



IgY-Technology: A New Frontier in the Veterinary Diagnosing Procedures



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Abstract

Domestic animals, as bovine and equine, are vulnerable to diseases caused by infectious pathogens. Unfortunately, the pathogens that infect animals can also infect humans. In this context, the public health demands a global effort to control zoonotic diseases in herds from developing countries. Serology tests are applied to diagnose the infected animals, protect the human population and implement the disease control procedures. Among the reagents used in diagnostic platforms, the antibodies, as the Immunoglobulin G (IgG) and IgY from mammals and chickens, respectively, are some of them. The IgY antibodies are available in egg yolk content from immunized chicken. According to the principles of welfare, the large amount of antibodies and the antigenic target recognition made IgY an alternative biologic reagent in veterinary diagnosing tests. Innovative platforms for serology assays are being produced using IgY-technology.

Introduction

The hens' immune system has been a model for a myriad of revolutionary discoveries through the last decades. The bursa of Fabricius was the keystone for the research in humoral response's immunology. The absence of blood circulating antibodies after a bursectomy highlighted the lymphoid tissues function for B cells maturation and, also, their crucial role in antibody production. Additionally, hens' blood circulating antibodies were demonstrated to be transferred to their off spring by transport molecules on the surface of yolk membrane during the folliculogenesis.

The discovery that the antibody repertoire is the same in hens' blood and egg yolk brought an alternative to antibody production. Extracting antibodies from egg yolk of immunized hens reduces the painful events to the producing antibodies' host. Approaches were developed aiming the extraction of these antibodies by low cost and high concentration of antibodies taking in account animal welfare.

The hens' genome was sequenced and there were found coding genes for three classes of antibodies: Immunoglobulin (Ig) M (IgM), IgA and IgY. All of them are blood circulating antibodies. However, in the egg, a large number of IgY were found in yolk while only traces of IgM and IgA were deposited in white during egg formation. In this context, egg yolk was targeted for obtaining hens' antibodies. The IgY antibodies are progressively receiving attention from the scientific community, due to their similarities in biological functions with the mammals' IgG and their higher yield and easier extraction. In addition, when using avian antibodies in diagnostic approaches

the undesirable effects such as false positive results can be avoided. This feature is due to IgY structural difference in its Crystallizable Fragment (Fc) compared to the same equivalent region in IgG.

IgY is a promising biological molecule for many applications. Avian antibodies have been applied to diagnostic approaches such as viral and bacterial infections. Also, they have been used for immunotherapy such as oral passive immunization. IgY has been conjugated with enzymes, fluorophores or nano particles and they also can be used for enzyme-linked assays, immune histochemistry procedures or immunochromatography's test as well.

The bovine and equine herds are both susceptible to infectious diseases, including some zoonosis, which must be prevented by precocious diagnosing approaches. The detection of circulating or tissue antigens and specific blood circulating antibodies, brings celerity to diseases' diagnostic. In this context, a source for a quality biological molecule is needed. The IgY antibody fits all requirements to be this biological molecule. Hence, they have been used to diagnose viruses and bacteria that, infecting bovine and equine herds, cause risk to public health. In this mini review, we shall present recently published data using IgY-based approaches (named IgY-technology) for some challenges in veterinary medicine.

IgY antibodies: an overview

The hens' immune system is organized in lymphoid tissues, immune cells and molecules [1]. Bursa of Fabricius is an antibody-producing related lymphoid tissue; therefore, the bursectomy

drops the antibody production [2]. The B-cells derived plasma cells are the producing antibody cells. Firstly, compromised lymphoid stem cells living in bone marrow differentiate to immature B cells. The cell's maturation occurs in the Bursa of Fabricius tissue and it must involve a selective process preventing the development of autoimmune diseases [3]. The mature B cell leaves bursa of Fabricius to homing peripheral lymphoid tissues such as mucosa-associated lymphoid tissue being examples: eyes-associated Harderian's gland and gut-associated lymphoid tissue [4]. In these places, B cell must be exposed to foreign substances (named antigens), activate its genetic program for clonal expansion, and finally differentiate in Plasma cell [5]. B cell-derived Plasma cells are the antibodies' producing "factories".

The hens have three classes of antibodies: IgM, IgA and IgY, while mammals have five classes, IgM, IgA, IgG, IgE and IgD [6]. Avian IgY is an ancestor of IgG and IgE antibodies. The antibody molecule is a heterodimer protein assembled with two light chains and two heavy chains linked by inter chain disulfide bonds. Both, avian and mammalian primary immune response are featured by higher titles of IgM antibodies while the secondary immune response has mainly IgY (hens) or IgG (mammals) antibodies production [7,8]. Antibodies' coding genes control the hens and mammals' humoral immune response repertoire and the phylogenetic distance contributes to an interesting difference between these two animal groups [9].

Maternal antibodies are an important protection for offspring. Avian maternal antibodies are transferred to its descendent by egg yolk IgY while transplacental IgG transference occurs in mammals. During the hens' folliculogenesis there is an IgY translocation from the blood circulating to the egg yolk by specific receptors while IgM and IgA antibodies are secreted to the egg white [6,10].

Egg yolk antibodies are a promising source for high quality immunobiologics. The extraction of IgY antibodies from egg yolk reduces the number of painful events to the animal host. In addition, annually, just one hen can produce the equivalent number of antibodies of four adult rabbits [9]. Avian antibodies do not bind to the rheumatoid factor or the mammal's Crystallizable Fragment (Fc) receptor, and, they do not activate mammalian complement system. Altogether, these findings made IgY an attractive possibility to avoid the false positive results to serologic diagnostic.

As a quality immune biologic reagent, IgY antibody presents specificity and sensitivity against immunogens such as native proteins, recombinant proteins, peptides, and haptens. Avian antibodies are found in high titles in the egg yolk because they have there, 2-fold the blood circulating concentration [6]. Furthermore, the translocation of IgY, from blood to egg yolk has a circadian rhythm. In addition, IgY has an equivalent affinity maturity to mammals' IgG antibodies [9]. Regarding the antibodies' antigen binding repertoire, polyclonal and monoclonal IgY antibodies have been produced for a wide range of antigenic targets [11,12].

IgY-Technology applied to domestic animals' diseases

The animals' immune response is featured by cellular and humoral immune response and both are the arms of the acquired immune response [13,14]. In mammals' humoral immune response, the IgM antibodies are predominant in the first exposure to the pathogens whilst the IgG antibodies are commonly produced in subsequent exposures [15]. Regarding the serologic diagnostic platforms, these post-pathogens contact blood circulating antibodies can be named "primary antibodies" and they binding to immobilized antigens attached on the diagnostic platforms forming the immune complexes [16]. The detection these immune complexes is primarily the function of immunodiagnostic approaches. In this context, appropriate tools for an immune complexes' detection have been demonstrated by heterologous, polyclonal or monoclonal antibodies, prepared against the host antibodies [9]. These heterologous antibodies are named secondary antibodies and they are featured by detecting class-specific and, also, specie-specific antibodies [17].

Those secondary antibodies are produced using a different specie host from the target specie. Frequently, the antibodies producing hosts are mammals such as rabbits, goats or mice. Thus, the major class of mammal' antibodies is the IgG, which is the same used as a secondary antibody detecting immune complexes. IgG antibody has been prepared as a high avidity biological reagent which has been conjugated with enzymes, fluorophores or metal nanoparticles, and they are suitable tools amplifying the presence of immune complexes detection [12].

At the 80's decade, the increased attention to animal welfare guided the proposition for an alternative source of IgG secondary antibodies. The IgG production includes the animal's immunization and bleeding for extraction of its blood antibodies. In this context, hens' egg yolk has been introduced as a source of high quality antibodies that have an extraction method free of hens' bleeding procedures [6].

The major egg yolk antibody is the IgY and it has equivalent immune properties to the IgG antibody [9]. IgY is the ancestor of mammal's IgG and it has two antigen binding sites as the IgG and, also, the whole IgY molecule is composed by light and heavy peptide chains. However, the IgG's hinge region is absent in IgY antibodies and they have a lengthy Crystallizable Fragment (Fc) than IgG [15]. In this section, we present the applications of IgY antibodies by IgY-technology approaches diagnosing animals' infectious diseases specially in bovine and equine.

Viral diseases

Bovine and equine herds are susceptible to viral infectious diseases, some of them have a zoonotic potential, and others have a negative impact in the country's agriculture system. For instance, rabies is a worldwide zoonotic disease and affects bovine and equine herds in the rural zone of many countries. Foot and Mouth

Disease Virus (FMDV) is a bovine vesicular disease that causes economy losses. In this context, the Infectious Equine Anemia (IEA) and equine Herpesvirus are financial troubles for equine herds.

Rabies

Rabies is a lethal zoonotic disease that infects mammals as bovines, equines and bats [18,19]. The rabies' control program includes periodically monitoring of wild reservoirs such as bats, massive herd vaccination and bats' population control at risk zones [19]. Rabies infection is confirmed by histological assays as Immune HistoChemistry (IHC) or immune peroxidase antigen detection [20]. In the direct antigen detection assay, cytoplasm Lyssavirus inclusions are detected by specific polyclonal IgG anti-rabies antibodies [21]. IgY polyclonal anti-rabies antibodies has been introduced as a promising immune biological for direct detection of Lyssavirus [22]. In addition, IgY antibodies are properly conjugated with peroxidase and fluorescein [23, 24]. Additionally, after the pepsin digestion, IgY antibodies produce two antigen binding fragments (Fab), however IgG antibodies are degraded into F(ab')₂ [25]. This structural difference, in pepsin-degraded antibodies, has been proposed to influence the tissues' antibody distribution in treatments in animal models [26]. Regarding the IgY use for rabies therapy, it has been suggested that it has higher immunogenicity than the IgG for mammals [27].

Foot and Mouth Disease Virus

Bovine vesicular diseases are very important for livestock because of foot-and-mouth disease caused by Foot and Mouth Disease Virus (FMDV) [28]. FMDV's control is based on vaccination of susceptible animals as bovine herds, for example. The differentiation from vaccinated bovines to FMDV infected bovines is crucial to monitor the herd' health status in a country during a FMD eradication program. Enzyme-linked immune sorbent assay (ELISA) sandwich and a competitive ELISA has been suggested to the FMDV detection [29]. In this context, mammal-based recombinant anti body fragments, like single-chain Fragment Variable (scFv), has been applied in FMDV ELISA platforms [30]. Based on the repertoire of antibody genes, SCFV (Single-Chain Fragment Variable) immune libraries have been constructed from antibody genes of B cells derived from immunized animals [31]. Hen's single-chain antibody fragment libraries are useful to diagnostic and research of biological reagents. The use of avian immunoglobulin genes simplifies the construction of such repertoires since far fewer primer sets are required to access the avian antibody genes of B cell than in mammals [32].

Infectious Equine Anemia Virus

Infectious Equine Anemia Virus (IEAV) is the cause of a disease with morbidity and mortality that infects members of Equidae. In Brazil, officially approved Agar Gel Immuno Diffusion assay (AGID) has been used to detect seropositive animals. Moreover, ELISA test and ImmunoBlot (IB) has been used for epidemiological studies [33]. Phylogenomic analysis suggested that the Brazilian EIAV forms a cluster with strains from the United States [34]. The capsid

protein (p26) is one the major immunogenic proteins during EIAV infection and it is widely used for detection of its specific antibodies. Antigen Capture ELISA (AC-ELISA), based on mammals' monoclonal antibodies, has been reported for quantification of the EIAV's p26 protein level [35]. Polyclonal IgY-based detection antibody has been developed to the human severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid protein for the AC-ELISA diagnosing test in which this avian antibody increased the sensitivity of the system [36].

Bacterial diseases

In this section we shall try to describe perspectives for the IgY antibody technology use in zoonotic bacterial diseases; bovine brucellosis, bovine tuberculosis and equine glanders. Bovine brucellosis is caused by *Brucella abortus* that negatively impacts livestock productivity in Brazil. For this reason, in 2001, Brazil launched a new national program aiming the eradication of animal brucellosis. A recently large-scale study was published and revealed important differences in herd prevalence among Brazilian regions. Regarding brucellosis epidemiology herd size and kind of bovine herd were considered significant risk factors [37]. Bovine tuberculosis is caused by *Mycobacterium bovis* which offers risk of infection to human consumers' of raw milk and homemade fresh cheese in Brazil [38]. Glanders is a highly contagious and often fatal zoonotic disease caused by *Burkholderia mallei* primarily often of solipeds and, already used as a biological weapon [39]. Glanders has regained the status of a re-emerging disease because of the numerous recent out breaks and the pre-symptomatic, or carrier animals, that are potential sources of infection and play a crucial role in the spreading of the infectious agent [40].

Bovine Brucellosis

Bovine brucellosis' diagnosing test has been improved to avoid false positive or false negative results. Recently it was proposed a bovine brucellosis diagnostic bead-based immunoassay by Luminex assay using mammal's monoclonal anti bodies against smooth LipoPolySaccharide (LPS) from *B. abortus* [41]. *B. abortus*' LPS has been the target for immune chromatographic test in bovine brucellosis diagnosing [42]. On a strip, immobilized antibodies bind to bacterial LPS and report an infected animal at field conditions. Other cell wall polysaccharides (M polysaccharide) of *B. abortus* were immunogenic for mammalian antibodies eliminating the cross reactivity in serologic diagnostic therefore, used as a biological reagent for bovine brucellosis' diagnostic [43]. Regarding the bacterial polysaccharide immunogenicity, LPS was a target to produce promising IgY antibodies that were used as a LPS-binding tool for endotoxemia's model therapy [44]. Also, polyclonal IgY against LPS from *B. abortus* have been prepared and conjugated with immune magnetic beads to detect the bacterial antigen [45]. Recently, transplastomic plants were transformed with high immunogenic *B. abortus* protein and then, these antigens from the bacterial-recombinant plants were extracted and used to produce high quality polyclonal IgY against *B. abortus* [46].

Bovine tuberculosis

Intradermal test, bacterial culture and molecular methods such as Polymerase Chain Reaction (PCR) and genome sequencing have been used to diagnose *Mycobacterium bovis* infected animals [47]. However, a sandwich ELISA-based interferon gamma (IFN- γ) detection assay has been suggested as a promising complementary method of diagnosing early stages of *M. bovis* infection [48]. Corroborating this findings, positive samples in the tuberculin skin test are, also, positive in the IFN- γ test [49]. Recently, it was developed a novel immune chromatographic-based test for *M. bovis* detection from fecal samples of reservoir wild animals by polyclonal and monoclonal IgG antibodies against *M. bovis* antigen [50]. In the context of *Mycobacterium* genus, specific and sensitive polyclonal IgY antibodies has been used as an immunobiological reagent for the capture of *Mycobacterium avium* antigen and attesting of the bacterial growth in liquid culture [51]. Also, FITC-labeled and immune magnetic beads-coated polyclonal IgY antibodies against *M. Avium* Paratuberculosis (MAP) enhance the bacterial diagnostic and avoid unspecific reactivity to MAP [51,52]. Engineered IgY antibodies from chicken scFv (“gallibodies”) with a more specific antigen binding fragment have been suggested as a promising tool for sandwich ELISA or immune chromatographic test detecting antigens of *M. bovis* [53].

Equine glanders

PCR-based methods have been used for diagnostic and differentiation among *Burkholderia* genus bacteria [54]. However, recombinant antigen-based immunodiagnostic such as ELISA is highlighting the Glanders’ serologic diagnostic [55]. In addition, indirect ELISA or competitive ELISA prepared with recombinant antigens from *B. mallei* were promising diagnosing equine glanders compared to the Complement Fixation Test (CFT) [56,57]. Commercially CFT has been applied to detection of specific antibodies in seropositive Equidae infected with *B. mallei*, however there are false positive tests and divergences in CFT results from different laboratories [58]. Western Blot test (WB) has been prepared to overcome the false positive results caused by high sensitive CFT, and, hence, WB must be considered a confirmatory test while CFT must be used as a routine test for glanders diagnostic in non-endemic areas [59].

Scientific effort has been focused in production of mammal’s monoclonal antibodies with bactericidal activity against *B. mallei* and, in this context, bacterial LPS was a better target for antibody binding than bacterial proteins, and these antibodies result in a superior protection after experimental infection [60]. A valuable strategy diagnosing *B. mallei* are the scFv antibodies from a phage-displayed libraries and these scFv has been demonstrated to have high specificity to discriminate among the pathogenic *Burkholderia* genus [61]. At the moment, there are not any published research with IgY antibodies and glanders. However, these avian antibodies have demonstrated potential for application in ELISA tests,

monoclonal and scFv antibodies approaches. For this reason, our group is developing IgY anti bodies for application in equine glanders’ diagnostic.

Conclusion

Bovine and equine herds are susceptible to infectious diseases that have zoonotic potential, and hence, they have received attention by the government authorities. Diagnostic approaches have been developed to increase specificity and sensitivity and to detect infected animals. ELISA, immune chromatographic test, fluorescent assays are improving continuously and all of them demand high quality antibodies. Overcoming false positive results, mammals’ antibodies have been prepared as monoclonal anti bodies or scFv from phage-displayed libraries. In this context, avian antibodies such as IgY antibodies are promising high-quality antibodies. The results of IgY research demonstrated the robustness of this immune biological reagent and, also, suggested a wide range for IgY applications in viral and bacterial diseases in bovine and equine. We believe IgY-technology is a new frontier for biological reagents applied to diagnostic approaches in veterinary medicine.

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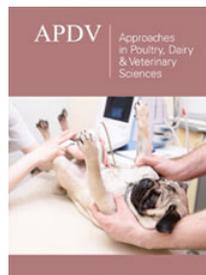
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