either immediately after treatment or up to 14 days after treatment. The use of coagulants to clarify oral fluids could improve and standardize oral fluid sample-handling characteristics. This line of research holds the potential to move oral fluid-based surveillance to the next level by providing a sample well-suited to testing in high-throughput laboratories.

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Table 3.1. Treatments evaluated for their effect on swine oral fluid PRRSV antibody detection

Component	Treatment formulations			
	Control	**A**	**B**	**C**
1X Phosphate buffer saline	-	97.9 ml	98.9 ml	98.9 ml
Bovine serum albumin[1]	-	0.5 g	0.5 g	0.5 g
Stock solution 0.1% xylene cyanol[2]	-	100.0 µl	100.0 µl	100.0 µl
Stock solution 1.0% chitosan[3]	-	1.0 ml	1.0 ml	-
Tween® 20[4]	-	1.0 ml	-	1.0 ml
Total components cost per sample	€ 0.0	€ 0.027	€ 0.026	€ 0.027
	$ 0.0	$ 0.033	$ 0.032	$ 0.033

[1] BSA (001-000-162, Jackson Immunoresearch, West grove, PA, USA)
[2] Xylene cyanol FF (X4126, Sigma-Aldrich, St. Louis, MO, USA)
[3] Chitosan oligosaccharide lactate (523682, Sigma-Aldrich)
[4] Polyethylene glycol sorbitan monolaurate (P1379, Sigma-Aldrich)

Table 3.2. Number of samples by day post-vaccination and qualitative PRRSV ELISA results

Specimen	Day post vaccination[1]														
	-7	-5	-3	0	3	6	7	8	9	10	14	21	28	35	42
Serum (no. samples)	17	-	-	17	4	9	4	9	4	9	17	17	17	12	12
PRRSV ELISA[2] (no. positive)	0	-	-	0	0	0	1	0	2	5	17	17	17	12	12
Oral fluid (no. samples)	16	16	17	15	15	13	16	16	15	15	16	15	12	12	12
PRRSV ELISA[3] (no. positive)[4]	0	0	0	0	0	0	0	3	8	14	16	15	12	12	12

[1] Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica.
[2] IDEXX PRRS X3 Ab Test, IDEXX laboratories Inc.
[3] IDEXX PRRS OF Ab Test, IDEXX laboratories Inc.
[4] Qualitative results on DPT 0 were identical among all treatments (NC, A, B, C) with the exception that one DPV 8 oral fluid sample (reported as positive above) was negative for treatments B and C.

Table 3.3. Quantitative effect of treatment on PRRSV oral fluid ELISA[1] sample-to-positive (S/P) ratios by day post-treatment (DPT)

	DPT	n	Negative Control S/P mean (95% CI)	Treatment A S/P mean (95% CI)	Treatment B S/P mean (95% CI)	Treatment C S/P mean (95% CI)
"Early" samples, i.e.,	0	108	0.06[a] (0.05, 0.08)	0.02[b] (0.01, 0.04)	0.05[a] (0.04, 0.07)	0.03[b] (0.01, 0.05)
samples collected ≤ 7	2	108	0.05[a] (0.03, 0.06)	0.00[b] (-0.01, 0.01)	0.04[a] (0.02, 0.05)	0.00[b] (-0.01, 0.01)
days post vaccination[2]	4	108	0.06[a] (0.05, 0.08)	0.01[b] (-0.01, 0.02)	0.09[a] (-0.01, 0.18)	0.01[b] (-0.01, 0.02)
	6	108	0.04[a] (0.03, 0.06)	0.00[b] (-0.02, 0.01)	0.02[a] (0.01, 0.04)	0.00[b] (-0.02, 0.02)
	14	32	0.04 (0.02, 0.06)	0.04 (0.01, 0.08)	0.02 (-0.01, 0.04)	0.03 (0.00, 0.06)
"Late" samples, i.e.,	0	67	7.21 (6.57, 7.86)	7.23 (6.58, 7.87)	7.16 (6.52, 7.79)	7.22 (6.57, 7.87)
samples collected ≥ 14	2	67	7.58 (6.93, 8.24)	7.59 (6.94, 8.24)	7.70 (7.03, 8.37)	7.61 (6.93, 8.29)
days post vaccination[2]	4	67	7.93 (7.17, 8.70)	7.91 (7.15, 8.67)	8.07 (7.32, 8.82)	8.08 (7.31, 8.85)
	6	67	7.54 (6.81, 8.27)	7.66 (6.93, 8.39)	7.74 (7.02, 8.46)	7.64 (6.91, 8.38)
	14	20	6.74 (5.30, 8.18)	7.16 (5.81, 8.51)	7.55 (6.21, 8.89)	7.07 (5.76, 8.38)

[1] IDEXX PRRS OF Ab Test, IDEXX laboratories.

[2] Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica.

[a,b] Treatment effect by DPT 0, 2, 4, 6, 14 was analyzed separately for oral fluid samples collected prior and after to the expected appearance of detectable antibody (Kruskal-Wallis test). Within DPT, differences ($p < 0.05$) between treatments are indicated by superscripted letters. Subsequent analysis of the complete dataset detected no significant interaction between storage time (DPT) and treatment (repeated measure multiple comparison test with Tukey adjustment).

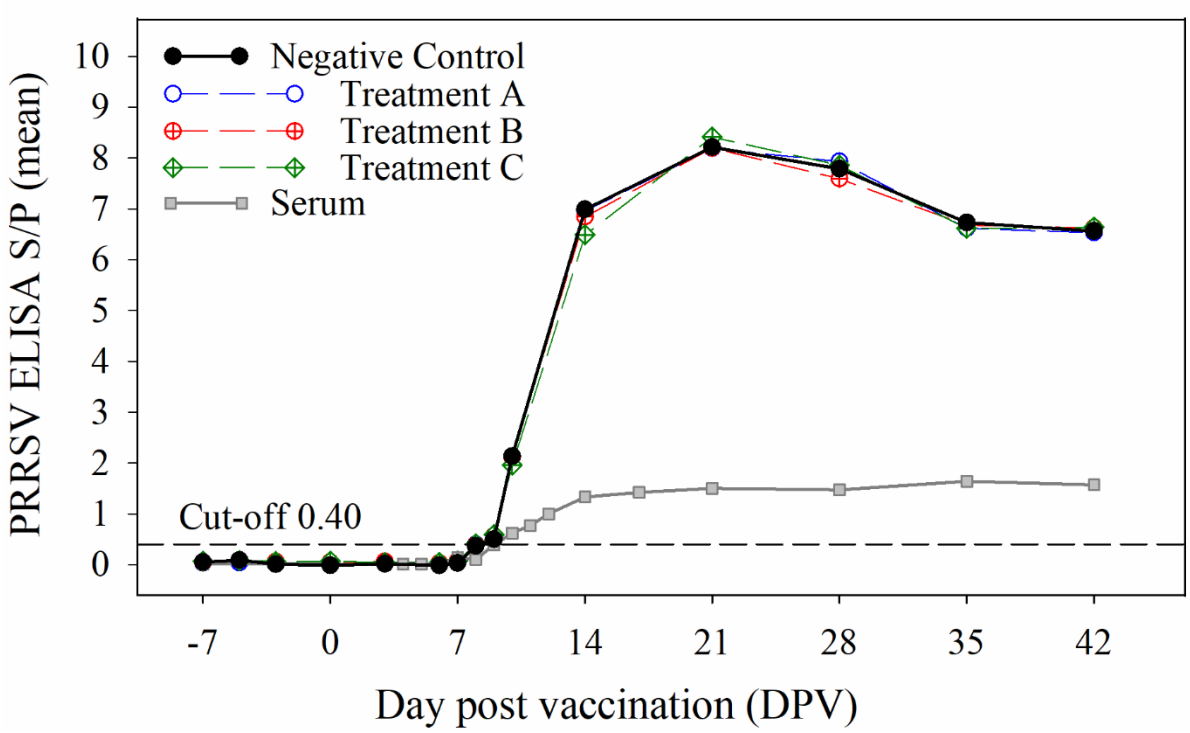

Figure 3.1. PRRS OF ELISA S/P results (mean) from serum[1] and oral fluid[2] samples by day post vaccination (DPV). Oral fluid samples were tested immediately following treatment (see Table 1). [1] IDEXX PRRS X3 Ab Test. [2] IDEXX PRRS OF Ab Test (IDEXX Laboratories Inc., Westbrook, ME, USA).

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GENERAL DISCUSSION

The use of antibody-based diagnostics methods started in the 1890's with the introduction of agglutination and/or precipitation assays for a variety of bacterial veterinary and/or zoonotic pathogens (Schoening and Creech, 1935; Merchant and Packer, 1956). From that starting point, it took nearly a century to elucidate the molecular structure, biological properties, and extravascular distribution of antibodies. That is, to open the door to the development of the antibody-based diagnostic technology (Berson et al., 1956; Coons, 1942; Yalow and Berson, 1960; Wide and Porath, 1966). Today, antibody engineering is a nascent science, with applications to a wide variety of diagnostics and primary treatments. As a result, the IgG molecule is probably the most studied of all proteins worldwide (Kohler and Milstein, 1975; Padlan, 1994).

At the beginning of the journey into antibody testing and for most of the time since, serum has had the primary role as diagnostic specimen. However, greater understanding of IgG corporal distribution and functionality has allowed other body fluids to become practical and affordable alternatives (Prickett et al., 2008; Rotolo et al., 2017). Saliva, better termed "oral fluids", was first used in livestock diagnostics beginning in the latter 1990’s (Smith et al., 2003; Wills et al., 1997). The simultaneous appearance of PRRSV antibodies in serum and swine oral fluids supported the importance of oral fluid in swine diseases surveillance in commercial herds (Kittawornrat et al., 2013; Kuiek et al., 2015; Ramirez et al., 2012). However, the oral fluid specimens often contain feed, feces, and environmental contaminants that may affect liquid handling and test performance in the laboratory.

The approach to clarify swine oral fluids evaluated in this research was based on an ancient technology that is still widely used in water treatment, food and beverage industries, and cutting-edge medical technologies (Alves and Mano, 2008; Angelakis and Zheng, 2015; Rinaudo, 2006; Zeng, et al 2008). The analyses of PRRSV IgG stability in swine oral fluid samples undergoing chemical treatments showed that it is possible to improve their innate characteristics, i.e., quickly and inexpensively remove suspended particulates, without affecting ELISA antibody responses.

However, the basic information about the physicochemical processes and main external factors that affect humans IgG stability is not sufficient, and information of interspecies and subclasses differences among IgGs is needed to better understand specific interactions of chemicals-antibody and its impact in diagnostics. Future efforts should include the evaluation of other coagulants and/or combinations of coagulants, e.g., chitosan in combination with others. In addition, the feasibility of using similar clarification approaches with other "polluted" diagnostic specimens like feces, colostrum or milk.

Sample clarification represents an affordable approach for the improvement of oral fluids, and probably other diagnostic specimens (Henao-Diaz et al., 2017). More broadly, further development of cost-effective, rapid clean-up technologies will expand our repertoire of diagnostic specimens, improve the sample-handling characteristics of these specimens in high-throughput laboratories, and create greater opportunities for the efficient collection of surveillance data. In summary, the research described in this thesis represents initial attempts at improving the characteristics of oral fluid as a diagnostic specimen, but likewise represents the beginning of a new line of research in the improvement, development, and/or expansion of veterinary diagnostic specimens.

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